Vaccine-Like Immunity against Malaria by Repeated Causal-Prophylactic Treatment of Liver-Stage Plasmodium Parasites

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Liver-stage development of Plasmodium parasites represents a dramatic expansion phase for the malarial parasite between vector transmission and onset of the pathogenic blood-stage cycle. Here, we report that repeated causal-prophylactic primaquine treatment of liver-stage Plasmodium parasites in rodents elicits vaccine-like protective immunity against sporozoite-induced malaria. This regimen differs fundamentally from those involving radiation- or genetically attenuated parasites, in which long-lasting immune responses are dependent on persistence of metabolically active parasites. Pharmacological inhibition of liver-stage parasites in the rodent malaria model offers a potential fast track toward development of a vaccine that targets parasites in preerythrocytic stages.

Malaria remains the world’s most important vector-borne infectious disease, affecting 500 million people worldwide and killing >1 million individuals annually. Transmission of malaria-causing Plasmodium parasites to the mammalian host occurs during the mosquito bite when motile sporozoites are injected into the skin. Malaria symptoms and disease are caused exclusively by parasites in the asexual blood stage, which rapidly multiply in erythrocytes. During the symptom-free prepatent period, sporozoites enter a suitable host hepatocyte, transform, and commence replication, resulting in the formation of thousands of infectious pathogenic merozoites.

In experimental animal models, immunizations with radiation-attenuated parasites induce robust, long-lasting protection against subsequent sporozoite challenges [1]. Notably, protection is ablated when immunizations are done with heat-inactivated and overirradiated sporozoites, indicating vital requirements for intrahepatic and metabolically active parasites, respectively. Limited studies involving men strongly corroborated use of live, attenuated whole parasites as the gold standard for malaria vaccine development [2]. Induction of sterile protection can be reproduced in the rodent malaria model parasite with genetically attenuated parasites [3]. Although proof-of-concept studies in the human malaria parasite are underway, an important direction toward translational research has emerged: are there fast tracks to induce protective immune responses against preerythrocytic-stage parasites?

Experimental immunization with live sporozoites followed by suppressive treatment of blood-stage parasites with chloroquine induced high levels of protection in mice [4–6]. Although not applicable in malaria-endemic countries because of the high prevalence of chloroquine-resistant Plasmodium falciparum strains, these studies confirmed that exposure to live sporozoites induces a protective response against parasites from a combination of life cycle stages, such as sporozoites, liver-stage parasites, and asexual blood-stage parasites. Notably, suppressive treatment with chloroquine was intended to exclude potential suppression of immune responses against preerythrocytic-stage parasites by an existing infection with asexual blood-stage parasites. More recently, robust induction of effector and memory CD8+ T cell responses was shown to occur even in the presence of parasites in the erythrocytic cycle [7].

Clinical studies of 8-aminoquinolines such as primaquine [8] and tafenoquine [9] for malaria chemoprophylaxis revealed an unusually prolonged delay to the onset of reinfection; in the case of tafenoquine, the delay was >6 times the plasma half-life. These observations, together with early indications of a protective effect of primaquine treatment [5], led us to hypothesize that repeated pharmacological ablation of liver-stage Plasmodium parasites has the potential to induce strong protective immune responses against sporozoite-induced malaria.

Materials and methods. Groups of C57Bl/6 and Balb/c mice (Charles River Laboratories) were immunized by intravenous injection of freshly dissected Plasmodium berghei and Plasmodium yoelii sporozoites, respectively, or by exposure of anes-
thetized mice to the bites of 10 infected *Anopheles stephensi* mosquitoes. Primaquine diphosphate (PQ; 60 mg/kg) was injected intraperitoneally into each animal either shortly after sporozoite inoculation (0 h) or at various time points up to 36 h thereafter. The first dose was given 0–36 h and the second 48 h after sporozoite injection. Sporozoite challenge was by intravenous injection of 20,000 wild-type sporozoites 14–21 days after the last immunization dose. Protection was independent of the time at which the first primaquine dose was received. Age-matched, naive control mice (n = 7) were treated with PQ but not infected with sporozoites. B, Sterile protection in sporozoite/PQ-immunized mice is maintained for at least 3 months. The immunization scheme was as in A, with PQ treatment 24 h and 48 h after sporozoite inoculations. The sporozoite challenge was done 3 months (black) or 6 months (grey) after the last boost by intravenous inoculation of 10,000–20,000 wild-type sporozoites. C, Protection is maintained upon sporozoite challenge via natural mosquito bite. Mice were immunized with 3 doses of 10,000 intravenously injected sporozoites, followed by PQ treatment 24 h and 48 h later. Sporozoite challenge was by exposure to 5 infected mosquitoes 39 days (black) or 90 days (grey) after the last boost. D, Substantial protection in animals that were immunized by 3 consecutive exposures to 10 infectious mosquito bites followed by standard PQ treatment. Immunized and control mice were challenged by exposure to 5 infected mosquitoes 35 days after the last boost. Note that 1 immunized animal developed infection with blood-stage parasites but remained free of symptoms indicative of cerebral malaria, whereas all control mice succumbed to cerebral malaria 7 days after challenge.

Results. We initiated our analysis with an immunization protocol that we previously used to establish protective immunity in genetically attenuated parasites [3]. C57bl/6 mice, which are highly susceptible to *P. berghei* sporozoite infection, were given 3 consecutive high-dose intravenous injections of *PbANKA* sporozoites, which consisted of 50,000 sporozoites for priming, followed by 2 doses of 25,000 sporozoites (figure 1A).

As expected, none of the animals developed infection with blood-stage parasites after subsequent PQ treatment (data not shown). Complete causal prophylaxis was achieved by treatment with a first dose either immediately, 12 h, 24 h, or 36 h after sporozoite injection, followed by a second dose 48 h after infection (data not shown). After PQ treatment, ~90% of the parasites had been eliminated 42 h after infection, as determined by quantitative RT-PCR (figure 2, which appears only in the electronic edition of the *Journal*). Furthermore, <1% of the parasites remained 72 h after infection in PQ-treated mice. The residual signal detected by PCR likely corresponded to nonviable
parasites because treated mice did not develop parasitemia after sporozoite inoculation. Importantly, when these animals were challenged intravenously with a high dose (10,000–20,000 *PbANKA* sporozoites) 14–21 days after receipt of the final boost, the majority (18 [95%] of 19) showed no development of blood-stage parasites (figure 1A). In contrast, all nonimmunized PQ-treated animals became patent 3–6 days after challenge and eventually developed symptoms of severe malaria 4 days thereafter (data not shown). Notably, sterile protection was independent of the PQ treatment scheme. This unexpected finding indicated that clearance of wild-type liver-stage parasites by PQ induced sterilizing immunity against subsequent sporozoite challenge.

The observed protection is expected to occur exclusively during development of preerythrocytic-stage parasites in the liver because these parasites are targeted by primaquine treatment. To confirm stage specificity, we challenged protected animals by intravenous injection of 1000 asexual blood-stage parasites. In agreement with our assumption, all sporozoite-immunized animals (*n* = 7) and sporozoite-naïve animals (*n* = 3) had positive results of blood smears 4 days later, developed high asexual parasite densities, and eventually had symptoms of cerebral malaria (data not shown). We next asked whether the sporozoite-specific sterilizing immunity observed by this immunization scheme is sustained beyond 1 month. We repeated the high-dose sporozoite inoculations and treated the animals with PQ 24 h and 48 h after infection. These mice were challenged 3 or 6 months later, again by a high-dose sporozoite injection (figure 1B). In agreement with our initial findings, none of the animals developed parasitemia over the 28-day survey period.

This analysis prompted us to test whether immunization protocols with lower parasite doses confer sterilizing immunity as well. We therefore reduced the immunization doses to 3 intravenous injections of 10,000 sporozoites followed by PQ-mediated ablation of liver-stage parasite development (figure 1C). Again, all immunized animals were completely protected from sporozoites during challenge exposure to 5 infected *A. stephensi* mosquitoes 1 month after the last boost. To test whether immunity can develop under natural transmission conditions, we exposed anesthetized animals to 10 infected mosquitoes 3 times and administered PQ treatment after each exposure (figure 1D). Three of 4 animals were completely protected. One animal developed infection with blood-stage parasites on day 4, which was 1 day later than infection onset in naïve control animals.

We then asked whether protection is sustained after receipt of lower-dose immunizations. When groups of animals that were immunized intravenously were challenged 3 months after receipt of the last boost, all animals had negative results of blood smears (figure 1C). Together, our findings indicate that, at least in the *P. berghei* model, vaccine-like immunity can be induced by a combination of exposure to unaltered sporozoites and causal-prophylactic treatment.

To test whether our results could be extended to another *Plasmodium* species and mouse strain, we repeated our experiments in the *P. yoelii*/*Balb/c* mouse model. We immunized mice by means of 3 doses of 10,000 *Py17XNL* sporozoites or 3 exposures to 10 infected mosquitoes (figure 3). Upon challenge 108 or 85 days, respectively, after receipt of the last boost, we again observed a substantial degree of sterile protection. All mice immunized intravenously and 3 of 4 mice immunized by exposure to infected mosquitoes had negative blood smear findings, indicating that they did not develop malaria during the observation period.

In conclusion, repeated causal-prophylactic treatment of liver-stage parasites in rodent malaria models elicited vaccine-like immunity in nearly all immunized animals.

**Discussion.** The most important finding of this study is the induction of sustained protection against natural malaria transmission by 3 rounds of immunization with liver-stage *Plasmodium* parasites from PQ-treated rodents. PQ and related
8-aminoquinolines lead to complete arrest of parasite development during the liver stage. Therefore, a regimen consisting of multiple sporozoite exposures followed by causal-prophylactic treatment differs fundamentally from immunizations with irradiation- or genetically attenuated parasites. In these experimental vaccines, persistence of metabolically active liver-stage parasites is essential to induce long-lasting CD8+ T cell–mediated responses [10, 11]. Here, we show that a similar vaccine-like immunity can be elicited by natural sporozoite inoculations in combination with causal-prophylactic treatment. Induction of protective immunity after ablation of liver-stage parasites by PQ also differs substantially from immunity elicited by chloroquine treatment to suppress blood-stage parasite infections in sporozoite-immunized animals [4–6]. Because chloroquine does not prevent the emergence of hepatic merozoites, the immune responses elicited under this regimen are directed against not only preerythrocytic-stage parasites but also blood-stage parasites. Important future research directions include identification of the underlying immune mechanisms that may differ substantially from liver-stage attenuated parasites and the site of immune priming and antigen display, which may include the liver or peripheral immunocompetent tissues, such as lymph nodes. In natural transmission immunizations, the draining lymph nodes may play a predominant role in priming the protective immune response, resulting in swift and robust induction of effector T cell populations [12].

PQ and the combination therapy atovaquone-chlorproguanil [13] are the only established treatments for liver-stage parasites. The use of PQ is limited by adverse effects, including crises of hemolytic anemia in individuals with deficiency of glucose-6-phosphate dehydrogenase activity and lesions of the gastrointestinal tract. The value of atovaquone-chlorproguanil is greatly reduced because of its propensity for rapid selection of atovaquone-resistant parasites. For these reasons, PQ and atovaquone-chlorproguanil are currently not recommended for prophylactic interventions in high-risk populations, such as young children living in malaria-endemic countries [8, 9]. A short, 3-day regimen of tafenoquine, another 8-aminoquinoline with exoerythrocytic activity, showed extended protection against P. falciparum reinfections in one study in Gabon [9]. This result can only partly be explained by the slow elimination of tafenoquine from the blood. When considered in light of our data, we hypothesize that an immune response to preerythrocytic-stage parasites can be elicited by extended causal-prophylactic treatment during repeated exposure to infected mosquitoes. Our findings are also supported by a recent study that tested coadministration of sporozoites with a toxic, and potentially carcinogenic, substance centanamycin [14]. Although this particular approach cannot be translated into a field application, the underlying mechanisms of robust induction of sterilizing immune responses due to chemical attenuation may be comparable to our model. Antisporozoite immunity alone, when acquired under continuous natural exposure conditions, is at best partial and does not prevent reinfections and malaria episodes [15]. Pharmacological ablation of the development of parasites in the liver stage, an otherwise fast and silent expansion phase of the parasite population, may therefore elicit improved immune responses.

On the basis of our findings in rodent malaria models, we propose to consider and specifically test in future clinical trials whether compounds that act against liver-stage Plasmodium parasites can substantially reduce reinfection rates over a prolonged period after cessation of treatment or prophylaxis. If its effectiveness is confirmed in populations exposed to high reinfection rates, causal-prophylactic treatment may serve a dual function by direct elimination of liver-stage parasites and thereby prevention of new infections during treatment and, additionally, by induction of an immune response against liver-stage parasites that acts beyond the treatment scheme. Alternative drugs to 8-aminoquinolines are urgently needed to enlarge our armamentarium against Plasmodium organisms and ultimately develop a safe causal-prophylactic protocol that offers many advantages over the current practice (i.e., continuous inhibition of blood-stage parasites) for preventing malaria.

In summary, a better understanding of the mechanisms of pharmacological elimination of preerythrocytic-stage Plasmodium parasites may ultimately lead to novel antimalarial interventions that may elicit previously unrecognized protective immune responses against reinfections.

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References


Figure 2. Primaquine (PQ) treatment eliminates liver-stage parasites. After intravenous injection of 100,000 sporozoites into C57bl/6 mice, livers were removed for RNA extraction. Seven mice were left untreated, 7 received a single PQ dose 24 h before liver removal at 42 h, and 6 received 2 doses 24 h and 36 h before liver removal. Four mice received 2 PQ doses as described above, but livers were removed at 72 h. Two mice were not infected. Values correspond to the expression levels of Pb18S normalized to mouse HPRT and are expressed as the percentage of the mean value for the untreated control group. The bars indicate mean infection levels.