INTRODUCTION

Malaria remains by far the most prevalent and fatal vector-borne infectious disease. The World Health Organization estimates that malaria causes 1 million deaths annually, mostly in young children in African countries (1). Globally, more than 3 billion people are at risk of infection (1). Recent regional successes in reducing the burden of malaria (2, 3) have prompted an ambitious revision of research priorities toward elimination of this ancient human disease (4). One major challenge will be the establishment of safe, affordable, and sustainable population-based interventions that (i) reliably protect against infections, (ii) prevent transmission, and (iii) induce long-term protective immune responses against reinfections.

Experimental vaccination with live-attenuated mosquito-transmitted Plasmodium parasite stages, termed sporozoites, can induce long-lasting and robust immunity (5). To date, immunization with radiation-attenuated Plasmodium sporozoites has been the gold standard for eliciting long-lived and potent protection against reinfection (6). Radiation-attenuated sporozoites retain their capacity to infect hepatocytes but developmental arrest occurs before the parasite replication phase, which normally results in the production of thousands of pathogenic blood-stage parasites, termed merozoites (7). Interest in whole-organism vaccines has been stimulated recently by studies with genetically and chemically attenuated parasites (8, 9). Although these proof-of-concept protocols offer enormous potential for the development of live-attenuated vaccines, major hurdles in production and delivery of live-attenuated parasites to populations at risk impede their translation into licensed products in the foreseeable future (10, 11). Moreover, the early liver-stage arrest of the attenuated sporozoite results in only one arrested parasite inside the host liver for every attenuated sporozoite inoculated. Therefore, high immunization doses, that is, natural sporozoite inoculation by >1000 irradiated mosquitoes, are needed to elicit sterile immunity in humans (12).

In contrast, infection treatment vaccination by three low-dose immunizations with 12 to 15 infected mosquitoes under continuous chloroquine treatment of emerging asexual blood-stage parasites is sufficient for sterile protection (13). This experimental strategy indicated that net expansion of the whole organism contained in the vaccine elicits stronger protective immune responses. Because of the ubiquitous prevalence of chloroquine-resistant parasite populations, however, this approach is limited to experimental situations. In an effort to translate these pharmacological parasite attenuation protocols into clinical interventions in malaria-endemic countries, we tested whether specific drug-induced attenuation of late liver-stage development has the dual capacity to prevent emergence of pathogenic merozoites while simultaneously building up protection against future reinfections.

We focused on inhibitors that target the apicoplast, a relict plastid-like organelle of parasites of the phylum Apicomplexa. On the basis of recent reverse genetics data, this organelle has been suggested as an attractive drug target in late liver-stage parasites (14). Biogenesis and inheritance of the apicoplast during the repeated cycles of host cell invasion and parasite replication can be inhibited by antibiotics, presumably by interfering with the prokaryotic-type protein translation machinery inside this organelle (15). With few notable exceptions (16), these antibiotics induce a distinctive “delayed-death” phenotype in apicomplexan parasites (17–19). This is characterized by the lack of an immediate effect of antibiotics on parasite survival during the first asexual replication cycle after start of drug exposure. Parasite growth, however, is arrested during the subsequent intracellular replication cycle, although drug may no longer be present.

We hypothesized that antibiotic administration during exposure to malaria transmission stages may generate robust protection against reinfection because the delayed death of liver-stage parasites during the exponential intrahepatocytic parasite expansion phase would maximize antigen diversity and parasite loads. We focused on two antibiotic
drugs as model compounds, namely, clindamycin and azithromycin, because of their proven anti-
*Plasmodium* activities (18, 20, 21). Moreover, both compounds have an excellent safety record in the most vulnerable populations at risk of malaria—young children and pregnant women (22, 23). In addition, azithromycin is slowly eliminated from the body and accumulates in liver tissue (20, 24), the site of the first and obligate life cycle stage of *Plasmodium* in its mammalian host.

**RESULTS**

**Antibiotics prevent *Plasmodium* blood infection without affecting liver-stage load**

We first established the in vivo regimen of clindamycin and azithromycin that prevents blood-stage infection after a high-dose infection with 25,000 sporozoites (Fig. 1A). Daily treatment of mice with clindamycin (250 mg/kg) or azithromycin (160 mg/kg) for 3 days during the clinically silent liver-stage development of the rodent malaria parasite *Plasmodium berghei* completely prevented patent blood-stage infections. To determine when azithromycin and clindamycin abort intrahepatic parasite replication, we compared the parasite loads 42 hours after sporozoite infection in livers isolated from untreated control mice and from mice under antibiotic cover (Fig. 1B). Liver parasite loads, as measured by parasite-specific 18S ribosomal RNA (rRNA), were comparable in all experimental groups, indicating that parasite inhibition occurred later in the parasite life cycle, during final maturation of liver stages. These in vivo findings differ fundamentally from those seen with irradiated or genetically attenuated liver-stage parasites in which arrest occurs before replication inside the host liver cells (5, 25). Therefore, antibiotic cover permits intrahepatic maturation of the malaria parasite, yet is fully effective in preventing pathogenic blood-stage infection in vivo.

**In vitro exposure to antibiotics does not prevent maturation of *P. berghei* liver-stage merozoites**

To expand our in vivo findings, we analyzed the effects of clindamycin and azithromycin on *P. berghei* liver-stage development in cultured hepato ma cells (Fig. 2). In agreement with in vivo–determined parasite loads, quantification of immature and mature liver-stage parasites 24 and 65 hours after infection revealed no differences compared to control (Fig. 2, A and B). *P. berghei* maturation in vitro occurred independent of antibiotic exposure, as shown by similar numbers of early and late liver-stage parasites (Fig. 2B). The morphology of clindamycin- and azithromycin-treated late liver-stage cells was indistinguishable.

![Fig. 2. In vitro exposure to antibiotics does not prevent liver-stage merozoite genesis. (A) Representative low-magnification images of infected hepato ma cells visualized with an antibody to PbHSP70 65 hours after infection. (B) Quantification of immature (24 hours) and mature (65 hours) liver stages in control (Ctrl), azithromycin (AZ)–treated, and clindamycin (CM)–treated infected hepatoma cells reveals indistinguishable parasite numbers (P > 0.4). (C and D) Representative immunofluorescence stainings of *P. berghei* liver-stage schizonts (C) and merozoites (D) demonstrating successful maturation. Hepatoma cells were infected with sporozoites and incubated for 48 and 72 hours, respectively, with 0.01% DMSO (control), 10 μM primaquine, 1 μM clindamycin, or 1 μM azithromycin. Intracellular liver-stage parasites were stained with an antibody to *PbHSP70*. Merosomes were visualized through endogenous GFP expression. Host nuclei are visualized with Hoechst 33342. Scale bars, 10 μm.](https://www.science translationalmedicine.org/content/2/40/40ra49)

**Fig. 1.** Antibiotic treatment of pre-erythrocyclic malaria parasites prevents blood-stage infections but does not inhibit liver-stage maturation in vivo. (A) Kaplan-Meier curves illustrate the time to patent blood-stage infections on three consecutive prophylactic doses of azithromycin (160 mg/kg) and clindamycin (250 mg/kg). (B) Quantification of parasite loads by real-time PCR in infected livers at 42 hours after sporozoite inoculation. Relative expression levels of the *Pb18S* gene were normalized to the mouse *GAPDH* gene (P > 0.4, Kruskal-Wallis rank test).

![Fig. 1.](https://www.science translationalmedicine.org/content/2/40/40ra49)
from that of nontreated controls (Fig. 2C). This lack of inhibition of maturation differs from treatment with the prophylactic drug primaquine, which aborts liver-stage development before the onset of nuclear divisions (26) (Fig. 2C). Final parasite maturation, as characterized by detachment of merozoite-containing infected liver cells, termed merosomes (27), occurred in treated and control cells (Fig. 2D). The viability of emerging merosomes was confirmed by expression profiling of signature genes expressed in liver-stage merozoites (Fig. 3A). Relative transcript abundance ranged from 20% (EXP1) to >90% (GAPDH) in treated versus untreated cell cultures, indicating metabolically active parasites.

**Merosomes emerging from antibiotic-treated cultures fail to establish blood-stage infections**

We next wanted to determine the ability of in vitro-generated merosomes to establish blood-stage infections in naïve recipient mice. We harvested culture supernatants from antibiotic-treated and untreated infected hepatoma cells and injected different numbers of viable merosomes into susceptible NMRI mice (Fig. 3, A and B). As expected, animals that received merosomes from nontreated hepatoma cells developed blood-stage infections and, as a consequence, malaria symptoms. The time to patency, defined as detection of blood-stage parasites in Giemsa-stained thick blood smears by light microscopy, was negatively correlated with the number of injected merosomes (4 and 6 days after injection of 500 and 100 merosomes, respectively) (Fig. 3B). In support of the fact that we had induced complete in vivo attenuation (Fig. 1A), mice that received identical numbers of merosomes from clindamycin- or azithromycin-treated cultures remained free of blood-stage infection (Fig. 3B).

**Azithromycin and clindamycin inhibit biogenesis and inheritance of the apicoplast**

To test whether clindamycin and azithromycin exert their delayed action by directly inhibiting apicoplast maturation in exposed liver-stage parasites, we generated antisera against a signature apicoplast protein, acyl carrier protein (ACP) (28). When we studied intrahepatocytic parasites 65 hours after sporozoite invasion, we observed markedly less apicoplast staining in treated than in control parasites (Fig. 3C). In the absence of drugs, apicoplasts in late liver-stage parasites typically consisted of complex branched subcellular structures, whereas in both clindamycin- and azithromycin-treated cultures, division of the apicoplast was apparently halted (Fig. 3C). To confirm the drug-induced defect in apicoplast segregation at the molecular level, we isolated genomic DNA (gDNA) from infected animals and cultured merosomes and compared the amount of two independent apicoplast markers: the nuclear-encoded ACP and the apicoplast-encoded EFTu gene (Fig. 3D). As expected, azithromycin-treated and untreated parasites had comparable numbers of the nuclear-encoded ACP gene (Fig. 3D). In marked contrast to this result but consistent with an apicoplast segregation defect, the numbers of the apicoplast-encoded EFTu gene were drastically reduced in the azithromycin-treated parasites (Fig. 3D). Collectively, these results demonstrate that antibiotic coverage during the pre-erythrocytic parasite expansion phase abolishes apicoplast biogenesis and inheritance but does not prevent intrahepatocytic parasite maturation. Antibiotic-induced liver-stage attenuation results in high numbers of arrested late-stage parasites per single-sporozoite invasion event in vivo and in vitro.

**Fig. 3.** Merosomes emerging from azithromycin- and clindamycin-treated cultures are noninfectious. (A) Analysis of relative RNA abundance in merosomes emerging from azithromycin-treated *P. berghei* liver-stage cultures as a marker of viability. Data are expressed as percentage of RNA abundance in untreated controls. Azithromycin-treated and untreated merosomes (70 hours after sporozoite infection of hepatocytes) were used to isolate total RNA and synthesize cDNA. The profile of signature liver-stage genes shows consistent, albeit reduced, RNA levels in azithromycin-treated merosomes. RNA abundance was normalized to GFP, expressed as a transgene under the *P. berghei* elongation factor 1α promoter. *AMA1*, apical membrane antigen 1; *EXP1*, exported protein 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSP70*, heat shock protein 70; *MS1*, merozoite surface protein 1; *18S*, ribosomal 18S rRNA. (B) Kaplan-Meier curves of merosome-infected mice. Animals were infected intravenously with merosomes harvested from untreated (control) and azithromycin- and clindamycin-treated infected hepatoma cells at the numbers indicated (three mice per group). (C) Inhibition of apicoplast maturation during liver-stage development. Shown are immunofluorescence images of 65-hour liver stages stained with antibody to *PbACP* (red) and to *PbHSP70* (green). Nuclei are stained with Hoechst 33342 (blue). Note the highly branched structure of mature apicoplasts in control parasites stained with an antibody to *PbACP* in contrast to azithromycin- and clindamycin-treated parasites. Scale bars, 10 μm. (D) Comparative analysis of *P. berghei* gDNA copy numbers of the nuclear-encoded, apicoplast-targeted ACP gene versus the apicoplast-encoded, apicoplast-localized elongation factor Tu (EFTu) demonstrates a significant reduction of EFTu copies in livers of azithromycin-treated animals and in vitro–cultured merosomes (*P* < 0.001, Kruskal-Wallis rank test). gDNA copy numbers were normalized against GFP copy numbers in GFP-transgenic *P. berghei* parasites (clone 507) (46).
Exposure to live sporozoites under antibiotic cover induces protection against a lethal sporozoite challenge

Table 1. Protection against reinfection induced by exposure to sporozoites under antibiotic causal prophylaxis. NA, not applicable.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Immunization dose(s)*</th>
<th>Time to high-dose challenge (days) †</th>
<th>n protected against blood infection/n at risk (%)</th>
<th>Time to detection of blood infection (days)</th>
<th>n protected against cerebral malaria/n at risk (%)</th>
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<tr>
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<td>40</td>
<td>5/5† (100)</td>
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<td>NA</td>
</tr>
<tr>
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<td>10,000 × 2</td>
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<td>5/5 (100)</td>
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<td>NA</td>
</tr>
<tr>
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<td>7</td>
<td>1/1</td>
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<tr>
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<td>58</td>
<td>5/10 (50)</td>
<td>7</td>
<td>5/5 (100)</td>
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<td>Clindamycin</td>
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<td>34</td>
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<td>5</td>
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<tr>
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<td>—</td>
<td>0/15 (0)</td>
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<td>—</td>
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<td>30</td>
<td>0/5 (0)</td>
<td>3–4</td>
<td>0/5 (0)</td>
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<tr>
<td>Clindamycin</td>
<td>—</td>
<td>30</td>
<td>0/5 (0)</td>
<td>3–4</td>
<td>0/5 (0)</td>
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</table>

*Immunizations were done by intravenous injection of salivary gland sporozoites at the numbers indicated. †High-dose challenge was done by intravenous injection of 10,000 freshly isolated salivary gland sporozoites, except for by-bite experiment (four to seven mosquitoes). ‡These animals were rechallenged 6 months later by intravenous injection of 10,000 sporozoites and remained sterile protected. §These animals were rechallenged 4 months later by intravenous injection of 10,000 sporozoites and remained sterile protected.
blood parasites via the intravenous route (Fig. 4A). All mice were fully susceptible to blood-stage inoculations, with no difference in time to patency as compared to age-matched control animals. This finding shows that sterilizing immunity induced by our infection and cure immunization protocol acts primarily against pre-erythrocytic stages.

Because of the known importance of CD8+ T cells in mediating immunity against the parasite liver stage (29), we asked whether depletion of CD8+ T cells would reverse protection. When immunized animals were treated with antibodies to CD8 before rechallenge, protection was completely abolished (Fig. 4B). In contrast, a majority of immunized B cell–deficient mice enjoyed sterile protection against a high-dose sporozoite challenge (Fig. 4C). These findings suggest that CD8+ T cells are the major immune effector cell population mediating protection, presumably by targeting infected hepatocytes.

To find out whether protection can be reversed by abrogating cytokine effector functions, we used mice that were previously immunized with 10,000 sporozoites under azithromycin cover. Forty-four hours after challenge, the parasite burden in the livers was quantified by real-time reverse transcription polymerase chain reaction (RT-PCR) (Fig. 4D). In these challenge experiments, protection against reinfection correlated with a large reduction of parasite load, irrespective of the immunization strategy, that is, immunizations with irradiated sporozoites or live sporozoite exposure during antibiotic administration (Fig. 4D). Neutralization of interferon-γ (IFN-γ), but not high doses of the tumor necrosis factor receptor (TNFR)–Fc fusion protein etanercept (anti–TNF-α), abrogated protection as indicated by high liver-stage burdens in animals treated with an antibody to IFN-γ. These results suggest a major role of IFN-γ–dependent CD8+ T cell responses in antiparasite defense.

**DISCUSSION**

Our results demonstrate that prophylactic administration of safe and affordable antibiotic drugs during exposure to intravenously and naturally transmitted live sporozoites can induce robust and long-lived immune responses against subsequent challenge with lethal sporozoite doses. We used the stringent *P. berghei/C57BL/6* mouse model to obtain conservative estimates of the anticipated degree of protection in humans (30). We describe the antibiotic-induced abrogation of *Plasmodium* stage conversion from the obligate but clinically silent liver phase to pathogenic blood-stage infections. This life cycle arrest correlated with the induction of CD8+ T cell–dependent protective immune responses against liver-stage, but not blood-stage, parasites. We show that this strategy is distinct from previous work on immunization, with live sporozoites under continuous suppressive treatment with the blood-stage–specific drug chloroquine (13, 31, 32). These studies with chloroquine have shown the potential of natural immunization under drug treatment, including in a human challenge trial (13), but the global prevalence of chloroquine-resistant parasites precludes the translation of chloroquine monotherapy–based protocols in malaria-endemic countries (33). Resistance of *Plasmodium* to antibiotics can be induced experimentally in vitro (15), but so far, there have been no reports of antibiotic-resistant *Plasmodium* infections. The short duration of antibiotic prophylaxis required for preventing blood-stage infection after an infective mosquito bite (34) may also facilitate the design of human intervention trials, especially in areas with highly seasonal, brief peaks of intense malaria transmission (35).

The short half-life of clindamycin (36) allowed us to unambiguously assess the protection afforded by our immunization protocol without potential interference from residual plasma drug concentrations. However, the short action and remaining concerns about clindamycin-associated colitis (22) preclude its wide use for prophylaxis, for instance, in mass drug administration. Azithromycin can be administered safely in infants and children and has already been used for mass treatment in trachoma control programs (37). A previously evaluated low-dose regimen of azithromycin was only partially protective in preventing blood-stage infections in individuals on chemoprophylaxis (34). Increased doses (38) and appropriate combination partner drugs for azithromycin (23) will need to be assessed in future clinical trials. Azithromycin-chloroquine, currently developed for intermittent preventive treatment in pregnancy (IPTpreg) (39), is a prime candidate for first proof-of-principle trials evaluating the safety and efficacy of periodic prophylaxis protocols during malaria transmission peaks.
There is considerable debate on whether frequent, but typically low-dose, sporozoite transmission to individuals taking continuous suppressive treatment can induce protective immune responses. Although the available literature has grown (40–43), the findings remain inconclusive and contradictory, not least because trial designs did not address bona fide immunoprophylactic or severe disease endpoints. Of relevance, none of these studies tested antibiotic drugs. The unique mechanism of action of antibiotic drugs permits exponential expansion of attenuated, but fully matured, liver-stage merozoites from single sporozoites. Our findings from natural transmission experiments showed that this strategy elicited a high degree of protection. Eighty-five percent of animals immunized by two rounds of exposure to infected mosquito bites under antibiotic cover remained protected against murine cerebral malaria, a key clinical development objective for first- or second-generation malaria vaccines (44).

In conclusion, we report that prophylactic use of antibiotic drugs induces exceptionally robust protection in an established rodent malaria model by blocking parasite life cycle progression. This approach may prove essential for preventing Plasmodium immune escape mechanisms that otherwise maintain susceptibility to lifelong reinfections. Our findings also point to the Plasmodium apicoplast as a previously unrecognized target for immunoprophylactic interventions and for identifying and testing suitable antibiotic drug candidates. If confirmed by comparative clinical trials in residents of malaria-endemic areas, particularly young children in high-transmission settings, induction of protective immune responses by natural infection under antibiotic coverage may offer a powerful shortcut toward a needle-free, whole-organism vaccination strategy.

MATERIALS AND METHODS

Experimental animals and parasites
Female NMRI, C57BL/6, and B cell–deficient Igh-6tm1Cgnf/ (μMT) (45) mice were from Charles River Laboratories. All animal work was conducted in accordance with European regulations and approved by the state authorities (Regierungspräsidium Karlsruhe). P. berghei ANKA sporozoites [clone 507, constitutively expressing green fluorescent protein (GFP)] (46) were isolated by dissection of salivary glands from infected female Anopheles stephensi mosquitoes.

In vitro drug susceptibility assay of P. berghei liver stages
To study in vitro activities of azithromycin (1 μM; Pfizer), clindamycin hydrochloride (1 μM; Sigma), and primaquine diphosphate (10 μM; MP Biomedicals) against Plasmodium hepatic stages, we seeded HuH7 cells in Lab-TekII slides (Nalge Nunc International), infected them with 10,000 P. berghei sporozoites in triplicate wells, and further incubated them in the presence or absence [control: 0.01% dimethyl sulfoxide (DMSO)] of drugs. Infected cells were fixed after 24 to 65 hours with cold methanol. Parasites were visualized with a monoclonal antibody to P. berghei 18SrRNA (GenBank identifier (GI), 28119965) (forward: 5′-AGAATAGATGCTGCATATATGG-3′; reverse: 5′-TGGATGCGACCATATCTCC-3′). Similarly, for profiling of apicoplast-specific genes, gDNA was isolated from infected livers and merozoites were harvested from infected hepatoma cells. The following gene-specific primers were used for the nuclear-encoded ACP gene (GI, 68067120) (forward: 5′-TCAAAATTGATTGATTCATCTCCAGAAGA-3′; reverse: 5′-GATATTGATTCATCTCCAGAAGA-3′); and the plastid-encoded EFTu gene (GI, 89512164) (forward: 5′-CGTCCCGTAGACAAAATGGT-3′; reverse: 5′-TTAGCTGTATTTTATCTTCT-3′), respectively. Real-time PCR was performed in triplicate, with 1 cycle of 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 60°C for 45 s. Relative copy numbers were determined with the ΔΔCt method.

Analysis of hepatic merozoite infectivity
To determine the infectivity of liver-stage merozoites, we infected HuH7 cells with 50,000 P. berghei ANKA sporozoites and further incubated them in the presence of clindamycin and azithromycin at the concentrations indicated above. Merozoites (merozoite-containing detached vesicles) were harvested from the cell culture supernatant 68 to 72 hours after infection, washed, and resuspended in drug-free medium. NMRI mice were injected with increasing numbers of treated and untreated merozoites.

Peptide synthesis and generation of an antibody to P. berghei ACP
Two peptides from the central portion (ASRRNEKTNFRNSDSFMEKK) and the C-terminal region (KINTVEDAINFIEKKNKPD) of P. berghei ACP (PbACP) were synthesized and used for immunization of rabbits (Eurogentec) (47). Antisera to PbACP were used at a 1:750 dilution in immunofluorescence assays to detect the apicoplast during liver-stage development.

Immunization of C57BL/6 mice with P. berghei sporozoites under antibiotic prophylaxis
For immunization protocols, C57BL/6 mice were intravenously injected with 1000 to 25,000 P. berghei ANKA sporozoites, as indicated in Table 1. In each experiment, untreated control mice were injected with equivalent numbers of sporozoites to confirm the infectivity of sporozoites used for immunization. At least >30 days after the last boost, immunized animals were challenged by intravenous inoculation of 10,000 P. berghei sporozoites. Mice were then examined for 28 days by daily Giemsa-stained blood films to detect the appearance of a patent parasitemia.

Quantification of parasite liver loads by real-time PCR
For quantification of the parasite loads in the liver by real-time RT-PCR, C57BL/6 mice were infected intravenously with 50,000 sporozoites, followed by intraperitoneal injection of clindamycin (250 mg/kg) or azithromycin (160 mg/kg) 1 and 24 hours later. Mice were killed 42 hours after infection, and livers were removed and homogenized. Total RNA was isolated with the RNaseasy kit (Qiagen), and complementary DNA (cDNA) was synthesized with the RETROscript kit (Ambion), according to the manufacturer’s instructions. Real-time PCR was performed with the ABI 7500 sequence detection system and Power SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer’s instructions, using gene-specific primers for the PbHSP70, followed by a goat Alexa Fluor 488–labeled antibody to mouse immunoglobulin G (IgG) and Hoechst 33342 to stain the nuclei.

Relative gene and transcript abundance in Plasmodium merozoites
The following primer pairs were used to compare transcript abundance in Plasmodium merozoites: PbGAPDH (GI, 68067510), 5′-AATTAAATGTTGCTGATTAGCCACGACATATCC-3′; and the plastid-encoded EFTu gene (GI, 89512164) (forward: 5′-CTGGATGCGACCATATCTCCAGA-3′; reverse: 5′-CTGGATGCGACCATATCTCCAGA-3′).
Neutralization of liver-stage immunity by antibodies

Immunized C57BL/6 mice received different antibodies for neutralization of possible mediators of anti-liver-stage immunity before challenge with 10,000 sporozoites. IFN-γ neutralization was performed by intraperitoneal injection of an antibody to IFN-γ (clone XMGI12, BioXcell) at days −1, 0, and +1 (dose per animal: 1.0, 0.75, and 0.75 mg, respectively) relative to the day of challenge. The TNFR-Fc fusion protein etanercept (Enbrel, Wyeth) was used at the same days as XMGI1.2 at a dose of 1 mg per animal. Antibodies to CD8+ were given to protected animals from days 3 to 1 as described before (48). Blood smear–negative animals received a fourth dose of the antibody on day 4. Protected control animals received antibody to IgG on the same days.

Confocal microscopy and software usage

Confocal pictures were obtained with an LSM510 confocal system (Zeiss). Confocal pictures were obtained with an LSM510 confocal system (Zeiss) equipped with visible and ultraviolet laser lines, and processed with Photoshop software (Adobe Inc.). Graphs were constructed with Prism software (version 5.0b, GraphPad Inc.). Statistical analyses were performed in STATA 10.2 (STATA Corp.). A P value threshold of 5% was used to indicate statistical significance.

REFERENCES AND NOTES


47. Abbreviations for the amino acids are as follows: A, Ala; D, Asp; E, Glu; F, Phe; I, Ile; K, Lys; M, Met; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; and V, Val.


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Competing interests: The authors declare that they have no competing interests.