Artesunate versus Chloroquine Infection—Treatment—Vaccination Defines Stage-Specific Immune Responses Associated with Prolonged Sterile Protection against Both Pre-erythrocytic and Erythrocytic Plasmodium yoelii Infection

Xiaohong Peng, Gladys J. Keitany, Marissa Vignali, Lin Chen, Claire Gibson, Kimberly Choi, Fusheng Huang and Ruobing Wang

*J Immunol* 2014; 193:1268-1277; Prepublished online 23 June 2014; doi: 10.4049/jimmunol.1400296
http://www.jimmunol.org/content/193/3/1268
Artesunate versus Chloroquine Infection–Treatment–Vaccination Defines Stage-Specific Immune Responses Associated with Prolonged Sterile Protection against Both Pre-erythrocytic and Erythrocytic Plasmodium yoelii Infection

Xiaohong Peng, Gladys J. Keitany, Marissa Vignali, Lin Chen, Claire Gibson, Kimberly Choi, Fusheng Huang and Ruobing Wang

Sterile protection against malaria infection can be achieved through vaccination of mice and humans with whole Plasmodium spp. parasites. One such method, known as infection–treatment–vaccination (ITV), involves immunization with wild type sporozoites (spz) under drug coverage. In this work, we used the different effects of antimalarial drugs chloroquine (CQ) and artemesunate (AS) on blood stage (BS) parasites to dissect the stage-specific immune responses in mice immunized with Plasmodium yoelii spz under either drug, as well as their ability to protect mice against challenge with spz or infected RBCs (iRBCs). Whereas CQ-ITV induced sterile protection against challenge with both spz and iRBCs, AS-ITV only induced sterile protection against spz challenge. Importantly, AS-ITV delayed the onset of BS infection, indicating that both regimens induced cross-stage immunity. Moreover, both CQ- and AS-ITV induced CD8+ T cells in the liver that eliminated malaria-infected hepatocytes in vitro, as well as Abs that recognized pre-erythrocytic parasites. Sera from both groups of mice inhibited spz invasion of hepatocytes in vitro, but only AS-ITV induced sterile protection against iRBCs. Finally, passive transfer of sera from CQ-ITV–treated mice delayed the onset of erythrocytic infection in the majority of mice challenged with P. yoelii iRBCs. Besides constituting the first characterization, to our knowledge, of AS-ITV as a vaccination strategy, our data show that ITV strategies that lead to subtle differences in the persistence of parasites in the blood enable the characterization of the resulting immune responses, which will contribute to future research in vaccine design and malaria interventions.

model of infection that CQ-ITV leads to Ab-mediated immunity against BS Ags resulting from transient exposure to BS parasites during the treatment regimen (19). In general, immunization with irr-spz or early-arresting genetically attenuated parasites (GAPs) exposes the host to a restricted range of spz and early liver stage (LS) parasite Ags, whereas immunization with IVT or late-arresting GAPs likely broadens the repertoire of Ags to include those expressed by late LS and early BS parasites (3, 20). This difference may explain why protection by CQ-ITV requires a much lower dose of wt spz as compared with irr-spz or early-arresting GAPs (21, 22).

To understand immune responses elicited by immunization with whole Plasmodium parasites in the presence and absence of BSs, we have used two drugs that have differential chemical effects on BS parasites. We identified a dose of AS-ITV that eliminates BSs, and compared it with a previously studied dose of CQ-ITV, which results in the persistence of BSs (8, 19, 23). This is the first time, to our knowledge, that these immunization strategies have been directly compared in terms of protection against different forms of the malaria parasite.

Materials and Methods

Mice and parasites
Female BALB/c or Swiss Webster mice (6–8 wk old) were purchased from Jackson Laboratory or Harlan, respectively. Animal handling was conducted following the protocol approved by Institutional Animal Care and Use Committee at Seattle Biomedical Research Institute. Both wt and GFP-lucerase Plasmodium yoelii 17XNL parasites were cycled between Swiss Webster mice and Anopheles stephensi mosquitoes. Infected mosquitoes were used to inoculate mice in the mock control group. Mice were treated with AS or CQ for 10 consecutive days starting on the same day that mice received the spz vaccination. Chloroquine diphosphate salt (Sigma-Aldrich) was dissolved in 5% sodium bicarbonate (Sigma-Aldrich) and was diluted in 1X PBS (Sigma-Aldrich), and artemisinin (Sigma-Aldrich) was substituted with 10% FBS RPMI 1640 medium containing anti-CD28 Ab (5 μg/ml; Biolegend) in FBS-2 (10 μg/ml; Promega). Cells were then transferred into a 96-well plate and incubated at 37°C in 5% CO₂ for 3 d. Next, cells were then transferred into a 6-well plate with 2 ml 10% FBS RPMI 1640 containing additional hIL-2 (20 ng/ml), and cultured for a further 3–4 d.

Primary hepatocytes were harvested from BALB/c mice 2 d before performing the killing assay, as previously described (18). Isolated hepatocytes were diluted in William’s E medium (Sigma-Aldrich) and supplemented with 10% FBS at a concentration of 400 cells/μl. Next, 100 μl of this cell suspension was added to each well of a 96-well tissue culture plate (Becton Dickinson Labware) and incubated overnight at 37°C in 5% CO₂. The next day, hepatocyte cultures were infected with 30,000 GFP-lucerase P. yoelii wt spz. Three hours p.i., plates were shaken gently, the supernatant was aspirated, and fresh culture medium was added. Infected cultures were further incubated for 24 h at 37°C in 5% CO₂. Various dilutions of expanded CD8⁺ T cells were added to the infected hepatocytes and incubated for an additional 16 h. Finally, luminescence units were measured using a Centro XS1 LB 960 Microplate Luminometer under the Bright-Glo Luciferase Assay System protocol (Promega). The percentage inhibition was calculated as 1 – OD of test sample/mean OD of medium.

Immunofluorescence assay and ELISA
Sera were collected from immunized mice on day 9 after the last immunization. Ab titers were determined by immunofluorescence assay (IFA) using paraformaldehyde-fixed spz, LS parasites, or IRBCs, respectively, as previously described (8). For the MS1 and schizont lysate ELISAs, Immunol 4 HBX 96-well plates were coated with 100 μl of 0.5 μg/ml yP.y., MSPI-19(XL)/VQ1 (MR1-48, deposited by D.C. Kaslow) or with 50 μl schizont lysate (5 μg/ml in PBS) prepared as previously described (26). Plates were incubated in blocking buffer (5% FBS in PBS + Tween 20; Fisher) for 1 h at room temperature. For the MSPI ELISA, serum samples were serially diluted in blocking buffer, and for the schizont lysate ELISA, serum samples were diluted at 1:10 in blocking buffer. After a 1-h incubation at room temperature, 100 μl rabbit anti-mouse IgG (Sigma) diluted 1:400 in blocking buffer was added to each well, and plates were incubated for an additional 30 min. For the CSP ELISA, plate-bound activated plates (Thermo Scientific Pierce) were washed with 2 ng/ml P/CSP peptide (OOGPGAOOGPGAPGAPGAP) in 100 μl of 1× PBS, and the ELISA was performed as per the manufacturer’s instruction. All plates were read using a SpectraMax M2 Microplate reader.

Inhibition of spz infection assay
Thirty thousand GFP-lucerase P. yoelii wt spz were coincubated for 20 min at room temperature with serum collected on day 9 after each
vaccination and diluted 1:10 in RPMI. Next, the serum-incubated spz were transferred into 96-well plates that were preseeded with 30,000 HepG2 cells the previous day. Forty hours p.i., cells were lysed by incubation with 100 μl Glo lysis buffer (Promega) containing 0.5% Triton X. Luminescence units were measured as described earlier.

### Passive transfer

Serum from AS-ITV or CQ-ITV mice immunized with three doses of wt *P. yoelii* spz (or mock immunized) was collected on day 9 after the last immunization and pooled. Naive mice were injected i.v. with 300 μl pooled serum and challenged i.v. with 10,000 *P. yoelii* spz or 20,000 *P. yoelii* iRBCs 24 h postinfection, as described previously (26). Parasitemia was examined daily by thin blood smear and Giemsa staining, starting on day 3 postvaccination.

### Statistical analysis

Data are presented as the mean ± SEM. Statistical analysis was performed using Prism software version 5.0 (GraphPad), and means were compared by two-tailed Student’s t tests or one-way ANOVA. A p value <0.05 was considered statistically significant. Analysis of FACS data was performed using FlowJo software (Tree Star).

### Results

**AS-ITV, but not CQ-ITV, eliminates BS infection after vaccination with wt *P. yoelii* spz**

First, we sought to select a chemoprophylactic dose of AS that eliminates BS forms. To do this, we infected BALB/c mice with 10,000 wt *P. yoelii* spz isolated from the salivary glands of infected *A. stephensi* mosquitoes by i.v. injection and treated them with either 0.3, 1.5, or 3.0 mg AS daily from days 1–10 p.i. (Fig. 1A). BS infection was monitored daily for 3 wk by thin blood smear microscopy. Mock-treated mice developed BS infection at day 4 p.i., with parasitemia levels peaking on day 11. Similarly, mice treated with 0.3 mg AS became patent on day 3, although parasitemia levels were lower overall and the peak of parasitemia was delayed by 3–4 d as compared with that of mock-treated mice. Mice treated with 1.5 mg AS displayed a low-level burst of patent parasitemia 4–5 d after drug withdrawal. Mice treated with 3 mg AS displayed no evidence of BS infection, indicating that 3 mg AS constitutes an optimal dose to eliminate BS infection.

We also treated mice with 0.8 mg CQ, the previously established chemoprophylaxis dose for this drug in the rodent malaria model (8). Similarly to the results observed with 1.5 mg AS, these mice developed a low-level burst of patent parasitemia 4–5 d after drug withdrawal (Fig. 1B). Doubling the dose to 1.6 mg CQ did not abrogate this low-level burst of parasitemia, in agreement with a recent report (19). This result agrees with the known mechanism of action of CQ, which only affects late trophozoites and schizonts, allowing for the persistence of ring and early trophozoites in the iRBCs during the CQ treatment phase that become patent after drug withdrawal (15, 19).

We repeated this experiment in additional groups of *P. yoelii*–immunized mice treated for 10 d with 0.8 mg CQ, 3.0 mg AS (or mock treated), and observed a close correspondence between the parasitemia levels determined by blood smear and the detection of *P. yoelii* 18S rRNA by qPCR on blood samples taken on days 11, 14, 18, and 24 postimmunization, as compared with that of mouse GAPDH (Fig. 1C).

In conclusion, we identified a dose of AS (3.0 mg) that completely abolishes BS forms as determined both by Giemsa staining of thin blood smears and by parasite RNA qPCR, as expected from the mode of action of this drug on BS *Plasmodium* parasites (15). Thus, next we compared the protective immunity induced by AS-ITV with that of CQ-ITV, which results in the exposure of the host immune system to replicating BS forms.

**AS-ITV induces protection only to pre-erythrocytic malaria infection, whereas CQ-ITV provides protection from both pre-erythrocytic and erythrocytic infections**

To characterize the protection resulting from immunization with wt *P. yoelii* spz under drug coverage, we vaccinated groups of 10 mice with 1–3 doses of 10,000 wt *P. yoelii* spz 1 mo apart and treated with either CQ or AS for 10 d starting on the day of vaccination (Fig. 2A). In agreement with the data presented in Fig. 1, 10 of 10 mice had BS parasitemia upon drug withdrawal after the first course of CQ-ITV, compared with 0 of 10 mice under AS coverage (Table I, Experiment 1). After subsequent courses of ITV, none of the mice had detectable BS parasitemia (Table I, Experiments 2 and 3). One month after each course of ITV, mice were challenged with 10,000 wt *P. yoelii* spz. As a control, a group of five mice were mock immunized with salivary gland extract from noninfected mosquitoes in the absence of drug treatment. None of the mice in either the CQ- or the AS-ITV groups displayed protection against challenge with wt *P. yoelii* spz 1 mo after the first course of ITV (Table I, Experiment 1, spz: 1 mo). However, 90% of the mice in each group were sterilely protected against spz challenge after the second course of ITV (Table I, Experiment 2, spz: 1 mo), and 100% of the mice in each group acquired sterile protection from spz challenge after the third course of ITV (Fig. 2B, Table I, Experiment 3, spz: 1 mo). In comparison, all mock-vaccinated mice had parasitemia on day 3 after challenge (Fig. 2B). To determine the length of the observed protection, we challenged additional groups of immunized mice with 10,000 wt *P. yoelii* spz 3 and 6 mo after the third course of CQ- or AS-ITV (Table I, Experiment 3, spz: 3 and 6 mo). Sterile protection was maintained for at least 6 mo after three courses of CQ- or AS-ITV, confirming that both treatments induce prolonged sterile protection against spz challenge (17).

Next, we analyzed whether AS- or CQ-ITV elicits immunity against BS forms of the malaria parasite by challenging mice i.v. with 20,000 malaria iRBCs 1 mo after three courses of CQ- or AS-ITV, as described earlier (Fig. 2A). We saw that the majority (8/13, or 62%) of the CQ-ITV treated mice were steriley protected against challenge with iRBC, whereas most of the remaining mice experienced a 1- to 4-d delay in the onset of parasitemia, as compared with the mice in the control group (Fig. 2C, Table I, Experiment 4, iRBC: 1 mo). In contrast, none of the mice under AS-ITV treatment acquired sterile protection. Nevertheless, the majority (8/11, or 73%) of the mice in the AS-ITV group experienced a 1- to 3-d delay in the onset of parasitemia after iRBC challenge, as compared with untreated, mock-immunized mice (Fig. 2C, Table I, Experiment 4, iRBC: 1 mo). Taken together, our data demonstrate that both CQ- and AS-ITV induced long-term sterile protection against pre-erythrocytic stages of malaria parasites. Moreover, CQ-ITV treatment also resulted in sterile protection against BS parasites, in agreement with previous reports (19). Interestingly, although unable to confer sterile protection, AS-ITV delayed the onset of BS infection, suggesting that although we were unable to detect BS in mice treated with AS, this regimen also induces some level of cross-stage immunity.

**CQ- and AS-ITV induce CSP-specific CD8+ T effector memory cells in the liver**

The long-lasting sterile protection against challenge with high doses of spz that we observed in mice subjected to three courses of CQ- or AS-ITV suggests that both treatments elicit strong immune responses against pre-erythrocytic stage parasites. Previously, it has been shown that CD8+ T cells play a major role in protection against pre-erythrocytic infection induced by vaccination with...
drug-attenuated spz (16, 17, 27). Moreover, protection against pre-erythrocytic infection has been recently demonstrated to correlate with the induction of malaria-specific CD8+ effector memory T cells (TEM) (28).

To assess the abundance of liver TEM and central memory T cells (TCM) in mice immunized three times with \textit{P. yoelii} spz under CQ or AS coverage (or mock-immunized, untreated mice), we purified liver lymphocytes on days 27, 55, 83, and 90, which correspond to

FIGURE 1. AS-ITV can control BS parasites, whereas CQ-ITV allows the persistence of BS parasites. (A) Groups of mice infected with 10,000 wt \textit{P. yoelii} spz by i.v. injection were mock treated (black circles, \( n = 3 \)) or treated with either 0.3 (red inverted triangles, \( n = 5 \)), 1.5 (blue triangles, \( n = 5 \)), or 3.0 mg (green squares, \( n = 5 \)) AS for 10 d after the infection (black bar). The y-axis shows the percentage of erythrocytes infected with parasites, as determined by microscopy of Giemsa-stained thin blood smears, and the x-axis corresponds to days p.i. (B) Groups of mice infected as described earlier were mock treated (black circles, \( n = 8 \)) or treated with either 0.8 (red triangles, \( n = 10 \)) or 1.6 mg (blue diamonds, \( n = 10 \)) CQ for 10 d after the infection (black bar). (C) Blood samples taken on days 11, 14, 18, and 24 p.i. with \textit{P. yoelii} spz as described in (A) were analyzed by thin blood smear microscopy and by qPCR quantitation of \textit{P. yoelii} 18S rRNA versus the mouse GAPDH to determine the presence of parasites. The left y-axis shows percentage of erythrocytes infected with parasites as determined by microscopy of Giensa-stained thin blood smears, the right y-axis shows the ratio of \textit{P. yoelii} 18S rRNA versus mouse GAPDH detected by qPCR, and the x-axis indicates days p.i.

FIGURE 2. CQ- and AS-ITV induce differential protection against challenge with \textit{P. yoelii} spz or iRBCs. (A) Experimental design. Mice received 10,000 wt \textit{P. yoelii} spz (or mosquito salivary gland debris) on days 1 (V1), 28 (V2), and 56 (V3), followed in each case by 10 d of treatment with 0.8 mg CQ or 3.0 mg AS. One month after the last dose, they were challenged with 10,000 wt \textit{P. yoelii} spz or 20,000 wt \textit{P. yoelii} iRBCs. Separate groups of mice were challenged on days 140 or 224 (3 and 6 mo after V3) with 10,000 wt \textit{P. yoelii} spz. (B and C) Patency curves of groups of mice immunized with three courses of CQ-ITV (solid line) or AS-ITV (dotted line), or untreated, mock-immunized, untreated mice, we purified liver lymphocytes on days 27, 55, 83, and 90, which correspond to

To assess the abundance of liver T_{EM} and central memory T cells (T_{CM}) in mice immunized three times with \textit{P. yoelii} spz under CQ or AS coverage (or mock-immunized, untreated mice), we purified liver lymphocytes on days 27, 55, 83, and 90, which correspond to
in the levels of CSP-specific CD8+ TEMs was observed be-
specific CD8+ TEMs were identified using an allophycocyanin- 
served a significant expansion of CSP-specific CD8+ TEMs in 
mice treated with either CQ- or AS-ITV 6 d after spz challenge. In summary, both CQ- and AS-ITV induce pre-
tween the mice under CQ or AS coverage either before or after 
izes 
P. yoelii 
of CQ-ITV resulted in a significant increase (percentage of CSP-specific CD8+ TEMs as compared with mock-
protection was quantified as previously described (29). We observed that addition of 5 × 10^5 liver CD8+ T cells from CQ- or AS-ITV–treated mice significantly inhibited parasite development (81.2 and 78.5%, respectively) as compared with those from mock-
immunized, untreated mice (Fig. 4A). The observed reduction in LS parasite development was specific, because inhibition was propor-
tional to the number of CD8+ T cells added to the coin-
cubation assay (Fig. 4B). These data confirm that CD8+ T cells induced by ITV have the ability to affect the development of Plasmodium-infected hepatocytes (Fig. 4B), although the mecha-
nism of killing remains to be fully elucidated.

It has been demonstrated that CD8+ T cells are cytotoxic to LS parasite-infected hepatocytes in vitro

It was found that the increase in the abundance of CD8+ TTEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.

We then determined whether the increase in the abundance of CD8+ TEMs observed on days 83 and 90 in the AS-ITV and 
CQ-ITV mice was specific for malaria pre-erythrocytic Ags by 
quantifying the abundance of TCM (CD127+CD62L+) and TEM (CD127+CD62L-) cells in the liver. We 
observed an increase in the abundance of TEMs only after the third course of AS- and CQ-ITV, as compared with mock-
immunized mice (Fig. 3B). This increase was most pronounced 
after challenge with P. yoelii spz. In comparison, the abundance of TEMs remained low throughout the course of the immunization, and we only observed an increase in TCMs in CQ-ITV mice after challenge.
To determine the functionality of the Ab response induced by CQ- and AS-ITV, we performed inhibition of spz invasion assays in vitro. Sera collected from mice immunized with P. yoelii spz under CQ or AS coverage on day 9 after the third course of ITV significantly inhibited (p < 0.0001) the invasion of HepG2:CD81 cells by luciferase-expressing P. yoelii spz (Fig. 6A). Furthermore, sera obtained from AS-ITV–treated mice showed significantly higher inhibition efficiency than sera from CQ-ITV mice (p = 0.024). Sera collected after each course of CQ-ITV inhibited the invasion of hepatocytes by spz with increasing efficiency (i.e., 7, 13, and 55% for vaccination doses 1, 2, and 3, respectively; Fig. 6B). Similarly, sera obtained from mice under AS coverage inhibited spz invasion at 15, 33, and 74% after vaccination doses 1, 2, and 3, respectively. In summary, both CQ- and AS-ITV elicit Abs that are capable of blocking spz invasion in vitro, and furthermore, successive vaccinations increase this functional Ab response.

Adoptive transfer of sera from mice treated with CQ-ITV, but not with AS-ITV, significantly delayed BS infection in naive mice challenged with P. yoelii iRBCs

Finally, to determine whether Abs from vaccinated mice could protect in vivo against spz or iRBC infection, we adoptively transferred sera from CQ-ITV– and AS-ITV–treated mice to naive mice. Twenty-four hours after receiving 300 μl donor sera by i.v. injection (26), mice were challenged with either 10,000 P. yoelii spz or 20,000 iRBC by i.v. injection. We observed that none of the mice that received sera from donor mice immunized under either CQ- or AS-ITV treatment acquired sterile protection against spz challenge (Fig. 6C). Interestingly, four of five mice (80%) demonstrate that mice immunized with P. yoelii spz under CQ coverage develop Abs that can recognize spz, LS, and BS forms of the malaria parasite, whereas sera from mice under AS-ITV treatment predominantly recognize spz and LS but display low reactivity to BS Ags.

Sera from both CQ- and AS-ITV–treated mice inhibit invasion of hepatocytes by P. yoelii spz in vitro
that received sera from donor mice from the CQ-ITV treatment group showed a 1- to 2-day delay of patency, as compared with control mice upon challenge with iRBCs (Fig. 6D). However, only one of five mice (20%) that received sera from AS-ITV–treated mice displayed delayed patency after iRBC challenge (Fig. 6D).

FIGURE 5. Sera from mice treated with CQ- or AS-ITV recognize pre-erythrocytic and erythrocytic Ags and parasites to different degrees. (A–C) Reactivity of serum collected from mock-immunized, untreated mice (mock, black bars), CQ-ITV–treated (CQ, gray bars), or AS-ITV–treated (AS, white bars) mice 9 d after the third immunization against pre-erythrocytic protein CSP B cell epitope QGPGAP (A), erythrocytic MSP 1 protein (B), or schizont lysate (C) by ELISA. The y-axis shows OD at 450 nm. The x-axis indicates the dilution factor of the sera used for CSP (A) and MSP1 (B); a 1:10 dilution of sera was used for the schizont lysate (C). Significance was analyzed using two-tailed Mann–Whitney tests. Error bars represent SEM. **p < 0.01. (D) Immunofluorescence assays with serum collected from mock-immunized, untreated mice (top panel), CQ-ITV–treated mice (middle panel), or AS-ITV–treated mice (bottom panel), on P. yoelii spz (left panel); LSs (middle panel); or BSs (right panel). Serum reactivity is shown in green (FITC), and DNA staining is shown in blue (DAPI). Merge images show a merge of the green and blue channels. Scale bar, 10 µm.

FIGURE 6. Sera from mice treated with CQ- or AS-ITV inhibits spz invasion of hepatocytes in vitro. (A) Quantification of HepG2 cell invasion and development after incubation of P. yoelii GFP-luciferase spz with serum collected from mock-immunized, untreated (n = 9), ITV-CQ–treated (n = 9), or ITV-AS–treated (n = 13) mice 9 d after the third dose. The y-axis shows luminescence units. Significance was determined by two-tailed Student t tests. Results are from two independent experiments. Error bars represent SEM. *p < 0.05, ****p < 0.001. (B) Percent inhibition of HepG2 cell invasion and development upon incubation of P. yoelii GFP-luciferase spz with sera obtained after the first (V1), second (V2), or third (V3) dose of CQ- or AS-ITV (AS), calculated as the percentage reduction in luciferase signal compared with that of the mock-immunized, untreated control. (C and D) Patency curves of naive mice challenged with wt spz (C) or iRBC (D) after adoptive transfer of immune sera from CQ-ITV–treated (solid line) or AS-ITV–treated (dotted line), or untreated, mock-immunized mice (dashed line). The y-axis shows the percentage of mice protected from infection at each time point, and the x-axis corresponds to the day of parasitemia onset after challenge.
Overall, the in vivo experiments described earlier demonstrate that Abs generated under CQ- or AS-ITV are not sufficient to confer sterile protection against either an i.v. spz or an i.v. BS challenge in a passive transfer experiment. Taken together, our data suggest that the protective immunity induced by CQ- and AS-ITV associates with the different effects of these drugs during the *Plasmodium* life cycle in the mammalian host.

**Discussion**

After decades of malaria vaccination studies in both animal models and malaria-naive humans, whole-parasite immunization is by far the most effective strategy in generating sterile protection, whether through irr spz, ITV, or GAP (reviewed in Refs. 22, 32). Although sterile protection can be achieved by immunization with a low number of bites of malaria-infected mosquitoes under CQ coverage, widespread resistance to this drug limits the applicability of the CQ-ITV approach in malaria control. Artemisinin-based combination therapies are currently the treatment of choice for uncomplicated *P. falciparum* and *P. vivax* malaria in adults and children in all endemic areas (33), although there have been several reports of drug resistance in the same geographic area where CQ resistance originated in the 1950s, where artemisinin and its derivatives have been used as a monotherapy for more than three decades at subtherapeutic doses (34, 35). Like CQ, AS prevents BS infection by blocking the ability of the parasite to complete the intraerythrocytic cycle. A major difference between CQ and AS is that CQ acts mainly on late BS parasites, whereas AS acts throughout the BSs, including early ring stages (reviewed in Ref. 15). In this work, we exploited the difference between the effects of AS and CQ on malaria erythrocytic parasites as a tool to define correlates of protection.

We first identified a dose of AS that completely blocks parasite development before the formation of pathogenic BSs (Fig. 1). By comparing the immune responses elicited by this AS-ITV strategy with those induced by CQ-ITV, we were able to characterize liver-stage immunity in the absence and presence of BS infection, respectively.

Consistent with the fact that neither CQ nor AS affect the pre-erythrocytic stages of malaria parasites (15), we observed that although a single course of either CQ- or AS-ITV was insufficient to induce sterile immunity against challenge with 10,000 *wt* *P. yoelii* spz, three courses resulted in sterile protection of all mice that lasted for at least 6 mo (Fig. 2, Table I). Our results agree with a recent study demonstrating that three courses of CQ-ITV induced long-lasting protective immunity against *P. falciparum* in malaria-naive volunteers (36). In contrast, two previous mouse studies reported that a single course of CQ-ITV is sufficient to generate 78–100% protection against spz challenge (19, 23). This apparent contradiction can be explained by the number of spz used in the challenge phase of each of these studies: Doll et al. (19), who report 100% sterile protection after a single course of CQ-ITV, used only 1000 spz for the challenge; Belnoue et al. (23), who report partial protection, used only 4000 spz; whereas our study used 10,000 spz. Therefore, these data suggest that the level of protection observed is inversely correlated to the number of spz used for the challenge. In addition to this, the level of protection from a particular immunization regimen has been shown to depend on the strain of mouse used for experimentation (22, 37). Therefore, the differences discussed earlier could also be attributed in part to the use of different mouse strains: whereas both Belnoue et al. (23) and this study used BALB/c mice, Doll et al. (19) used C57BL/6 mice.

Sterile protection induced by CQ-ITV in the mouse model has been shown to depend on immune responses against both pre-erythrocytic and BS Ags that involve CD8+, CD4+ T cells, and Abs (17, 19, 23). CD8+ T cells have been shown to be required for protection against malaria LSs (16, 18, 20, 38, 39). In agreement with this, we observed that repeated exposure of mice to malaria parasites under either CQ or AS coverage resulted in the induction of total and malaria-specific liver CD8+ TCM8, whereas CD8+ TCM levels remained low (Fig. 3). Challenge with spz further induced the levels of CD8+ TCM8 in CQ- and AS-ITV mice, as well as slightly increasing CD8+ TCM8 in CQ-ITV mice. These data agree with previous reports suggesting that CD8+ TCM8 levels correlate with protection (16, 40), and that passive transfer of CD8+ TCM8 into naive mice results in sterile protection against spz challenge (28).

Previous studies from our group and others have shown that CD8+ T cells induced by whole parasites are capable of killing *Plasmodium*-infected hepatocytes in vitro and in vivo (18, 30, 31). In this study, we determined that ITV-induced liver CD8+ T cells are also capable of interfering with the development of malaria-infected hepatocytes in vitro (Fig. 4). Several mechanisms have been implicated in the killing of infected hepatocytes by CD8+ T cells, including the production of proinflammatory cytokines such as IFN-γ and TNF-α, the production of cytotoxic molecules such as perforin and granzyme, and the clustering of CD8+ T cells around infected hepatocytes (18, 31, 39).

Although the role of CD8+ T cells in immunity against whole parasites is well established, humoral immunity also plays a crucial role in controlling malaria infection (7, 19, 40–42). Thus, we investigated the Ab response mounted against the predominant pre-erythrocytic protein, CSP, by mice immunized with wt *P. yoelii* spz under CQ and AS coverage. Both CQ-ITV and AS-ITV induced similar levels of Abs against pre-erythrocytic Abs.
(Fig. 5). Furthermore, whereas both CQ- and AS-ITV induced functional Abs that were capable of blocking invasion of hepatocytes by \textit{P. yoelii} spz in vitro, passive transfer of sera from mice immunized under CQ-ITV or AS-ITV did not have any protective effect against challenge with wt \textit{P. yoelii} spz by i.v. injection (Fig. 6). The difference observed between in vitro and in vivo assays could be because of the route of challenge used in our experiments, as it was recently shown that challenge by mosquito bite instead of i.v. not only allows Abs to act against the skin traversal phase of parasite infection, but also gives them sufficient time to block hepatocyte invasion (26).

Previous studies using CQ-ITV and late LS arresting GAP parasites have suggested that cross-stage immunity plays a role in protection against malaria challenge (19, 20, 23). This immunity has been attributed to expression of shared Ags by LS and BS parasites, in addition to exposure to low levels of BS parasites in the case of CQ-ITV. Similarly to what was recently reported by Doll et al. (19), we saw that CQ-ITV resulted in sterile protection against BS challenge in >60% of mice. This protection was shown to be dependent on the presence of transient BS parasites after drug withdrawal, because a prolonged treatment that prevented the reoccurrence of these parasites abolished cross-stage protection (19). In contrast, we observed that a dose of AS that completely blocks the development of BSs did not result in sterile protection against BS challenge, although the majority of the AS-ITV–treated mice displayed a delayed patency, suggesting that AS-ITV induces some level of cross-stage immunity (Fig. 2). This could be caused by an immune response to Ags expressed by late LS parasites (20, 43), or alternatively by transient exposure to BS Ags expressed by merozoites as they exit the liver and briefly exist in the circulation before invading RBCs and being cleared by AS. Similarly to AS-ITV, late-arresting GAPs also result in cross-stage protection that is likely mediated by exposure to BS Ags during late LS (20).

Although some reports have suggested that CD8+ and CD4+ cells are involved in BS immunity (44, 45), most studies indicate that Abs are the main mediators of protection (reviewed in Ref. 41). Consistent with the mode of action of CQ on late BS parasites, we saw a high level of reactivity of sera from CQ-ITV–treated mice to MSP1 and schizont lysate and iRBCs (Fig. 5). In addition, passive transfer of Abs from CQ-ITV–treated mice into naive mice delayed the onset of parasitemia after BS challenge. These results suggest that Abs could contribute to the sterile protection against BS challenge observed in CQ-ITV mice (Fig. 2). In contrast, sera from AS-ITV–treated mice did not react against MSP1 (Fig. 5B), although it showed low levels of reactivity against schizont lysate and iRBCs. Although this low level of cross-stage AB response elicited by AS-ITV did not result in protection against challenge with iRBC, it could partially explain the delay in patency observed. Further studies are required to establish whether BS-specific CD8+ and CD4+ cells are involved in cross-stage protection in the AS-ITV model, as well as to determine the identity of shared liver and BS Ags that may associate with cross-stage immunity.

In summary, we showed that the ability of ITV to induce protection against LS and BS parasites depends on the stages of the malaria parasite affected by the drug used to cover the spz infection (Fig. 7). Furthermore, although both CQ- and AS-ITV induce cross-stage immune responses against BS, only CQ-ITV results in sterile protection. This suggests that exposure to a significant load of BS Ags during immunization is essential to confer sterile protection. Therefore, although pre-erythrocytic vaccines have the advantage that they act during a bottleneck in the parasite life cycle and before the large LS and BS expansions in parasite numbers, to be fully protective they might need to also elicit cross-stage protection that is able to block any break-through infection. Thus, vaccines that rely on ITV or on late-LS arresting parasites and allow expression of LS and BS Ags might constitute the most efficient means to confer anti-infection and antidiisease immunity.

Acknowledgments
The following reagent was obtained through the MR4 as part of the Bio-defense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases, NIH: \textit{P. yoelii} yPy, MSPI-19 (XL)/VQ1, MRA-48, deposited by D.C. Kaslow. The allopurinyl-conjugated CSP (SYVPSAEQI) tetramer was obtained from the NIH Tetramer Core Facility. We thank Drs. Brandon Sakk and Ashley Vaughan for critical review of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Gating strategy for sorting CD8\(^+\) cells from mouse livers used in ILSDA. Liver lymphocytes were initially gated based on forward and side scattering (top left panel). Dead cells were excluded based on propidium iodide staining (top right panel). CD3\(^+\) T cells (bottom right panel) were further separated into CD4\(^+\) and CD8\(^+\) T cells (bottom left panel).