Induction of Anti-Malaria Immunity by Pyrimethamine Prophylaxis
during Exposure to Sporozoites is Curtailed by Parasite Resistance

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Abstract

Each year, infections with the protozoan parasite *Plasmodium falciparum* kill one million people, mostly children in Africa. Intermittent preventive treatment (IPT) with sulfadoxine-pyrimethamine (SP) reduces malaria incidence and aims to prevent mortality in infants, children and pregnant women. There is contradictory evidence whether this strategy may generate additional protection against reinfection beyond the limited duration of the intervention. Previous work established that ablation of either liver stage maturation or subsequent life-cycle conversion by causal prophylactic drugs elicits protective immune responses against reinfections when drugs are no longer present. Here, we show in the rodent malaria model that pyrimethamine, a component of SP, inhibits liver stage development *in vitro* and *in vivo*, and, hence, confirms its causal prophylactic activity. Repeated exposure to high doses of *Plasmodium berghei* sporozoites during pyrimethamine prophylaxis induced complete protection in C57bl/6 mice against challenge 35-199 days later with high doses of sporozoites delivered intravenously. Immunizations by infectious mosquito bites induced limited and inoculation-dependent protection against subsequent infected mosquito bite challenge, but provided partial protection against experimental cerebral malaria. Short-term pyrimethamine prophylaxis during intravenous transmission of sporozoites from a pyrimethamine-resistant strain delayed, but did not prevent, blood stage infection. Our data provide a rationale for the notion of sustained protective efficacy of IPT based on the capacity of arrested, drug-sensitive liver stages and/or suppressed blood stages to mount lasting protection.
Introduction

Malaria, caused by *Plasmodium falciparum*, is one of the most pernicious parasitic diseases, mostly affecting children and pregnant women in sub-Saharan Africa (2, 20). For many decades chloroquine, arguably the safest, most affordable, and globally available antimalarial drug was the first-line treatment throughout Africa (10). After the global spread of chloroquine-resistant *P. falciparum*, national malaria control programs changed the first-line treatment to a co-formulation of sulfadoxine and pyrimethamine (SP) in the early-mid 1990s (10). Coinciding with this deployment of an efficacious replacement drug, a substantial decline of malaria morbidity and mortality was reported from several study sites (1, 13, 26, 28, 36). Unfortunately, parasite resistance to pyrimethamine has spread rapidly (11, 34, 40), leading to the introduction of artemisinin-based combination therapy (ACT) in all endemic countries (http://www.who.int/malaria/am_drug_policies_by_region_afro/en/index.html). However, there is still consensus on the benefits of SP in intermittent preventive treatment (IPT) programs for infants, pregnant women, and, to a lesser extent, children (18, 41).

The aim of IPT is to reduce the frequency of malaria episodes without preventing every blood stage infection. IPT offers protection by clearance of pre-existing, mostly asymptomatic, infections, followed by a short period of post-treatment chemoprophylaxis (41) and permits the natural acquisition of immunity against the pathogenic blood stages (38). Thereby, IPT avoids the rebound effect that was initially seen in extended continuous chemoprophylaxis, where individuals experienced higher post-intervention attack rates (3, 17, 23). An intriguing finding of a follow-up study on one of the first IPTi trials was that the protective efficacy of SP appeared to extend into the second year, when the drug was no longer administered (35).

Experimental studies in the rodent malaria system demonstrated the potential for induction of lasting protection against re-infection by inoculation of live sporozoites during prophylactic administration of antimalarials (6, 7, 16, 32). The drugs tested so far in studies of immunoprophylaxis act at different points in the *Plasmodium* pre-erythrocytic cycle: primaquine kills intrahepatic parasites (32), antibiotics, such as azithromycin, lead to a
delayed death inside the infected hepatocyte, resulting in emergence of non-infectious liver stage merozoites (16), and chloroquine cover results in suppression of emerging blood stage infections (6, 7). These experimental findings were validated by a recent proof-of-concept study in human volunteers. In this study exposure to 12-15 infected mosquitoes during chloroquine prophylaxis elicited sterile protection against challenge by infected mosquito bites (33).

The inhibitory effect of the antifolate drug pyrimethamine on hepatic stages of the malaria parasite was first described more than four decades ago by direct and indirect observations (8, 24). In this study we wanted to test whether causal prophylaxis with pyrimethamine during inoculation of mice with sporozoites can generate immune-mediated protection against challenge with sporozoites when the drug is no longer present. Secondly, we studied the prophylactic efficacy of pyrimethamine against a pyrimethamine-resistant Plasmodium strain and the effect of pyrimethamine-resistance on potential secondary, immune-mediated protection against reinfection with sporozoites (38). We hypothesized that the results may also help explain some of the controversial results from IPT trials.
**Material and Methods**

All animal research was conducted in accordance with European Union and German regulations and approved by the state authorities (Landesamt für Gesundheit und Soziales, Berlin).

**Sporozoite inoculation in mice during pyrimethamine prophylaxis**

Groups of C57bl/6 mice (Charles River Laboratories) were inoculated by intravenous injection of freshly dissected *Plasmodium berghei* ANKA [clone 507, constitutively expressing green fluorescent protein (GFP)] sporozoites, or by exposure of anesthetized mice to the bites of infected *Anopheles stephensi* mosquitoes. Infected mosquitoes used for infected mosquito bite, hereafter termed ‘by-bite’, experiments were anaesthetized on ice, presorted under an epifluorescence microscope and put into individual cups one day before the inoculation or challenge (see subsequent section) was performed. Pyrimethamine (Sigma) was administered orally with the drinking water for 42 hours at a concentration of 70 µg/ml or injected on three consecutive days intraperitoneally at the doses indicated. The irradiation dose for the experiments with irradiated sporozoites was 12,000 cGy. The intraperitoneal treatment with chloroquine diphosphate salt (Sigma) dissolved in sterile PBS consisted of 1.6 mg/mouse for 7 days until mice were parasitemia-free as assessed by Giemsa-stained blood smears. To assess the chemo-prophylactic efficacy of pyrimethamine, animals were monitored for asexual blood stages over a period of at least 14 days by microscopic examination of Giemsa-stained blood smears.

**Challenge with *Plasmodium* sporozoites**

Mice that had been subjected to the inoculation/treatment protocol and age-matched control mice were challenged by intravenous injection of freshly dissected sporozoites or by exposure to bites by infected mosquitoes. Control mice were not age-matched at the intermediate (day 64) and latest challenge time point (day 199) and in the re-challenge
experiments. In these experiments, 6 to 8 weeks old control mice were used. Mice were checked daily for the appearance of blood-stage parasites by microscopy of blood smears, starting at day 3 after sporozoite injection and for symptoms of cerebral malaria (i.e., ataxia, paraplegia, deviation of the head, and coma).

**In vitro liver stage development**

For determination of the *in vitro* activity of pyrimethamine on pre-erythrocytic forms, 100,000 hepatoma (HuH7) cells were seeded in 24 well plates one day before addition of 40,000 ANKA wild type or *sera1(-)* sporozoites (31). After adding the sporozoites, the plates were centrifuged at 2,000 rpm for 5 minutes and incubated for 2 hours at 37°C. After washing infected cells three times with sterile HBSS medium, they were treated with trypsin and transferred to chamber slides (Lab-Tek, Nunc) for incubation with serially diluted pyrimethamine (concentration range: 1 µM – 100 µM) in complete DMEM medium containing 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin. Cells were fixed 48 hours after infection with cold methanol and stained using an anti-\(Pb\)HSP70 antibody (39) and Hoechst 33342 to determine the size and number of liver stages. Inhibition of parasite growth was determined by comparing sizes of pre-erythrocytic forms in relation to the host cell nucleus size and by counting parasites per slide.

**Quantification of parasite loads in the liver**

To determine inhibition of liver stage development by either direct drug action (causal prophylaxis) or by immune responses against liver stage parasites generated by the inoculation/treatment protocol (immunization), parasite loads in the livers of infected C57bl/6 mice were determined by quantitative real-time PCR 42 hours after intravenous injection of 10,000 or 25,000 sporozoites as previously described (9, 16).
Results

Pyrimethamine blocks intrahepatocytic replication of *Plasmodium berghei* liver stages

We first established that oral pyrimethamine treatment of C57bl/6 mice within the first 42 hours after intravenous application of 10,000 *Plasmodium berghei* ANKA sporozoites prevented asexual blood stage infection even after prolonged monitoring (0/9), whereas untreated controls showed parasitemia at day 3 after sporozoite inoculation (4/4) (Fig. 1A). In order to differentiate between a suppressive and causal-prophylactic effect on *P. berghei* infections, we administered 25,000 sporozoites to C57bl/6 mice (n=5) under pyrimethamine cover for 42 hours. Control animals (n=5) received DMSO in water. In mice that received oral pyrimethamine treatment, the hepatic liver burden was reduced by 99% as determined by quantitative PCR compared to the untreated controls (Fig. 1B; unpaired t-test; \( P<0.01 \)). We therefore confirm that pyrimethamine acts as a true causal-prophylactic drug, by inhibiting liver stage maturation.

To further characterize the action of pyrimethamine on *P. berghei* liver stage development, we used an established *in vitro* assay. We infected cultured hepatoma cells (HuH7) with 10,000 *P. berghei* sporozoites and measured liver stage development by immunofluorescence assay (IFA) using an anti-*PbHSP70* antibody (39). Forty-eight hours after infection liver stages in cultures that were exposed to pyrimethamine at concentrations of 1, 3.33, 10 and 100 \( \mu \)M were viable but significantly reduced in size as compared to the controls (Fig. 1C and Fig. 3C) suggesting intrahepatocytic persistence of metabolically active but growth arrested parasites.
Protection against sporozoite challenge in mice after repeated exposure to live sporozoites during pyrimethamine prophylaxis

We next wanted to test whether sporozoite inoculation during parallel administration of pyrimethamine can induce protective immune responses against re-infection with sporozoites (Table 1). First, animals received three consecutive sporozoite doses during oral administration of pyrimethamine. For this protocol 10,000 sporozoites were injected intravenously into age-matched naïve C57bl/6 mice. Three groups of animals were then challenged intravenously with 10,000 sporozoites in the absence of pyrimethamine at three different time points after the last inoculation/treatment step (Table 1): (i) an early time-point, i.e. day 35 (n=9), (ii) an intermediate time interval, two months later (n=4), and (iii) in a long-term protection experiment, 199 days (n=4) later. Regardless of the time to challenge, none of the challenged animals developed parasitemia after 5 months of follow-up. All control animals developed blood stage infections and symptoms of cerebral malaria on days 3-4 and 7-8 after sporozoite challenge, respectively. We conclude that intravenous inoculation of high doses of live, drug-sensitive sporozoites during pyrimethamine administration induced sterilizing protection against homologous challenge with equally high doses of sporozoites.

Protection is long-lasting and liver stage-specific

We next re-challenged protected mice 5 months after the last challenge by intravenous injection of 10,000 *P. berghei* ANKA sporozoites (Table 1). All control animals became positive at day 3 (n=5) and 4 out of 5 mice developed symptoms of cerebral malaria 7 days after challenge. In the test group, all previously exposed mice remained steriley protected against re-challenge with the homologous *P. berghei* ANKA strain (n=9). We conclude that pyrimethamine-mediated ablation of liver stage development has the potential to induce lasting protection, similar to an equivalent protocol with primaquine (32).

To test whether sterile protection elicited by the sporozoite/pyrimethamine regimen is liver stage-specific, we infected 3 protected animals with 7,500 *P. berghei* ANKA-infected red blood cells (Table 1). The period to patency was 5 days in both control and immunized mice.
indicating that the protective immune mechanisms were targeting the liver phase of the parasite.

Incomplete and dose-dependent protection by sporozoite inoculation via mosquito bite during pyrimethamine prophylaxis

To assess whether inoculation of mice with sporozoites via the natural transmission route, that is, via infected mosquito bites, during pyrimethamine prophylaxis can elicit protective immunity, we inoculated animals (n=20) by bites of *P. berghei* ANKA-infected *Anopheles stephensi* mosquitoes during oral administration of pyrimethamine. The infectivity rate of the mosquitoes’ salivary glands was determined by epifluorescence microscopy. We only used mosquito batches where ≥70% of insects were infected. The protocol consisted of three consecutive exposures to 10 mosquitoes/mouse during pyrimethamine prophylaxis as previously described (32). The by-bite challenge in the absence of pyrimethamine prophylaxis was performed 49 days after the last by-bite exposure, again by exposure to 10 homologous *P. berghei* ANKA-infected mosquitoes (Fig. 2A). 5 out of 20 immunized and 2 out of 5 control mice stayed malaria-free, indicating that not all inoculated animals progressed to a blood-stage infection. Notably, the time to patency was not significantly increased in immunized vs. control mice (mean prepatent period 5.3 vs. 4.3 days, respectively; Gehan-Breslow-Wilcoxon test; *P*=0.09). All three infected animals in the control group developed symptoms of cerebral malaria, compared to two out of the five infected mice in the inoculation/prophylaxis group (Gehan-Breslow-Wilcoxon test; *P*< 0.05) (Fig. 2B).

We next tested whether three inoculation/prophylaxis immunizations with higher mosquito numbers (11-15 bites/mouse) would increase the degree of protection against reinfection. The challenge with 5-10 infected mosquitoes was carried out 40 days after the last immunization (Fig. 2A). Simultaneously increasing the immunization dose and decreasing the challenge dose resulted in substantial protection. In this experiment, 4 out of 5 animals stayed parasitemia-free whereas all controls became patent three days after exposure to infected mosquito bites and subsequently, developed cerebral malaria (Fig. 2B).
Incomplete attenuation of pyrimethamine-resistant *P. berghei* by pyrimethamine
during intrahepatocytic development delays, but does not prevent, blood stage
infection and confers limited protection against challenge

In the next set of experiments we aimed to find out whether (i) pyrimethamine retains partial
activity against liver stages from a *P. berghei* strain that has been selected for high
pyrimethamine resistance during blood stage infections (31) and (ii) the degree of
attenuation, if any, can delay or even prevent patent blood stage infections. Finally, in an
exploratory experiment we wanted to find out whether break-through blood stage infections
after sporozoite inoculation during pyrimethamine prophylaxis can alter the permissiveness
to subsequent sporozoite challenge (29, 30). Since drug-mediated clearance of *P. berghei*
infections renders C57Bl/6 mice refractory against a second blood stage infection, immunity
needs to be determined by measuring parasite load in the liver shortly after challenge. The
use of *P. berghei sera1(-)* parasites for this experiment also gave us the opportunity to
specifically test the prophylactic efficacy of our sporozoite inoculation/ pyrimethamine
prophylaxis protocol in the context of drug resistance. We used irradiated *P. berghei*
sporozoites for boosting potentially protective immune responses after a single
inoculation/prophylaxis step. Irradiated sporozoites, the gold standard for live malaria
vaccines (27), are reliably growth-arrested after hepatocyte invasion (22).

We infected mice with either 25,000 wild type (WT) sporozoites or 25,000 sporozoites
from a clonal *P. berghei* strain, which contains a chromosomally integrated *Toxoplasma
gondii* dihydrofolate reductase/thymidylate synthase resistance cassette with two amino acid
replacements (Ser$^{36}$$\rightarrow$Arg, Thr$^{83}$$\rightarrow$Asn), that, if inserted in *P. falciparum*, confers high-level
resistance of blood stage infections to pyrimethamine (15). This genetically engineered
parasite clone was previously selected in mice during blood stage infections due to
uninhibited growth during treatment with oral pyrimethamine (31). These parasites, denoted
here as *sera1(-)*, are indistinguishable from WT parasites during liver stage development. We
treated infected mice with oral pyrimethamine for 42 hours and then determined the relative
Pb18S rRNA transcript levels to assess hepatic parasite burden (Fig. 3D). We detected a significant 80% reduction of the relative parasite burden in livers of pyrimethamine-treated animals that were infected with the pyrimethamine-resistant parasites compared to WT inoculations (unpaired t-test; \(P=0.03\)).

We next studied the causal prophylactic activity of pyrimethamine against the sera1(-) clone by determining the time to blood stage patency in treated (70 µg/ml) vs. untreated mice (n=5, each) (Fig. 3A). As previously reported (32), the time to patency in untreated control animals was 3.2 days for both WT and sera1(-) parasites. In pyrimethamine-treated animals, the prepatent period of sera1(-) parasites almost doubled compared to untreated control animals (5.8 days vs. 3.3 days; two way ANOVA; \(P<0.001\)). Pyrimethamine-treated animals infected with wild type parasites remained blood stage parasite-negative (Figs. 1A, 3A). We also confirmed this finding \textit{in vitro} in cultured hepatoma cells (Fig. 3B, C). Exposure of pre-erythrocytic forms from the sera1(-) clone to concentrations of pyrimethamine of \(\geq 3.3\) µM led to growth inhibition. Together, our findings indicate that parasites selected for high-level pyrimethamine resistance during blood stage infections display partial susceptibility in the brief pre-erythrocytic phase.

To study the role of parasite resistance and/or blood stage breakthrough infections on the development of liver stage immunity, we infected animals with 10,000 sporozoites from the sera1(-) clone (n=13) or WT ANKA strain (n=5) during administration of subtherapeutic doses of pyrimethamine (three intraperitoneal injections of 0.3 to 5 mg pyrimethamine) (Fig. 4). Fourteen out of 18 mice developed blood stage parasitemia (12/13 sera(-), 2/5 WT). All animals, including the four that remained blood stage-negative, received a curative treatment regimen with chloroquine between day 14 and day 21 and shortly thereafter on day 24, a boost of hepatic stage immunity by inoculation of 10,000 irradiated WT sporozoites. On day 43, all animals were challenged by IV inoculation of 10,000 \textit{fully viable} WT sporozoites (Fig. 4). On day 45, the liver stage burden was significantly reduced in all animals that had received a sporozoite infection during pyrimethamine prophylaxis (unpaired t-test; \(P<0.001\)).
However, animals that previously developed a blood stage infection had a higher relative liver load than animals that remained blood stage-negative (unpaired t-test; $P<0.05$).
Discussion

Attenuation of *Plasmodium* liver stages or complete suppression of subsequent blood stage infections has shown that exposure of the immune system to a requisite number of intrahepatocytic parasites induces potent and long-lasting protection against challenge with sporozoites. Irradiated or genetically attenuated sporozoites that can invade but not fully mature inside hepatocytes are being developed as whole organism vaccine candidates (22,25,27). Interestingly, similar effects can be achieved by unaltered wild type sporozoites delivered naturally by mosquito bites during exposure to certain drugs (6,7,16,22,32,33). The use of pyrimethamine, a drug with causal prophylactic activity, as a component of SP in extensive IPT programs prompted us to study the tantalizing question of whether pyrimethamine-mediated attenuation of liver stages could also generate protective immune responses against re-infection. As an experimental model we choose the *P. berghei*/C57Bl/6 as an appropriate parasite-host pair in which sterile protection is difficult to achieve (21).

We found that (i) pyrimethamine blocks the intrahepatocytic replication of *Plasmodium berghei* liver stages *in vitro* and *in vivo*, (ii) pyrimethamine retains partial activity against liver stages from a *P. berghei* strain that has been selected for high pyrimethamine resistance during blood stage infections, (iii) prophylactic administration of pyrimethamine during repeated intravenous inoculation of mice with live sporozoites induces complete protection against sporozoite challenge but this effect was less pronounced when mice were ‘immunized’ naturally by infected mosquito bites, warranting further exploration in future experiments, and finally, (iv) incomplete attenuation of pyrimethamine-resistant *P. berghei* by pyrimethamine during intrahepatocytic development delayed, but did not prevent, blood stage infection and conferred only limited protection against challenge (Fig. 3 and 4).

Although not a focus of this study, the protection observed after repeated inoculations with wild type sporozoites during administration of oral pyrimethamine, appears to be akin to the immunological mechanism and degree of protection afforded by irradiated sporozoites, by certain genetically-modified sporozoites, or by primaquine/sporozoite ‘immunization’.

Using these attenuation protocols, sporozoites are able to glide and invade, but growth arrest
occurs at a relatively early stage during liver-stage development. Intrahepatic development of malaria parasites is a prerequisite for allowing the immune system to mount protective responses, since heat-killed sporozoites that cannot invade hepatocytes do not elicit robust protection against sporozoite challenge (12, 19, 37). In contrast, attenuation by pyrimethamine permits hepatocyte invasion but appears to block intrahepatocytic replication (Fig. 1 and 3). This could be explained by the inhibitory effect of the antifolate drug pyrimethamine on DNA synthesis. We do not know whether and for how long pyrimethamine-exposed liver stages may persist in this apparently growth-arrested but metabolically active state in vivo. However, this pyrimethamine-mediated attenuation is clearly different from the lethal injury of intrahepatocytic parasites by primaquine.

What could be the implications of our findings for IPT with SP or potential replacement drugs with equivalent anti-liver stage activity? More generally, are mouse model data relevant for the interpretation of IPT studies and possibly, valuable for monitoring IPT programs? Even though it may be tempting to speculate on a potential added immunoprophylactic benefit in individuals who received IPT there are a number of important gaps in our knowledge that preclude any firm conclusions. Firstly, only a single, if very visible, study demonstrated sustained protection during one year of follow-up in children who had received IPT in their first year of life (35). Subsequent studies were unable to replicate this intriguing finding and in some instances, even suggested a rebound effect (4,14).

Secondly, it is controversial whether homologous challenge in the P. berghei/ C57Bl/6 model reflects the effect of protective immune responses in human infections with P. falciparum. Finally, we do not know whether the typically low dose inoculation of approximately 100 P. falciparum sporozoites by Anopheles spp. under natural transmission conditions can provide the requisite ‘immunization’ dose that permits the immune system to mount protective responses, even in high transmission areas. The inoculation dose-dependency seen in this study definitely seems to suggest so. Perhaps, a more practical implication of our findings in a P. berghei clone that had been selected for high-level pyrimethamine resistance in the blood stage but which remained partially susceptible to pyrimethamine during liver stage
development is that it may help explain the somehow surprising protective efficacy of IPT in infants and children with SP (4, 14). It also indicates that an adjustment of the currently inappropriate SP dose (5) may entail a disproportional increase in the chemoprophylactic efficacy after each IPT dose.
Acknowledgments

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References


TABLE 1. Protection against re-infection by exposure to sporozoites under pyrimethamine causal-prophylaxis

<table>
<thead>
<tr>
<th>Drug</th>
<th>Immunization doses</th>
<th>Time to challenge</th>
<th>animals protected/a</th>
<th>Prepatency (days)</th>
<th>Time to re-challenge</th>
<th>animal protected/c</th>
<th>PP (days)</th>
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<td>(8)</td>
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<td>-</td>
<td>0 / 5 (0%)</td>
<td>3</td>
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* Pyrimethamine was administered orally with the drinking water for 42 hours at a concentration of 70 µg/ml to C57bl/6 mice.

* Immunization was done by intravenous injection of sporozoites in the numbers indicated. Boosts were given at 2-4 weeks intervals under oral pyrimethamine.

* Challenge was done by intravenous injection of 10,000 sporozoites or 5-10 infectious mosquitoes. Re-challenge was done by intravenous injection of 10,000 sporozoites.

* Animals were monitored for parasitemia for at least 14 days after challenge.

* Prepatency is defined as the time until detection of the first blood stage parasite.

* Control animals are displayed from combined experiments. For all challenges age-matched naïve control animals (at least n=2) were infected in parallel.

* Animals were challenged with 7,500 infected red blood cells and developed parasitemia the same day as the controls (day 5), indicating liver stage specific protection of the sporozoite immunization protocol.

N/A, not applicable.
Figure Legends

Figure 1. Pyrimethamine treatment of pre-erythrocytic malaria parasites prevents blood stage infection, and inhibits growth of susceptible and pyrimethamine-resistant parasites *in vitro* and *in vivo*. (A) Kaplan-Meier curve shows the time to patent blood stage (BS) infection after oral treatment with pyrimethamine for 42 hours. (B) Quantification of parasite loads by real-time PCR in infected livers at 42 hours after sporozoite inoculation under oral drug treatment with pyrimethamine. WT parasites were used for infection of C57bl/6 mice. Relative expression levels of the *Pb18S* gene were normalized to the mouse *GAPDH* gene. (C) The *in vitro* development of treated (1 µM pyrimethamine) and untreated pyrimethamine-susceptible exoerythrocytic forms (EEFs, green) are shown in low magnification (big picture) and in higher magnification (lower right corner). Scale bar, 10 µm.

Figure 2. Immunization of mice with mosquito bites under pyrimethamine cover leads to a delay in the onset of blood stage infection or protection against re-infection compared to non-immunized mice. (A) Immunizations were done by the bite of infectious *Anopheles* mosquitoes in two independent experiments. In the first experiment, three consecutive immunizations were done by exposure to 10 mosquitoes/mouse, whereas in the second experiments three immunizations with higher mosquito numbers (11-15 bites/mouse) were used. (B) The time to patency of immunized and control animals after by bite challenge is shown. (C) The percentage of immunized and control animals developing cerebral malaria is shown.

Figure 3. Mice infected with pyrimethamine-resistant sporozoites show a delay in the onset of blood stage infection after oral treatment with pyrimethamine. (A) Kaplan-Meier curve shows the time to patency of pyrimethamine-resistant and susceptible sporozoites. Animals were treated orally with pyrimethamine for 42 hours. For all experimental groups, 5 C57bl/6 mice were used. (B) *In vitro* development of pyrimethamine-resistant exoerythrocytic forms
(EEFs) under different drug concentrations. Scale bars, 10 µm. (C) The effect of pyrimethamine on the growth of pyrimethamine-resistant and susceptible parasites is illustrated by comparing EEF size to the host cell nucleus. 50 EEFs were counted for each drug concentration. nd, none determined. (D) Quantification of parasite loads by real-time PCR in infected livers at 42 hours after inoculation of pyrimethamine-resistant parasites with or without oral pyrimethamine prophylaxis. Relative expression levels of the *Pb18S* gene were normalized to the mouse *GAPDH* gene.

**Figure 4.** Break-through blood stage infections curtail pre-erythrocytic immunity. (A) Animals (*N*=18) were infected with pyrimethamine-resistant (*N*=13) and -sensitive (*N*=5) sporozoites under suboptimal pyrimethamine cover. All animals were monitored for parasitemia and cured with 1.6 mg/day chloroquine for 7 days, starting at day 14. 14 animals developed parasitemia between day 3 and 10 after infection, and 4 stayed malaria-free. All animals and untreated control animals (*N*=4) were immunized with 10,000 irradiated sporozoites at day 24. Finally, all mice were challenged with 10,000 WT sporozoites at day 43. Mice were sacrificed 42 hours after the WT challenge and livers were removed for RNA extraction and cDNA synthesis. (B) Shown are quantitative RT-PCR data grouped into control mice (black circles; *N*=4), blood stage-negative Pyr/spz. mice (green circles; *N*=4), and blood stage-positive Pyr/spz. mice (red circles; *N*=14). Relative expression levels of the *Pb18S* gene were normalized to the mouse *GAPDH* gene. * P<0.05: *** P<0.001.