Trimethoprim-Sulfamethoxazole Prophylaxis During Live Malaria Sporozoite Immunization Induces Long-Lived, Homologous, and Heterologous Protective Immunity Against Sporozoite Challenge

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Trimethoprim-sulfamethoxazole (TMP-SMX) is widely used in malaria-endemic areas in human immunodeficiency virus (HIV)-infected children and HIV-uninfected, HIV-exposed children as opportunistic infection prophylaxis. Despite the known effects that TMP-SMX has in reducing clinical malaria, its impact on development of malaria-specific immunity in these children remains poorly understood. Using rodent malaria models, we previously showed that TMP-SMX, at prophylactic doses, can arrest liver stage development of malaria parasites and speculated that TMP-SMX prophylaxis during repeated malaria exposures would induce protective long-lived sterile immunity targeting pre-erythrocytic stage parasites in mice. Using the same models, we now demonstrate that repeated exposures to malaria parasites during TMP-SMX administration induces stage-specific and long-lived pre-erythrocytic protective anti-malarial immunity, mediated primarily by CD8⁺ T-cells. Given the HIV infection and malaria coepidemic in sub-Saharan Africa, clinical studies aimed at determining the optimum duration of TMP-SMX prophylaxis in HIV-infected or HIV-exposed children must account for the potential anti-infection immunity effect of TMP-SMX prophylaxis.

Keywords. HIV; malaria; antiretroviral therapy; liver stages; eradication; children; trimethoprim-sulfamethoxazole; immunity.

Human immunodeficiency virus (HIV) infection and malaria geographically overlap, and each enhances the pathogenicity of the other. With increasing roll out of antiretroviral therapy, the population of HIV-exposed, HIV-uninfected children is growing [1]. Understanding how drugs used in the management of both HIV-infected patients and HIV-uninfected, HIV-exposed patients, particularly in malaria-endemic areas, requires further study.

Trimethoprim-sulfamethoxazole (TMP-SMX) is used for opportunistic infection prophylaxis in these patients. TMP-SMX also reduces the risk of clinical malaria in areas of varying transmission intensity and antifolate resistance [2]. TMP and SMX target dihydrofolate reductase and dihydropteroate synthase, respectively, resulting in disruption of folate synthesis and ultimately, DNA replication [3]. However, as with many other

malaria-prevention strategies, the question remains how TMP-SMX influences the acquisition of malaria-specific immunity and whether such an influence contributes to the reduced case burden observed during TMP-SMX prophylaxis. Such questions have public health implications, and field studies examining the optimum duration of TMP-SMX prophylaxis and its impact on anti-malarial immunity are ongoing [4–6].

The life cycle of *Plasmodium* parasites begins with the female anopheline mosquito infecting the mammalian host with sporozoites, the infective form of the parasite. Sporozoites then travel to the liver, invade hepatocytes, and develop into liver-stage parasites, or exoerythrocytic forms. These events constitute the pre-erythrocytic stages and are clinically silent. Once established in hepatic merozoites, infected hepatocytes release merozoites, which then invade erythrocytes, and this marks the erythrocytic phase of infection, which correlates with the presence of malaria clinical symptoms. Most antimalarials target parasites in this symptomatic asexual blood stage. Naturally acquired immunity to malaria only develops after a number of disease episodes, and is imperfect as it fails to protect against further infection [7]. Asexual blood-stage infection may prevent development of effective anti-infection immune responses [8]. In contrast, complete protective anti-malarial anti-infection immunity is induced with attenuated malaria parasites that are

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arrested during liver-stage development [9]. Anti-infection immunity is also observed in mice that are infected with sporozoites and then given drugs that kill liver-stage parasites [10, 11] and in animal models and humans that are infected with wild-type parasites while receiving chloroquine, a drug that kills blood-stage parasites [12, 13]. This long-lasting sterile immunity is likely based on responses to pre-erythrocytic stage parasites [14]. The use of drugs to prevent or suppress blood-stage parasite infection while permitting antigen exposure to induce immunity is referred to as chemoprophylaxis vaccination, or CPS. Taken together, these data indicate that exposure to liver-stage parasites and avoidance of blood-stage parasites can induce highly effective anti-infection immunity.

TMP-SMX reduces the incidence of clinical malaria episodes [2], presumably because of an anti-blood-stage parasite effect, but whether an anti-liver-stage parasite effect occurs in humans and how this may contribute to anti-infection immunity is unknown. The degree of effect will likely depend on transmission intensity, preexisting immunity, antifolate resistance, and duration of TMP-SMX prophylaxis.

We have shown that TMP-SMX at prophylactic doses can arrest development of liver-stage parasites in animal models [15], and we speculated that TMP-SMX prophylaxis during repeated malaria parasite exposures would induce protective long-lived sterile immunity by targeting pre-erythrocytic stage parasites in mice. To understand what happens in the field with naturally exposed populations receiving TMP-SMX prophylaxis, we set out to model and investigate the effects of repeated live sporozoite administration in mice receiving TMP-SMX as causal prophylaxis at levels approximating what is achieved in children receiving TMP-SMX prophylaxis.

METHODS

Mice

Female BALB/c or B-cell knockout (KO; Igh-Jtm1Dhu N⁺N2) mice, aged 4–6 weeks and weighing 20–25 g, were purchased from Taconic or the National Institutes of Health. Experiments using mice were approved by the institutional animal care and use committees of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Mice were age and sex-matched for all experiments.

Parasites and Mosquitoes

Anopheles stephensi mosquitoes were allowed to feed on mice infected with *Plasmodium yoelii* (17XNL) or *Plasmodium berghei* (ANKA), and sporozoites were harvested on days 14–18 (*P. yoelii*) or days 21–26 (*P. berghei*) by mosquito dissection. All experiments were performed with *P. yoelii* 17XNL, except the heterologous challenge experiments, which involved *P. yoelii* 265BY and *P. berghei*. All mosquitoes had been allowed to feed on infected mice, and parasites had not undergone serial blood passages because this was demonstrated elsewhere to modulate infection virulence [16].

TMP-SMX Formulation and Dose

TMP-SMX was used in its commercially available suspension form and was dosed either at 6 hours prior to infection or at 18, 24, 36–38 hours post-infection (or for [Supplementary Figure 1](#), from time zero which would have paralleled sporozoite infection). We had previously established that these regimens did not result in hepatotoxicity in mice by assessing serum alanine transaminase levels in uninfected, treated mice, and drug dosing and pharmacokinetics were established to approximate what is achieved in children receiving TMP-SMX prophylaxis [15]. Additionally, we established that the TMP-SMX regimen described above did not contribute to suppressing blood stage parasites based on our prior experiments [15], pharmacokinetics, and experiments in which we dosed during liver stages only and then infected with infected red blood cells (iRBCs), and subinoculation (the latter 2 described herein). We previously established that this TMP-SMX regimen inhibits development of liver-stage parasites in vitro and prevents patent parasitemia [15, 17].

Immunization Schedule, Challenge, and Protection Assessment

Unless otherwise specified, mice were injected 3 times, at 14–21-day intervals, with 10 000 *P. yoelii* 17XNL sporozoites, based on previously used regimens (one experiment used 200 sporozoites per infection, and one used infected-mosquito bites [iMBs]) [18]. For the iMB experiments, infectious bites were quantified by assessing the number of mosquitoes that had blood and sporozoites in the salivary gland, with mosquitoes sourced from cartons in which the prevalence of infection was, on average, $\geq 80\%$.

Starting the same day as sporozoite inoculation, immunized and control animals were given a 60- μ L dose of TMP-SMX by oral gavage. During the immunization regimen, the absence of parasitemia in immunized mice was confirmed by examination of Giemsa-stained slides of tail blood specimens collected every other day. In addition, in separate experiments, 100 μ L of blood per mouse was collected from mice on day 7 after live sporozoite immunization with TMP-SMX prophylaxis and subinoculated into naive mice to confirm that the regimen blocked patent parasitemia. In another set of experiments, groups of mice were immunized with 2–3 doses of 10 000 irradiated *P. yoelii* 17XNL sporozoites, 2 weeks apart, and challenged intravenously with 200 nonirradiated *P. yoelii* 17XNL sporozoites 4–12 weeks after immunization [19, 20].

Mice immunized with live sporozoites that received TMP-SMX prophylaxis were rested for a minimum of 4 weeks after the last sporozoite and drug administration. The mice were then challenged intravenously with 200 sporozoites (the minimum number required to reliably and reproducibly infect mice in our system; data not shown), unless otherwise specified (intravenous challenge with 10^6 iRBCs or with 10 000 sporozoites for liver-stage parasite burden by polymerase chain reaction [PCR] for experiments as indicated) [18]. The presence of sporozoites in the mosquitoes that had fed was verified by

microscopy of their dissected salivary glands. Mice were challenged as early as 4–6 weeks to 10–12 weeks (referred to as “early” and “late” time points, respectively) after the last immunization and as late as 16 months.

Parasitemia, where reported, was determined by counting the number of iRBCs per 50 fields (15 000 erythrocytes). Smears were reevaluated by an expert blinded to the findings from the initial evaluation. To assess protection, mice had Giemsa-stained smears performed every day from day 3 to day 15 after challenge to assess prepatent period [21], or by quantitative reverse-transcription PCR analysis of the liver-stage parasite burden in harvested livers, as previously described, with few modifications [22, 23]. Briefly, 40 hours after sporozoite inoculation, livers were harvested, and total RNA was isolated using TRI reagent (Molecular Research Center), in accordance with the manufacturer’s instructions. Reverse transcription was performed using 1 µg of total RNA and random hexamers. After this, real-time PCR was performed using primers that recognize *P. yoelii*-specific sequences within the 18S ribosomal RNA (rRNA) gene and the QuantiTect SYBR Green PCR Kit (Qiagen). Ten-fold dilutions of a plasmid construct containing the *P. yoelii* 18S rRNA gene [22, 24] were used to create a standard curve. Differences between the mean amounts of parasite 18S rRNA in the livers or cells of treated mice and controls were analyzed for statistical significance.

Mice were challenged with homologous *P. yoelii* 17XNL strains unless otherwise described, in which case they were challenged with *P. yoelii* P265BY, which is genetically different from *P. yoelii* 17XNL [25], or with *P. berghei* ANKA [26].

The regimen depicted in Figure 1 was used for experiments unless otherwise specified. Controls were used at every immunization and challenge time point, to ensure sporozoite infectivity. Controls (naive BALB/c mice, unless otherwise specified as B-cell-KO mice) received either drug and no immunization

with challenge, or challenge only. Controls for flow cytometry and immunophenotyping experiments were unimmunized (ie, naive) animals (Figure 4).

In Vivo Depletions

Purified control rat antibodies were purchased from BioXCell (New Hampshire). Rat immunoglobulin G2b (IgG2b) anti-CD4⁺ monoclonal antibody (clone GK 1.5) and rat IgG2b anti-CD8⁺ monoclonal antibody (clone 2.43) were used. Immunized mice were injected intraperitoneally with 500 µg of anti-CD8⁺, anti-CD4⁺ monoclonal antibodies with or isotype control (LTF-2) on days 1 and 0 before challenge. More than 98% of blood CD8⁺ or CD4⁺ T cells were depleted by this procedure, as verified by flow cytometry.

For interferon γ (IFN-γ) neutralization, on days 2, 1, and 0 before and days 1 and 2 after challenge, mice received a single intraperitoneal dose of 2 mg of anti-IFN-γ (clone XMG-1.2) antibodies or isotype control (HRPN) antibodies. The efficacy of IFN-γ antibodies was verified in a separate experiment as previously described [18], in which anti-IFN-γ monoclonal antibody treatment was able to reverse protection in mice immunized with 3 doses of irradiated sporozoites (10 000 RAS-Py sporozoites/dose) [19].

Cell Preparation and Stimulation

Single-cell suspensions from spleens were obtained by means of previously described methods, with few modifications. Briefly, splenic cells were buffer. Cells were washed twice with Hank’s balanced salt solution (HBSS, Sigma Aldrich, St. Louis, Missouri) and suspended in complete medium (Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, glutamine, Pen Strep, and HEPES) [27]. Hepatic lymphocytes were obtained by a method described previously with few modifications. Livers from euthanized mice were perfused with cold

Figure 1. Live sporozoite immunization under trimethoprim-sulfamethoxazole (TMP-SMX) chemoprophylaxis vaccination. BALB/c mice were injected intravenously with 10 000 *Plasmodium yoelii* 17XNL sporozoites (Spzs) 3 times, with 14–21-day intervals between each injection, unless otherwise indicated. Starting the same day as Spz inoculation, immunized and control animals were given TMP-SMX by oral gavage 18, 24, and 36–38 hours after the first infection, a regimen that prevents patent parasitemia without affecting blood-stage parasites. During the immunization regimen, the absence of parasitemia in immunized mice was confirmed by examination of Giemsa-stained slides of tail blood specimens obtained every other day. Mice immunized with live Spzs that received TMP-SMX prophylaxis were rested for a minimum of 4 weeks after the last Spz and drug administration. Mice were then challenged IV with Spz; the single asterisk (*) indicates that the challenge was 200 Spz IV with homologous *P. yoelii* 17XNL unless otherwise indicated. The presence of Spzs in the mosquitoes that had fed was verified by microscopy of their dissected salivary glands, and controls were implemented at each immunization and challenge to confirm the infectivity of mosquitoes. Blood-stage infection was determined by the presence of parasites in Giemsa-stained blood smears prepared daily during days 3–15 after challenge, or in one experiment, via liver parasite burden quantification by RT-qPCR. Abbreviation: PCR, polymerase chain reaction.

HBSS prior to excision. Single-cell suspensions were made by gentle mashing of the liver through a 70- μ M mesh screen, with suspensions then centrifuged at 400 $\times$ g for 7 minutes. The pellets were suspended in 15 mL of a 35% Percoll solution and centrifuged at 25°C at 500 $\times$ g for 10 minutes. The supernatant was then discarded, and the pellet was suspended in 5 mL of ACK lysing buffer for 3 minutes. HBSS was added, and the cells were centrifuged at 400 $\times$ g for 7 minutes twice, followed by suspension in complete medium [27].

With other whole-organism sporozoite immunization regimens, such as irradiated sporozoite immunization or other chemoprophylaxis vaccination regimens, mice develop protective CD8⁺ T-cell responses [11, 18, 28–30]. Specifically, circumsporozoite protein (CS)-specific T cells have been shown as sufficient to confer protection against sporozoite challenge in the irradiated sporozoite model [27]. To identify CD8⁺ T cells responding to TMP-SMX chemoprophylaxis vaccination, we used the H-2K^d-restricted immunodominant *P. yoelii* CS epitope SYVPSAEQI peptide (derived from *P. yoelii* CS₂₈₀₋₂₈₈) [31] as an indicator by measuring IFN- γ release by responding cells. Cells isolated from livers and spleens of euthanized mice that had undergone the regimen outlined in Figure 1 were stimulated in vitro with SYVPSAEQI peptide. Cells underwent surface staining and were fixed, permeabilized, and then stained for cytokines in the presence of permeabilization buffer. Responding CD8⁺ T cells were quantified by flow cytometry 4–6 weeks and 12–14 weeks after the third immunization, which parallels “early” and “late” challenge time points, as previously described [29].

Because CD8⁺ T cells involved in protection likely recognize *Plasmodium* antigens in addition to CS epitopes [32], we used the previously described CD11a^{hi}CD8 α phenotype, which has been validated as a surrogate for identification of the global CD8⁺ T-cell response to whole-organism vaccination [27].

Vaccine-induced CD8⁺ T-cell populations were characterized using the following methods, as previously described [27, 33]. Cells were first stained with Violet LIVE/DEAD fixable dead cell stain (Invitrogen) to exclude dead cells from the analysis. Fluorochrome-labeled antibodies against the following antigens were used to surface stain cells: CD90.2(30-H12) Brilliant Violet 570 (Biolegend), CD4 (RM4-5) V500 (BD Biosciences), CD8 (53–6.7) Brilliant Violet 650 (Biolegend), CD62L(MEL-14) APC/Cy7 (Biolegend), CD44 (IM7) PerCP/Cy5.5 (Biolegend), and CD11a (M17/4) FITC (eBioscience). Following fixation/permeabilization, IFN- γ (XMG1.2) was used in intracellular staining. Samples were analyzed on a BD LSR II flow cytometer, and data were analyzed with FlowJo software (Treestar).

Effects of Plasma From Immunized Mice on Parasite Development In Vivo

Sporozoite neutralization assay was performed as previously described, with few modifications [18]. Four thousand sporozoites

that had been preincubated with immune or control plasma (1:10 dilution) for 15 minutes at 37° were injected into each naive mouse. Parasitemia was monitored from days 3 to 15 after challenge.

Statistical Analysis

Kaplan–Meier survival curves statistics are descriptive [10, 18, 34]. Differences in parasite burden were compared using the Mann–Whitney or Kruskal–Wallis test with Dunn’s post test. Statistical analyses were performed using GraphPad Prism software (version 7).

RESULTS

TMP-SMX Prevents Patent Parasitemia by Killing Pre-erythrocytic Stage Parasites When Administered up to 36–38 Hours After Infection During Liver-Stage Parasite Development

Subinoculations of 100 μ L of blood per mouse were collected from mice on day 7 after live sporozoite immunization with TMP-SMX prophylaxis and injected into naive mice without resultant parasitemia (data not shown). In addition and in separate experiments, mice treated with TMP-SMX at 18, 24, and 36–38 hours from time 0 which would have paralleled sporozoite infection were infected with 10⁵ iRBCs at 46 hours, and no significant differences in parasitemia levels between control and treated groups on day 5 [15] were detected ($P > .05$; Figure 1 and Supplementary Figure 1).

TMP-SMX Chemoprophylaxis Vaccination Results in Induction of Sustained, Anti-infection, Stage-Specific Immunity Against Infectious Sporozoite Challenge

Mice receiving TMP-SMX during live sporozoite immunization with 3 doses (minimum 2) of 10 000 sporozoites intravenously (TMP-SMX chemoprophylaxis vaccination as per Figure 1) develop protection against challenge with 200 sporozoites intravenously at the early time point (4–6 weeks), as reflected by a reduced liver burden after challenge (Figure 2A). Protection against challenge at 4–6 weeks and challenges at later time points (including up to 8, 9, and 16 months) was also observed (Figure 2B). Immunization with only 1 dose of 10 000 sporozoites with TMP-SMX or with as few as 200 sporozoites 3 times did not result in any protection (data not shown). Immunity is stage specific as immunized mice challenged with iRBCs were not protected (Figure 2C). Of note, mice receiving TMP-SMX only and no live sporozoite immunization (ie, drug controls) had no significant difference in their challenge responses, compared with naive mice that received neither drug nor sporozoites (data not shown).

Protective Immunity Due to TMP-SMX Chemoprophylaxis Vaccination Is Primarily Dependent on CD8⁺ T-Cell Effector/Effector Memory Responses but Is Not Solely Dependent on IFN- γ Production

Depletion of CD8⁺ T cells prior to challenge resulted in the abrogation of protection induced by TMP-SMX chemoprophylaxis vaccination, whereas neither the depletion of CD4⁺ T cells at early or late challenge time points (Figure 3A) nor the depletion of IFN- γ at the early challenge time point (Figure 3B) abrogated

Figure 2. Trimethoprim-sulfamethoxazole (TMP-SMX) chemoprophylaxis vaccination results in the induction of sustained, anti-infection immunity against infectious sporozoite challenge directed against pre-erythrocytic parasites only. *A*, Groups of mice receiving TMP-SMX chemoprophylaxis vaccination as described in Figure 1 had protection assessed by examining the liver-stage parasite burden by real-time quantitative polymerase chain reaction (qPCR). Forty hours after sporozoite infection, livers were harvested, and total RNA was isolated. A significant difference was detected in the mean amount of parasite 18S ribosomal RNA in the livers from mice that received TMP-SMX chemoprophylaxis vaccination, compared with control mice ($P < .0001$, by the Mann–Whitney test). Results represent 1 experiment with 10 TMP-SMX chemoprophylaxis vaccination recipients and 20 controls, with a specimen from each recipient evaluated in triplicate by PCR. *B*, Groups of mice receiving TMP-SMX chemoprophylaxis vaccination as described in Figure 1 were challenged at 4 different time points (4–6 weeks and 8, 9, and 16 months), with protection assessed by determining the time to positive Giemsa-stained smears after challenge with 200 sporozoites intravenously; smears were examined daily on days 3–15 after challenge. Results represent pooled data from 4 different challenge time points (50 control mice are represented together in this figure, pooled from the 4 different challenge time points depicted). Mice challenged at 4–6 weeks and then again at 8, 9, and 16 months remained smear negative for parasites, compared with controls. *C*, Groups of mice received TMP-SMX chemoprophylaxis vaccination as described in Figure 1 but were then challenged with 10^5 infected red blood cells (iRBCs). Protection assessed by determining the time to positive Giemsa-stained smears after challenge with 200 sporozoites intravenously indicated that none of these animals were protected against iRBC challenge. Results represent pooled data of 2 separate experiments. Asterisks (*) indicate that these animals were challenged again. Abbreviations: CPS, chemoprophylaxis vaccination; rRNA, ribosomal RNA.

Figure 3. Protective immunity due to trimethoprim-sulfamethoxazole (TMP-SMX) chemoprophylaxis vaccination is primarily dependent on CD8⁺ T-cell responses but not on interferon γ (IFN- γ) production. *A*, Groups of mice receiving TMP-SMX chemoprophylaxis vaccination as described in Figure 1 also received anti-CD4, anti-CD8, or isotype control antibody, after which T-cell depletion was confirmed by flow cytometry. Depletion of CD8⁺ and CD4⁺ T cells by 98% was confirmed before challenge with 200 sporozoites (spz) intravenously. Protection was abrogated in mice receiving anti-CD8 antibody, as measured by determining the time to positive Giemsa-stained smears, which were performed daily from days 3 to 15 after challenge at early time points (4–6 weeks). In contrast, mice that received TMP-SMX chemoprophylaxis vaccination and anti-CD4 antibody did not differ in the time to development of patent parasitemia, compared with controls. Results shown represent pooled data from 2 separate experiments. Separate experiments with challenge at late time points (12–14 weeks) demonstrated similar results (data not shown). *B*, Groups of mice receiving TMP-SMX chemoprophylaxis vaccination as described in Figure 1 received either anti-IFN- γ antibody or isotype control, with confirmation of anti-IFN- γ depletion in a separate, parallel experiment that demonstrated reversal of protection by irradiated sporozoite immunization. Protection as assessed by determining the time to positive Giemsa-stained smears, performed every day from days 3 to 15 after challenge, demonstrated no significant difference between mice that received isotype control and those that received anti-IFN- γ antibody at the early challenge time points (4–6 weeks). *C*, Challenges with 200 spz IV at the early (4–6 week) timepoint were assessed and compared in groups of B-cell-knockout (KO) mice that were unimmunized or which received TMP-SMX chemoprophylaxis vaccination as described in Figure 1, and BALB/c mice which had undergone TMP-SMX chemoprophylaxis vaccination with parallel controls in parallel. B-cell-KO mice that received TMP-SMX chemoprophylaxis vaccination experienced only partial protection. Separate experiments with challenge at late time points (12–14 weeks) demonstrated similar results (data not shown).

Figure 4. Mice which have undergone trimethoprim-sulfamethoxazole (TMP-SMX) chemoprophylaxis vaccination have circumsporozoite protein (CS)-specific CD8⁺ T-cell effector/effector memory responses, as well as global CD11a^{hi}CD8 α responses. Given the significance of CD8⁺ T-cell responses, we further characterized these responses by using cells isolated from livers and spleens of mice that received TMP-SMX chemoprophylaxis vaccination [CPS] (Figure 1) and control mice. *A*, Shown is flow cytometry of CS-specific CD8⁺ T cells or CD8⁺ T cells identified by stimulation with CS SYVPSAEQI peptide and interferon γ (IFN- γ) production, characterized as having CD8 CD11a^{hi} CD62L^{lo} CD44^{hi}, or an effector/effector memory phenotype based on CD62L expression (cells isolated from spleen at time point parallel to early challenge time points (4–6 weeks)). These cells were absent in the spleens of naive mice but were present in those of immunized mice (top and bottom sections of panel *A*, respectively). Parallel results were found for cells isolated from spleens of mice at time points paralleling late challenge (12–14 weeks) and also from livers at time points paralleling early and late challenges (data not shown). *B*, Production of IFN- γ by these CD8⁺ T cells was absent from naive mice but evident in livers and spleens obtained from mice that underwent TMP-SMX chemoprophylaxis vaccination at time points paralleling early and late challenge (4–6 and 12 weeks, respectively), as represented by percent of CS-specific CD8⁺ T cells, calculated from CD8⁺ T cells identified by stimulation with CS SYVPSAEQI peptide and IFN- γ production/Total T cells (spleen, $P = .1499$; liver, $P = .3929$). *C*, Examination of the global CD8⁺ T-cell response (ie, CD11a^{hi}CD8 α T cells/Total CD8 T cells), revealed that cells isolated from livers and spleens of immunized mice had larger CD8⁺ T-cell responses, compared with control mice, at time points paralleling early challenge time points (4–6 weeks; spleen, $P \leq .01$; liver, $P = .0357$). Data are not shown for separate experiments on cells isolated from mice at time points that paralleled late challenge time point (12 weeks).

protection. B-cell-KO mice that were immunized were partially protected at early and late challenge time points (Figure 3C; data from the early challenge time point only are shown). Mice injected intravenously with sporozoites pre-incubated with plasma from immunized mice had no difference in the time to patent parasitemia, compared with controls (data not shown).

Given the significance of CD8⁺ T-cell responses, we further characterized these responses by using cells isolated from livers and spleens of TMP-SMX chemoprophylaxis vaccination-immunized mice, compared with control mice. CS-specific CD8⁺ T cells (ie, CD8⁺ T cells identified by stimulation with CS SYVPSAEQI peptide and IFN- γ production in the spleens of immunized mice but not naive mice) were characterized as having CD8 CD11a^{hi} CD62^{lo} CD44^{hi}, or an effector/effector memory phenotype based on CD62L expression (cells isolated from spleens of naive and immunized mice at time point parallel to early challenge time point, shown, Figure 4A; parallel results found for cells isolated from spleens of mice at time points

paralleling late challenge and also liver at time points paralleling early and late challenges, data not shown). Production of IFN- γ by these CD8⁺ T cells was evident in livers and spleens obtained 4–6 and 12 weeks after TMP-SMX chemoprophylaxis vaccination from immunized mice only (spleen, $P = .1499$; liver, $P = .3929$; Figure 4B). In addition, when examining the global CD8⁺ T-cell response (ie, the percentage of CD11a^{hi}CD8 α cells, calculated by dividing the number of CD11a^{hi}CD8 α T cells by the total number of CD8⁺ T cells), cells isolated from livers and spleens of immunized mice were noted to have larger populations of responding CD8⁺ T cells, compared with control mice, at both 4–6 and 12 weeks (spleen, $P \leq .01$; liver, $P = .0357$; Figure 4C).

TMP-SMX Chemoprophylaxis Vaccination Generates an Immune Response That Confers Protection Against Heterologous Challenge

Mice that were immunized with TMP-SMX chemoprophylaxis vaccination and challenged with the heterologous strains of *P. yoelii* 265BY and *P. berghei* were protected when challenged at early (shown) and late time points (Figure 5A and 5B), compared with controls.

TMP-SMX Chemoprophylaxis Vaccination Also Generates Protective Immunity When Infected Mosquitoes Are Used to Immunize

Incomplete protection was observed after 3 TMP-SMX chemoprophylaxis vaccination immunizations with iMBs, but complete protection was observed after 4 immunizations (Figure 6). The number of iMBs per immunization is summarized in [Supplementary Table 1](#).

DISCUSSION

Here, we have demonstrated in rodent models of malaria that repeated exposure to malaria parasites during TMP-SMX

Figure 5. Trimethoprim-sulfamethoxazole (TMP-SMX) chemoprophylaxis vaccination generates an immune response that confers protection against heterologous challenge. A, Groups of mice receiving TMP-SMX chemoprophylaxis vaccination as described in Figure 1 underwent heterologous challenge with 200 *Plasmodium yoelii* 265BY IV sporozoites. All mice were protected against heterologous challenge at early time points (4–6 weeks). Separate experiments with challenge at late time points (12–14 weeks) demonstrated that all mice were also protected (data not shown). B, Groups of mice receiving TMP-SMX chemoprophylaxis vaccination as described in Figure 1 underwent heterologous challenge with 200 *Plasmodium berghei* IV sporozoites. All mice were protected against heterologous challenge at early time points (4–6 weeks). Separate experiments with late challenge time points (12–14 weeks) demonstrated that all mice were also protected (data not shown).

Figure 6. Trimethoprim-sulfamethoxazole (TMP-SMX) chemoprophylaxis vaccination with infected mosquito bites generates an immune response that confers protection against homologous challenge. Mice were immunized with the bites of infected mosquitoes 3 or 4 times under TMP-SMX prophylaxis, with the timeline and TMP-SMX prophylaxis regimen for immunizations as described in Figure 1. Challenge at early time points (4–6 weeks after the third immunization or, in separate groups, 4–6 weeks after the fourth immunization) which shown. Mice were incompletely protected after immunization involving 3 bites under TMP-SMX prophylaxis but were completely protected after immunization involving 4 bites. The numbers of infected-mosquito bites per mouse are displayed in [Supplementary Table 1](#).

prophylaxis, or TMP-SMX chemoprophylaxis vaccination, induces long-lived protective antimalarial immunity directed against homologous and heterologous pre-erythrocytic-stage parasites that is mediated primarily by CD8⁺ T-cell-dependent responses. Our study is the first to evaluate the influence of clinically relevant concentrations of TMP-SMX prophylaxis on malaria parasite infection and immunity during live sporozoite administration in an animal model. Moreover, our study is the first to explore the induction of a malaria-specific immune response by use of a drug that is already widely used in malaria-endemic areas and in the highly vulnerable HIV-infected and HIV-exposed pediatric populations.

The in vivo depletion studies and the immunophenotyping of activated T cells performed with our TMP-SMX chemoprophylaxis vaccination model demonstrate similar immunity to that observed with irradiated sporozoite vaccination [14, 28, 35, 36]. While CS epitope-recognizing CD8⁺ T cells (effector/effector memory cells) and the global T-cell response (ie, CD11a^{hi}CD8 α cells) are both likely to be important for protection against sporozoite challenge [14, 28, 37–39], depletion of IFN- γ in our experiments yielded more mixed results, indicating that this cytokine may not be the sole mediator of protection in this model. We cannot rule out the possibility that direct lytic activity of CD8⁺ T or CD4⁺ T cells, or CD4⁺ T-cell mediated help for antibody production, as shown in humans and other rodent models [40], may play some role. There was partial loss of immunity in the B-cell-KO mice in our model, suggesting that the B-cell response may contribute to the protection observed. However, as B-cell-deficient mice have been shown to have a large expansion of $\gamma\delta$ T cells in the *Plasmodium chabaudi* malaria model [41, 42], it is possible that it was this response, rather than antibodies, that contributed to protection. Indeed, sporozoites preincubated with plasma from immunized mice resulted in the same prepatent period as in the control. The induction of heterologous protection and the longevity of immune protection we observed parallels findings in other models of whole-organism vaccination [14, 43], although the degree to which humans immunized with irradiated sporozoites [44] or chloroquine chemoprophylaxis vaccination [45] are protected against heterologous strains, or how our model translates into humans, requires further study.

We observed that delivery of fewer sporozoites intravenously did not induce immunity, although delivery of fewer sporozoites by iMBs did. These data are in agreement with previously published work and suggest that the immunity we observed is dose dependent [18] and that other mechanisms of protective immunity may be induced when sporozoites are delivered by mosquito bite [46]. Additionally, in contrast to chloroquine chemoprophylaxis vaccination, TMP-SMX chemoprophylaxis vaccination does not induce cross-stage protection [47, 48]. This may be due to differences in the antigens expressed at the time points at which the drugs inhibit the parasite:

TMP-SMX kills parasites at mid-to-late hepatic stages, whereas chloroquine kills emerging hepatic-stage merozoites [14].

Although it is known that TMP-SMX reduces clinical malaria burden [2], limited data are available examining how TMP-SMX prophylaxis and discontinuation affects malaria immunity [4]. A recent study described a rebound in malaria incidence in HIV-exposed, HIV-uninfected children who received TMP-SMX prophylaxis for 4 but not 2 years [4]. This could be attributed to the failure of TMP-SMX in preventing breakthrough infections (ie, from the liver to the bloodstream) due to high transmission and antifolate resistance in the area, resulting in inhibition of cell-mediated protective immune responses [8] and/or resultant T- and B-cell exhaustion [49].

Such studies, coupled with our data, highlight the need for further study on how TMP-SMX prophylaxis modifies anti-malarial immunity. Transmission intensity, preexisting immunity, antifolate resistance, and duration of TMP-SMX prophylaxis itself might influence this effect. Determination of the optimum timing and duration of TMP-SMX prophylaxis in this context should be prioritized.

Supplementary Data

Supplementary materials are available at <http://jid.oxfordjournals.org>. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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