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Vaccination with Live *Plasmodium yoelii* Blood Stage Parasites under Chloroquine Cover Induces Cross-Stage Immunity against Malaria Liver Stage

Elodie Belnoue,*† Tatiana Voza,‡§ Fabio T. M. Costa,*†** Anne Charlotte Grüner,*†,†† Marjorie Mauduit,*† Daniela Santoro Rosa,*† Nadya Depinay,*† Michèle Kayibanda,*† Ana Margarida Vigário,*† Dominique Mazier,§†† Georges Snounou,‡§† Photini Sinnis,* and Laurent Rénia2*†,††

Immunity to malaria has long been thought to be stage-specific. In this study we show that immunization of BALB/c mice with live erythrocytes infected with nonlethal strains of *Plasmodium yoelii* under curative chloroquine cover conferred protection not only against challenge by blood stage parasites but also against sporozoite challenge. This cross-stage protection was dose-dependent and long lasting. CD4+ and CD8+ T cells inhibited malaria liver but not blood stage. Their effect was mediated partially by IFN-γ, and was completely dependent of NO. Abs against both pre-erythrocytic and blood parasites were elicited and were essential for protection against blood stage and liver stage parasites. Our results suggest that Ags shared by liver and blood stage parasites can be the foundation for a malaria vaccine that would provide effective protection against both pre-erythrocytic and erythrocytic asexual parasites found in the mammalian host. The Journal of Immunology, 2008, 181: 8552–8558.

Malaria infection is initiated by the inoculation of sporozoites by an infected anopheline female mosquito. Sporozoites are injected into the dermis of the mammalian host where the majority takes several hours before entering the bloodstream (1). From the blood circulation, sporozoites are arrested in the liver where they invade hepatocytes and develop into liver stage parasites. These two parasite forms constitute the pre-erythrocytic stages. The merozoites released from the mature hepatic schizont into the circulation invade erythrocytes and initiate the cyclical asexual erythrocyte cycle responsible for the pathological manifestations of the infection.

The experimental induction of sterile protection has been primarily achieved when whole parasite preparations were used as Ags (2). Immunization with whole parasites has been principally conducted with live sporozoites classically attenuated by irradiation (3, 4) and more recently through genetic modification (5–7). In both cases hepatocytes are invaded by the attenuated sporozoites but none develop to maturity. The sterile protection induced is solely due to the destruction of the pre-erythrocytic stages of the challenge parasites because immunized individuals remain fully susceptible to challenge with blood stage parasites (3–7). These observations, which have been consistently obtained in rodents and humans, helped to establish the stage specificity of acquired immunity as a rule for malaria (8). However, demonstration that the immunity acquired against erythrocytic parasites has no influence on the pre-erythrocytic stages is as yet unavailable. First, until recently it was inherently difficult to demonstrate formally that hosts successfully immunized specifically against erythrocytic stages do not thereby acquire some protection against pre-erythrocytic parasites. Only a few observations were made in this context and these were consistent with stage-specific immunity (9, 10). Second, it had been generally assumed, though not necessarily demonstrated, that the Ags that induce protective immune responses against one parasite stage would not be expressed in the other.

Recent omic analyses of the proteomes and transcriptomes of the distinct parasite stages observed during the life cycle, have revealed that many *Plasmodium* proteins are expressed during different stages of the life cycle (11–18), and pertinently that in many cases these included Ags expressed by both pre-erythrocytic and erythrocytic parasites (19–26). This response has raised the possibility that some of these proteins could become the target of parasite-induced immune responses that would then lead to some degree of cross-stage immunity in the mammalian host (24, 25). In the present study, we have investigated the impact of an immunization protocol based on
Materials and Methods

Mice and parasites

BALB/cJ female mice were purchased from Harlan Laboratories. Breeding pairs of Ab-deficient homozygous BALB/CJ Ht mice, originally from Tac- tonic Farms, were given by V. Nussenzweig (New York University, New York, NY). All experiments and procedures conformed to the French Min-
istry of Agriculture 1987 regulations for animal experimentation. Plasmo-
dium yoelii yoelii sporozoites of the uncloned line of the 265BY nonlethal strain (Py265), and of clone 1.1. 17XNL nonlethal strain expressing con-
stitutively GFP (Py17X) (18), were obtained by dissection of the salivary glands of infected Anopheles stephensi mosquitoes 16–21 days after an infected blood meal. Infected RBC (iRBC) were obtained from mice in-
lected 8–12 days before with P. yoelii strains 265BY, 17XNL clone 1.1, or P. berghii ANKA (clone Bds) blood stage parasites. Blood samples were

whole erythrocytic parasites on the pre-erythrocytic stages re-
sulting from a sporozoite challenge.

Immunization schedule and chloroquine treatment

Mice were i.v. injected once or twice with different amounts of P. yoelii 265BY live iRBC (Py-iRBC) or a matching number of normal RBC. Mice were also i.p. injected with 100 µl of 8 mg/ml chloroquine (Sigma-Aldrich) diluted in saline, daily for 10 days starting the day of iRBC injection. In one experiment, chloroquine treatment was extended to 15 days. At the end of the chloroquine treatment, absence of parasites on Giemsa stained blood smears or by cytofluorometry was confirmed in all treated mice.

Challenge and protection assessment

Mice immunized with iRBC and treated with chloroquine were rested for a minimum of 15 days after the last chloroquine injection to allow complete elimination of the drug. The mice were then challenged i.v. with 100 P. yoelii 17XN clone 1.1 sporozoites (highly infectious), 4000 P. yoelii 265BY sporozoites, or 105 or 106 Py-IRBC. Blood stage infection was determined by the presence of parasites on Giemsa-stained blood smears or by cytofluorometry on days 4–10 postchallenge. Quantification of parasite load over time was performed starting the day of iRBC injection. In one experiment, chloroquine treatment was extended to 15 days. At the end of the chloroquine treatment, absence of parasites on Giemsa stained blood smears or by cytofluorometry was confirmed in all treated mice.

Ab assays

Blood from immunized mice was collected the day before sporozoite or iRBC challenge by retro-orbital puncture. Ab titers in individual mouse serum or pooled sera were determined by an indirect immunofluores-
cence assay, as previously described (31) on methanol-fixed slides con-
taining immobilized sporozoites, cultured liver stage parasites or air-dried iRBC. Titers were expressed as the reciprocal of the highest positive serum dilution and presented as the mean ± SD for sporozoites and blood stages parasites. Inhibition of sporozoite and liver stage development assay was performed as previously described (32). Briefly, sera (1/10 dilution) from P. yoelii iRBC-immunized mice were added to hepatocyte cultures at the time of sporozoite inoculation and removed 3 h later. Sera from control mice immunized with adjuvant alone were used as control. Schizont numbers were assessed in triplicate cultures by immunofluorescence assay using hyperimmune sera recognizing P. yoelii liver stages as previously de-
scribed (31). The percentage of inhibition was calculated by comparing the number of parasites in the experimental cultures with the number of para-
sites in control wells.

In vivo Ab treatment

Purified control rat Abs were purchased from Sigma-Aldrich. Rat IgG2a anti-CD4 mAb (clone GK1.5; American Type Culture Collection (ATCC) TIB 207), and rat IgG2b anti-CD8 mAb (clone 2.43; ATCC TIB 210) were used. The rat IgG2a anti-IL-12 mAb (clone C17.8.20) cell line was pro-
vided by Dr. G. Trinchieri (National Cancer Institute, Frederick, MD) (33). The rat IgG1 anti-IL-6 mAb (clone MP5-20F3) and the rat IgG1 anti-IFN-γ (clone XMG-1) (34) cell lines were provided by Dr. P. Minoprio (Institut Pasteur, Paris, France). Abs were purified from culture supernatant by am-
omium sulfate precipitation. Immunized mice were injected i.p. with 500 µg of anti-CD9 or anti-CD4 mAb or control rat IgG on days −1 or 0 before challenge. The >98% of blood CD8+ or CD4+ T cells were depleted by this procedure as verified by cytofluorometry (FACScan; BD Biosciences) using anti-CD4 (clone H129-19; Sigma-Aldrich) and anti-CD8 (clone 53-6.7; BD Pharmingen) mAbs that recognized epitopes different from those recognized by the depleting mAbs. IFN-γ neutralization on days −2, −1, 0, +1, and +2 after challenge, mice received a single i.p. dose of 2 mg of the anti-IFN-γ mAb XMG-1. IL-12 neutralization was achieved by i.p. administration of 1 mg of the anti-IL-12 mAb 12 h before and 3 h after challenge. Efficacy of the batches of Abs used in this study was verified previously (35). Because rat IgG1 and IgG2a have a maximal half-life of around 2 and 4 days (36), respectively, the treatment would only effectively neutralize IL-12, IL-6, or IFN-γ at a time when liver stage parasites are present in the animals. Inhibition of NO production was achieved by i.p. injection of a single dose of 100 µg of S-methylisothiourea (SMT; Sigma-
Aldrich) in 0.1 ml of PBS, a potent inhibitor of inducible NOS (37), on days 0, +1, and +2 after sporozoites challenge.

IFN-γ ELISA

The 96-well plates (Nunc) coated with anti-IFN-γ (clone R4-6A2; BD Pharmingen) mAb were incubated overnight at 4°C, then the sera were added and the plates incubated overnight at 4°C. Detection was made after adding of second biotinylated Ab (clone XMG1.2; BD Pharmingen) followed by incubation for 45 min at room temperature. Extravidin-phos-
tase alkaline was then added and plates were incubated for 1 h at room temperature. The phosphatase activity was measured by adding 4-methylumbelliferyl phosphate (Sigma-Aldrich) as substrate, and read-
ing fluorescence at 360/460 nm using a spectrophotometer. Dilutions of known quantity of IFN-γ were used for the standard curve. The detec-
tion limit was 30 pg/ml.

Results

Natural infection induces sterile immunity against blood but not pre-erythrocytic stages

BALB/c mice infected with P. yoelii 265BY sporozoite developed a patent blood infection 3–4 days postinoculation. They controlled the infection and eliminated blood parasites by day 20 (data not shown). Mice were rested for 15 days and then challenged with homologous sporozoites. All the mice that had self-cured after the initial infection were completely protected, whereas naïve control mice were not (Fig. 1A). When parasite loads were assessed, using quantitative PCR targeting the parasite’s small subunit ribosomal RNA genes (29), in the livers of mice previously infected with

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sporozoites and then challenged with homologous sporozoites after self-cure, the development of hepatic parasites was not significantly different from that of control mice (Fig. 1B). This demonstrated that normal sporozoite-induced infection in mice conferred protection against blood but not against liver stage parasites.

**Immunization with live iRBC under chloroquine cover induces sterile immunity**

It has been shown that that patent blood infection could have a suppressive effect on the immunity directed against pre-erythrocytic parasites (38, 39), in which case the development of cross-stage immunity would be hampered. To ascertain whether cross-stage immune responses could be induced, we treated mice immunized with live iRBC under chloroquine cover with nonviable and thus unable to initiate a blood infection, or that viable parasites initiated a blood infection that was then very effectively curtailed by anti-blood stage immune responses. Therefore, immumized with a single dose of $10^6$ Py-iRBC were still completely protected from infection by a sporozoite challenge (5/5 in the immunized group vs 0/5 in the control mice). However, sterile immunity against a similar challenge was not observed in all the mice immunized with a single dose of $10^6$ Py-iRBC (Table I); nonetheless, in the mice where parasitemia was observed, it was of shorter duration and parasite levels were lower than in control animals (Fig. 2A). However, because full protection was also obtained in the mice immunized with $10^6$ Py-iRBC and challenged with Py-iRBC (Table I), it was not possible to ascertain whether a component against the pre-erythrocytic stages contributed to the protective immunity observed.

**Immunity induced with live iRBC inhibits hepatic parasites**

We next assessed by quantitative PCR the liver stage parasite loads in the livers of mice immunized with $10^6$ Py-iRBC under chloroquine cover and then challenged with homologous sporozoites. It was clear that in the number or the development of hepatic parasites were drastically curtailed as liver parasite loads in the immunized mice were decreased by $>95\%$ as compared with those found in control mice (Fig. 2, B and C). The inhibition of hepatic parasites induced by Py-iRBC immunization was not due to nonspecific immune mechanisms because no significant reduction was observed in animals immunized under chloroquine cover with normal RBC (Fig. 2B) or with *P. berghei* ANKA iRBC (Fig. 2C).

The absence of a full inhibition of liver stage parasites suggested either that those present in the liver of immunized mice were not viable and thus unable to initiate a blood infection, or that viable liver parasites initiated a blood infection that was then very effectively curtailed by anti-blood stage immune responses. Therefore,

**Table I. Protection in BALB/c mice after immunization with Py-iRBC**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Dose</th>
<th>No. of Doses</th>
<th>Challenge</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iRBC</td>
<td>$10^6$</td>
<td>1</td>
<td>Spz</td>
<td>0/10 100%</td>
</tr>
<tr>
<td>iRBC</td>
<td>$10^6$</td>
<td>1</td>
<td>Spz</td>
<td>7/12 41.7%</td>
</tr>
<tr>
<td>nRBC</td>
<td>$10^6$</td>
<td>1</td>
<td>Spz</td>
<td>10/10 0</td>
</tr>
<tr>
<td>iRBC</td>
<td>$10^6$</td>
<td>2</td>
<td>Spz</td>
<td>0/5 100%</td>
</tr>
<tr>
<td>nRBC</td>
<td>$10^6$</td>
<td>2</td>
<td>Spz</td>
<td>5/5 0</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Spz</td>
<td>10/10 0</td>
</tr>
<tr>
<td>iRBC</td>
<td>$10^6$</td>
<td>1</td>
<td>iRBC($10^6$)</td>
<td>0/5 100%</td>
</tr>
<tr>
<td>nRBC</td>
<td>$10^6$</td>
<td>1</td>
<td>iRBC($10^6$)</td>
<td>5/5 0</td>
</tr>
</tbody>
</table>

* Mice were i.v. injected with live Py-iRBC or normal RBC (nRBC) and treated with daily doses of 0.8 mg of chloroquine from day 0 to 10. Data represent pooled results from two experiments using $n = 5$ mice per group in each experiment.

* Mice were challenged i.v. 15 days after the end of the chloroquine treatment with 4000 sporozoites (Spz) or $10^6$ iRBC of *P. yoelii* 265BY.

* p < 0.05 between immunized and control mice.

**Table II. Role of B cells in the protection conferred by immunization with iRBC under chloroquine cover**

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Immunogen</th>
<th>Infected/Injected</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>None</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>WT</td>
<td>Py17X-iRBC</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td>BKO</td>
<td>None</td>
<td>5/5</td>
<td>0%</td>
</tr>
<tr>
<td>BKO</td>
<td>Py17X-iRBC</td>
<td>3/3</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Wild-type (WT) and BKO BALB/c mice were immunized with $10^6$ *P. yoelii* 17XNL clone 1.1 iRBC (Py17X-iRBC) treated with daily doses of 0.8 mg of chloroquine from day 0 to 15.

* Mice were challenged by i.v. injection with 4000 sporozoites.

* p < 0.05 between immunized WT and BKO mice determined by Fisher’s exact test at day 10 postchallenge.
we subinoculated 100 μl of blood collected 6 days after the sporozoite challenge from the immunized mice into naive recipient mice. One of the recipient mice developed patent parasitemia (Supplemental Table S1), demonstrating that some of the remaining liver parasites were viable and capable of initiating a very low grade parasitemia in the immunized mice. When 100 μl of blood were collected at day 7 after sporozoite challenge, patent parasitemia was not observed in the recipient mice, indicating that emerging blood stage parasites were rapidly eliminated in the immunized mice (data not shown).

**Effect of Abs in mice immunized with iRBC**

Abs present in the sera of mice immunized with 10⁶ Py-iRBC under chloroquine cover reacted against air dried *P. yoelii* 265BY sporozoites (mean IFA titer of 880 ± 440, n = 5 mice), liver stage parasites (pooled sera IFA titer of 3200), and blood stage parasites (mean IFA titer of 2560 ± 870, n = 5 mice). Equal amounts of IgG1 and IgG2a Abs specific for sporozoites or for blood stage parasites were detected (data not shown). However, the sera from the immunized animals had no detectable effect on in vitro liver stage development when they were added along with *P. yoelii* 265BY sporozoites to primary hepatocyte cultures (data not shown). This result indicated that the anti-sporozoite and anti-liver stage Abs in the sera were not functional in vitro. BALB/c B cell-deficient (BKO) mice devoid of Abs were then used to assess whether Abs played a role in cross-stage protection in vivo. As expected, BKO that were not treated with chloroquine were unable to control an infection initiated by the blood stages of the nonlethal *P. yoelii* 17X clone 1.1 (Py17X-iRBC) and mice died with high parasitemia between days 43 and 50 (5 of 5 BKO mice vs 5 of 5 wild-type mice). This confirmed that B cells were crucial for anti-*P. yoelii* blood stage immunity (41). Furthermore, chloroquine cover for 10 days in BKO mice was not sufficient to suppress the immunizing 10⁶ Py17X-iRBC because parasites were found in the blood of 4 of 5 BKO mice 7 days after the last drug injection.

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*The online version of this article contains supplemental material.*
and immunized mice treated with anti-IFN-γ by sporozoites. These mice were collected 42 h after challenge with 2500 sporozoites, from control mice or mice immunized with 10^6 Py-iRBC or normal RBC under 10-day chloroquine cover starting on the day of immunization. Sera were collected 42 h after challenge with 35,000 sporozoites. Results are expressed as mean ± SEM of n = 5 mice. *, p < 0.05 vs control mice using ANOVA followed by Tukey’s test. B. Groups of BALB/c mice were injected with 10^6 Py-iRBC and treated with chloroquine from day 0 to 10 starting the day of Py-iRBC inoculation. They were challenged i.v. with 4000 sporozoites at least 15 days after the last chloroquine injection. Immunized mice were injected with the rat Abs control rat IgG or anti-IFN-γ. Data are cumulative results of two experiments with n = 4–5 mice per experiment. All control mice treated with chloroquine alone (n = 10) developed patent blood stage parasitemia after sporozoite challenge. Protection was defined as the percentage of the mice that did not develop patent blood stage over 10 days postchallenge in the group that were challenged by sporozoites. **+, p < 0.05 between immunized mice treated with rat IgG and immunized mice treated with anti-IFN-γ Abs, for values on day 10, as calculated by Fisher’s exact test. Number in brackets represents the percentage of protection.

When chloroquine cover was extended for 15 days, the immunizing parasites were cleared in 3 of 5 BKO animals. Upon challenge with sporozoites 15 days after the last chloroquine injection, these three immunized BKO mice, as well as five control BKO mice that received chloroquine for 15 days, developed patent blood stage parasitemia (Table II). Altogether, these observations demonstrated that Abs to erythrocytic but not to pre-erythrocytic parasite forms were essential for sterile protection.

CD4⁺ or CD8⁺ T cells are involved in protection induced by Py-iRBC against liver but not blood stage

Treatment of BALB/c mice immunized with 10⁶ Py-iRBC under chloroquine cover with anti-CD4⁺ T cell, anti-CD8⁺ T cell Abs fully reversed liver stage inhibition, which was not the case when the mice were treated with control rat IgG (Fig. 3A). Nonetheless, the immunized mice depleted of CD4⁺ or CD8⁺ T cells were still protected because parasite could not be detected by microscopy in the blood after challenge (Fig. 3B). These mice were also completely protected against a challenge with blood stage parasites (Fig. 3C). This result indicated that our immunization protocol induced two types of protective immunity, one mediated by T cells that were inhibitory to liver stage parasites and the other mediated by Abs inhibitory to blood stage parasites.

Role of cytokines and NO in the in vivo inhibition of the pre-erythrocytic stages

High levels of circulating IFN-γ were first detected in mice immunized with 10⁶ Py-iRBC under chloroquine treatment 42 h after challenge with homologous sporozoites (Fig. 4A), at a time where blood stage parasites were yet to emerge from the liver. In Py-iRBC-immunized mice, treatment with anti-IFN-γ mAb on the days before and after sporozoite challenge reduced the proportion of fully protected animals (35%) as compared with that obtained in immunized animals (85%) treated with a control Ab (Fig. 3B). The incomplete abrogation of sterile protection by IFN-γ neutralization suggested that other cytokines were involved in the protection. However, Ab neutralization of IL-12 or IL-6, which were previously shown to have anti-liver stage activity (42–45), did not abrogate sterile protection in immunized mice (5 protected of 5 mice in each group).

To evaluate the extent with which a known terminal effector of hepatic stage inhibition, NO, contributed to the sterile protection observed, immunized mice were treated with SMT, a potent and selective inhibitor of inducible NO synthase (37). SMT treatment fully abrogated protection after sporozoite challenge but not after blood stage parasites challenge (Table III).

Discussion

In this study we provide evidence that runs counter to the notion that acquired immune responses to Plasmodium parasites are primarily stage-specific. Using mice that had acquired sterilizing immunity solely through exposure to blood stage parasites, we demonstrated that the immune responses included some that have potent inhibitory effects on the pre-erythrocytic stages of challenge parasites. Immunization simply consisted of administering blood stage parasites under chloroquine cover. The efficiency with which sterile immunity was induced using this protocol was dependent on the number of doses and the quantity of immunizing parasites. It was nonetheless possible to obtain full protection reproducibly after a single inoculation of a million parasitized RBC. Under the optimal immunization protocol, sterile protection was observed irrespective of the parasite form used for the challenge: iRBC or sporozoites. The induction of strong inhibitory responses against the pre-erythrocytic stages was clearly revealed by measurements of parasite loads in the livers of immunized mice after sporozoite challenge. Although a 99% reduction in liver load was obtained, some of the liver parasites were still viable and able to lead to a transient blood stage parasitemia that was undetectable by microscopy because it was rapidly inhibited by the immunity acquired to the blood stages. Thus, the sterile immunity observed results from the combined action of immunity against both liver and blood stage parasites.

Our results provided confirmation that patent blood stage infection suppresses the development of cross-stage immunity (38, 39) because in mice infected with sporozoites and challenged with sporozoites after self-cure of the blood stage infection no effect against the liver stages could be observed (Fig. 1), whereas sterile immunity against blood stage parasites was induced. It is likely that this phenomenon accounts for the lack of acquired protective

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Treatment*</th>
<th>Parasite Form</th>
<th>Infected/Injected Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nRBC None</td>
<td>Spz 10/10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>iRBC None</td>
<td>Spz 2/9</td>
<td>78*</td>
<td></td>
</tr>
<tr>
<td>iRBC SMT</td>
<td>Spz 10/10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>nRBC None</td>
<td>iRBC 5/5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>iRBC None</td>
<td>iRBC 0/5</td>
<td>100*</td>
<td></td>
</tr>
<tr>
<td>iRBC SMT</td>
<td>iRBC 0/5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were immunized with 10⁶ normal RBC (nRBC) or 10⁶ Py-iRBC following daily treatment with 0.8 mg of chloroquine for 10 days. Control and immunized groups are the same as in Fig. 3B.

* Mice were treated with 100 μg of SMT on days 0, +1, and +2 after sporozoite (Spz) challenge.

* Mice were challenged by i.v. injection with 4000 homologous sporozoites.

* p < 0.05 vs mice immunized with normal RBC.
immunity against natural reinfections in inhabitants of endemic areas. Indeed, depending on the endemic site, 80–100% of adults radically cured of blood stage infections get reinfected within a 3-mo period (46, 47).

When the immune responses induced were investigated, it could be demonstrated that both CD4+ and CD8+ cells were necessary to mediate the immunity to liver stage parasites because depletion of either cell type nearly fully reversed the inhibition of liver stage development in the immune mice. The fact that partial regression of liver stage inhibition was not observed suggested either that the two T cell subsets acted in synergy to eliminate liver stage parasites, or that one subset was important for the expression of the inhibitory activity of the other, for example by controlling migration of effector T cells to the infected liver or of cytokine production. This intriguing phenomenon deserves further studies. Depletion of either of the two T cell subsets did not reverse the protection acquired against blood parasites, either after sporozoite or blood stage parasite challenge, demonstrating that sterile immunity induced by our protocol against the blood stages is T cell-independent.

When the Ab responses were analyzed, it was clear that they played a central role in the elimination of erythrocytic parasites because sterile protection could not be obtained when the immunization was conducted in BKO mice. These observations confirmed previous reports of a crucial role for B cells in the control of blood stage parasitemia (41) or in enhancing parasite elimination during treatment with antimalarial drugs (48). However, although the Abs induced in immunized mice recognized pre-erythrocytic parasites, in vitro functional tests provided no indication that they had an inhibitory activity on sporozoite invasion, or on parasite development in hepatocytes. Nonetheless, it remains to be tested whether the B cells induced were involved in the quality of the T cell responses, a role already demonstrated for P. chabaudi blood stage malaria (48). In a recent study, the sterile protection obtained by immunization with genetically attenuated sporozoites, whose development is arrested in the liver and are, therefore, incapable of leading to a blood stage infection, was equally observed in BKO mice (49). If the innate parasite immune effector mechanisms induced by genetically attenuated sporozoites and via immunization with live sporozoites under chloroquine are broadly similar, the abrogation of protection we observed in BKO mice would be due to the incapacity of the immunized mice to clear the erythrocytic parasites that would have emerged from the few liver parasites that escaped the cross-stage protective responses we observed. The problems inherent to interpreting data on protection against hepatic stages in hosts fully immune to the blood stages have also been encountered in another recently reported work in which mice were immunized with blood stage of the otherwise lethal P. yoelii YM that was genetically attenuated to give self-limiting infections (50). Following a primary self-cured infection, the mice were equally fully protected from sporozoite or erythrocytic parasite challenges. In that work, however, hepatic parasite loads in the liver were not assessed, and thus it was not possible to ascertain whether a component against the pre-erythrocytic stages contributed to the protective immunity induced.

Previous work had shown that hepatic parasites were susceptible to radical nitrogen intermediates induced by different cytokines (IFN-γ, IL-6, or TNF-α) (51, 52), or after immunization with irradiated sporozoites (53, 54). We showed that the inhibition of pre-erythrocytic parasites induced by blood stage immunization under chloroquine cover was strictly dependent on NO production. Although IFN-γ was shown to play a role against the hepatic parasites in the immunized mice, the levels of protection were not fully reduced by effective Ab neutralization of this cytokine, suggesting the involvement of another mediator. However, we found that the cytokines IL-12 and IL-6 that have been shown previously to mediate the inhibition of liver stage development (42–45), were not to be implicated in protection. Thus, our results imply that, in our immunization model, other cytokines may lead to NO-mediated killing, and identifying them deserves further studies.

Our demonstration of cross-stage immunity relied on measurements of the parasite load in the livers of sporozoite-challenged animals as well as on experimental deletions of defined immunological effectors. These investigations could not be envisaged in humans on ethical grounds. However, in essence the immunization protocol we adopted, inoculation of low parasite numbers whose growth was checked by drug treatment is akin to the immunization with ultra low-dose blood stage parasites used to induce sterile protection in humans against challenge with P. falciparum blood stage parasites (55). It would be interesting to determine whether protective immunity in the human volunteers might extend to pre-erythrocytic parasites. However, demonstrating such protection would require a massive P. falciparum sporozoite challenge, a prerequisite for the quantification of the parasites emerging from the liver into the bloodstream.

Our demonstration of effective cross-stage immunity strongly indicates that parasite Ags expressed in both blood stage and pre-erythrocytic stage parasites are implicated, though the humoral and cellular immune responses induced appear to act differentially on the two parasite stages. It is at present unclear what Ag leads to the acquisition of the cross-stage immunity demonstrated in this study. A different repertoire of Ags might be responsible for the sterile protection induced by irradiated or genetically attenuated sporozoites because the resulting hepatic forms are arrested at an early stage of development. Although the nature of the Ags responsible for sterile immunity remains speculative, the possibility to develop a vaccine effective against both blood and hepatic stage parasites justifies the efforts that would be needed to identify and characterize the cross-stage protective Ags.

Disclosures

The authors have no financial conflict of interest.

References


Table S1. Subinoculation of blood from immunized and challenged into recipient mice

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Infected mice/Injected mice *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microscopy</td>
</tr>
<tr>
<td>10^6 Py-iRBC</td>
<td>0/5</td>
</tr>
<tr>
<td>None</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* Mice were challenged with 4 000 *P. yoelii* 265 BY sporozoites and blood infection was determined by microscopy using Giemsa-stained blood smears or by subinoculation of 100 microliters of blood (corresponding to 5 × 10^7 RBC) from immunized and control mice into normal recipient mice.