Genetically Attenuated *Plasmodium berghei* Liver Stages Persist and Elicit Sterile Protection Primarily via CD8 T Cells

Ann-Kristin Mueller,* Martina Deckert,[†]
Kirsten Heiss,* Kristin Goetz,* Kai Matuschewski,*
and Dirk Schlüter[‡]

From the Department of Parasitology,* Heidelberg University School of Medicine, Heidelberg; the Department of Neuropathology,[†] University of Cologne, Köln; and the Institute for Medical Microbiology,[‡] OvG Universität Magdeburg, Magdeburg, Germany

Live-attenuated *Plasmodium* liver stages remain the only experimental model that confers complete sterile protection against malaria. Irradiation-attenuated *Plasmodium* parasites mediate protection primarily by CD8 T cells. In contrast, it is unknown how genetically attenuated liver stage parasites provide protection. Here, we show that immunization with *uis3*(–) sporozoites does not cause breakthrough infection in T and B-cell-deficient *rag1*^{–/–} and IFN- γ ^{–/–} mice. However, protection was abolished in these animals, suggesting a crucial role for adaptive immune responses and interferon- γ . Although *uis3*(–) immunization induced *Plasmodium*-specific antibodies, B-cell-deficient mice immunized with *uis3*(–) sporozoites were completely protected against wild-type sporozoite challenge infection. T-cell depletion experiments before parasite challenge showed that protection is primarily mediated by CD8 T cells. In good agreement, adoptive transfer of total spleen cells and enriched CD8 T cells from immunized animals conferred sterile protection against malaria transmission to recipient mice, whereas adoptive transfer of CD4 T cells was less protective. Importantly, primaquine treatment completely abolished the *uis3*(–)-mediated protection, indicating that persistence of *uis3*(–)-attenuated liver stages is crucial for their protective action. These findings establish the basic immune mechanisms underlying protection induced by genetically attenuated *Plasmodium* parasites and substantiate their use as vaccines against malaria. (Am J Pathol 2007, 171:107–115; DOI: 10.2353/ajpath.2007.060792)

Live-attenuated *Plasmodium* parasites remain the gold standard for malaria vaccine development because they confer long-lasting sterile protection against natural malaria transmission. In a rodent model and in human volunteers, immunization with irradiated sporozoites results in robust protective immune responses against subsequent challenges with infectious sporozoites.^{1–3} Presumably, attenuation of irradiated sporozoites occurs by a set of random mutations that eventually lead to a block in liver stage development. Because every immunized individual receives a different set of genetically nondefined parasites, this approach is limited only to experimental vaccine studies. In addition, if under-irradiated, sporozoites remain competent for liver stage development and induce blood stage infection, and if over-irradiated, sporozoites fail to induce protection.^{4,5} Despite these technical hurdles, immunization with irradiated sporozoites is an invaluable model system to dissect the effector and memory immune mechanisms that result in sustained sterile protection against malaria transmission.⁶ Ultimately, these studies and the identification of potential protective antigens may lead to the generation of potent pre-erythrocytic stage vaccines.⁷

Protection induced by irradiated sporozoites is essentially mediated by noncytolytic, interferon (IFN)- γ -producing CD8 T cells.^{8–13} Although liver stage-specific CD4 T cells and antibodies are also induced, they play a minor protective role compared with CD8 T cells.^{9,10,14,15} Interestingly, protection induced by irradiated sporozoites seems to be dependent on the per-

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Address reprint requests to Kai Matuschewski, Department of Parasitology, Heidelberg University School of Medicine, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany; or Dirk Schlüter, Institut für Medizinische Mikrobiologie, OvG Universität Magdeburg, Leipzigerstrasse 44, 39120 Magdeburg, Germany. E-mail: kai.matuschewski@med.uni-heidelberg.de and dirk.schluter@medizin.uni-magdeburg.de.

sistence of parasites in the liver, and there is evidence that this persistence provides the stimulus to maintain parasite-specific T cells in the liver.^{16,17} The importance of the local intrahepatic immune response is underscored by the finding that both adoptive transfer of intrahepatic and spleen-derived CD8 T cells isolated from mice immunized with irradiated sporozoites confers protection in naïve recipients challenged with virulent sporozoites.¹⁷

Recently, we developed defined genetically attenuated *Plasmodium* (GAP) parasites that constitute a reproducible and standardized source of potent live attenuated parasites.^{18,19} In these parasites, the liver stage-specific genes *UIS3* and *UIS4* were deleted, resulting in a block of complete maturation of intrahepatic parasites and thus preventing the development of the pathogenic blood stages. Importantly, mice immunized with *uis3*(-) sporozoites were completely protected against challenge infection with wild-type (WT) sporozoites.¹⁸

However, before GAPs can be translated to human malaria parasites and delivered as a vaccine to those in need in endemic countries, fundamental safety issues, such as occurrence of breakthrough infections in immunodeficient individuals, have to be addressed.²⁰ This is particularly important in endemic areas, where many individuals are immunocompromised because of the high prevalence of human immunodeficiency virus. To address these questions and to identify the underlying immune effector mechanisms that confer sterile protection after immunization with *uis3*(-) sporozoites, we performed experimental studies in immunodeficient *rag1*^{-/-}, B-cell-deficient *Igh-6*^{tm1Cgn/J} (μ MT), IFN- γ ^{-/-}, and immunocompetent mice.

Materials and Methods

Experimental Animals

For all experiments, C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) and isogenic *rag1*^{-/-},²¹ B-cell-deficient *Igh-6*^{tm1Cgn/J} (μ MT),²² and IFN- γ ^{-/-} mice (all from The Jackson Laboratory, Bar Harbor, ME), at the age of 50 to 80 days, were used. Animal care and experimental procedures were performed according to European regulations and approved by the state authorities (Regierungspräsidium Karlsruhe).

Immunization and Challenge Experiments

For transmission experiments, *P. berghei* sporozoites (NK65 strain) were extracted from the salivary glands of infected *Anopheles stephensi* mosquitoes. Sporozoites (10,000 and 50,000) were injected intravenously (i.v.) into mice. Immunization with *uis3*(-) parasites was performed for three times at 14-day intervals between each immunization. Challenge infections of *uis3*(-) immunized mice with WT sporozoites were performed 14 days after the final *uis3*(-) boost. The presence of blood stage parasites was determined by daily examination of Giemsa-stained blood smears and followed for at least 28 days. All immunization and challenge experiments were performed at least twice.

Primaquine (PQ) Treatment

At the indicated time points after *uis3*(-) immunization, animals were cleared of liver stage parasites by PQ treatment. PQ diphosphate (60 mg/kg; ICN Biochemicals, Aurora, OH) was injected subcutaneously for 3 consecutive days. *uis3*(-) immunized and PQ-treated animals were subsequently challenged with a dose of 50,000 live WT sporozoites 17 or 32 days later.

Isolation of Leukocytes from Liver and Spleen

Splenic leukocytes were isolated from sacrificed mice by passing spleens through a 70- μ m cell strainer (BD Biosciences, Heidelberg, Germany), and erythrocytes were lysed with ammonium chloride. Before isolation of hepatic leukocytes, animals were anesthetized with ketamine/xylazine (Essex, München, and Bayer Vital, Leverkusen, Germany) and intracardially perfused with 0.9% NaCl to remove contaminating intravascular leukocytes from the liver. Thereafter, liver tissue was minced through a 100- μ m cell strainer, and leukocytes were separated by Percoll gradient centrifugation (GE Healthcare, Freiburg, Germany) followed by lysis of erythrocytes with ammonium chloride.

Flow Cytometry

Liver-derived leukocytes were stained with rat anti-mouse CD4-fluorescein isothiocyanate (clone RM4-5) and rat anti-mouse CD8-phycoerythrin (clone 53-6.7). Control staining was performed with isotype-matched control antibodies. All antibodies were obtained from BD Biosciences. Flow cytometry was performed on a FACScan (BD Biosciences), and the data were analyzed with WinMDI or Cell Quest software (BD Biosciences).

T-Cell Depletion Experiments

For depletion of CD4 and/or CD8 T cells, mice were treated with either rat anti-mouse CD4 (clone GK1.5) and/or rat anti-mouse CD8 (clone PC61) antibodies, respectively. Antibodies were purified from tissue culture supernatants by protein G chromatography, adjusted to a concentration of 2.5 mg/ml in 0.1 mol/L phosphate-buffered saline (PBS), sterile-filtered, and stored at -80°C until used. Control mice were treated with rat IgG (Sigma Chemical Co., St. Louis, MO). Anti-CD4 and anti-CD8 antibodies were injected intraperitoneally at a concentration of 0.5 mg/ml per mouse at the indicated time points after injection. For the first 3 days of treatment, antibodies were injected daily. Thereafter, anti-CD4 and/or anti-CD8 antibodies were injected every 3rd day. Efficacy of CD4 and CD8 T-cell depletion was >95% as controlled by flow cytometry.

Adoptive Cell Transfer

Isolated spleen cells from *uis3*(-) immunized animals were either i.v. transferred as bulk splenocytes or as

purified CD4 plus CD8 T cells, purified CD4 T cells, or CD8 T cells to naïve recipient mice. Purification of T cells was performed with the Midi-MACS system (Miltenyi, Bergisch-Gladbach, Germany) using Pan-T-cell, CD4 T-cell, or CD8 T-cell isolation kits, respectively, as recommended by the manufacturer. The purity of the isolated T-cell population was >90% as controlled by flow cytometry.

Immunofluorescence Titration and Circumsporozoite (CSP) Enzyme-Linked Immunosorbent Assay (ELISA)

For titration of *P. berghei* sporozoite-specific antibody levels, salivary gland-associated sporozoites were incubated on glass slides and fixed. Mouse serum was titrated, and bound primary antibody was detected with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). CSP-specific antibodies were detected in mouse serum by a CSP-based ELISA. Microtitration plates (Immunolon 4HBX; Nunc, Wiesbaden, Germany) were coated with 15 µg/ml peptide (DPPPPNPN)₂D in PBS at 4°C overnight. Plates were then washed four times and blocked with 5% bovine serum albumin/PBS/0.05% Tween 20 at 37°C for 1 hour. Duplicates of sera were added in a 1:100 dilution in 2.5% bovine serum albumin/PBS/0.05% Tween 20 and incubated at 4°C overnight. A monoclonal CSP-specific antibody (clone 3D11) was used as control. Peroxidase-conjugated goat anti-mouse IgG (Fc-specific) (1:7000; Sigma Chemical Co.) was used as secondary antibody, followed by detection of bound antibody with tetramethylbenzidine (Sigma Chemical Co.) at 450 nm using an ELISA reader (Dynex Technologies, Berlin, Germany).

Histopathology

Immunohistochemistry was performed on frozen liver sections as described previously.²³ In brief, sections were stained with rat anti-mouse CD45 (clone M1/9.3.4.HL.2), Ly-6G (Gr-1; clone RB6-8C5), CD4 (clone G.K.1.5.), or CD8 (clone 2.43; all antibodies from the American Type Culture Collection, Manassas, VA) followed by peroxidase-linked sheep anti-rat IgG F(ab')₂ (GE Healthcare). In addition, sections were stained with rat anti-mouse F4/80 (clone F4/80; American Type Culture Collection), biotinylated mouse anti-rat IgG F(ab')₂ (Dianova, Hamburg, Germany), and streptavidin-biotin complex (DAKO, Hamburg, Germany). Peroxidase reaction products were visualized using 3,3'-diaminobenzidine and H₂O₂ as cosubstrate. Some sections were gently counterstained with hemalum.

Figure 1. The protective effect of *uis3*(-) sporozoites depends on host lymphocytes and IFN-γ. **A:** *Rag1*^{-/-}, *IFN-γ*^{-/-}, and C57BL/6 mice were infected i.v. with 10,000 *uis3*(-) or 10,000 WT sporozoites. Parasitemia was monitored by microscopy of Giemsa-stained blood smears at daily intervals for 28 days after injection. **B:** *Rag1*^{-/-}, *IFN-γ*^{-/-}, and C57BL/6 mice were immunized i.v. with three doses of 10,000 *uis3*(-) sporozoites or were left nonimmunized. Two weeks after the last immunization, both immunized and nonimmunized mice were challenged i.v. with 50,000 WT sporozoites. After infection with WT sporozoites, parasitemia was monitored by microscopy of Giemsa-stained blood smears at daily intervals for 28 days after infection. In **A** and **B**, each experimental group consisted of at least four animals

Results

Immunization with Genetically Attenuated Parasites Induces No Parasitemia in *rag1*^{-/-} and *IFN-γ*^{-/-} Mice but Fails to Confer Protection

We first addressed the question whether mice that are deficient in their adaptive immune responses or IFN-γ, the major protective cytokine in the model of irradiated sporozoites, are permissive for genetically attenuated parasites. Therefore, we infected *rag1*^{-/-}, *IFN-γ*^{-/-}, and isogenic immunocompetent C57BL/6 mice with 10,000 *uis3*(-) sporozoites i.v. and monitored blood stage parasitemia (Figure 1A). Importantly, *rag1*^{-/-} and *IFN-γ*^{-/-}

mice remained blood stage-negative throughout the monitoring period (28 days). In contrast, infection with WT *P. berghei* sporozoites resulted in 100% parasitemia in *rag1*^{-/-}, IFN- γ ^{-/-}, and WT mice. These findings indicate that breakthrough infections with *uis3*(-) parasites are unlikely to occur in individuals that lack functional B and T cells or IFN- γ .

These results permitted us to test whether sterile protection is achieved in *rag1*^{-/-} and IFN- γ ^{-/-} mice. We used an immunization protocol that confers 100% protection against WT *P. berghei* challenge infection in C57BL/6 mice¹⁸ (Figure 1B) and immunized *rag1*^{-/-} and IFN- γ ^{-/-} mice with two (Figure 1B) or three (data not shown) boosts of 10,000 *uis3*(-) sporozoites. Again, all *rag1*^{-/-} and IFN- γ ^{-/-} mice remained malaria-free, corroborating our findings above (data not shown). Importantly, when challenged with a high dose of 50,000 WT sporozoites 14 days after the final *uis3*(-) boost, all *rag1*^{-/-} and IFN- γ ^{-/-} animals developed blood stage parasitemia irrespective of prior *uis3*(-) immunization. In contrast, only nonimmunized immunocompetent C57BL/6 mice became patent (Figure 1B). The corresponding prepatent periods of *rag1*^{-/-} mice were delayed compared with naïve C57BL/6 mice, whereas 50% of IFN- γ ^{-/-} mice became patent already at day 3 after challenge. Collectively, these findings suggest that protection elicited by immunization with GAP parasites is mediated by adaptive immune responses and dependent on IFN- γ .

GAP Immunization Induces Sporozoite-Specific Antibodies, but B Cells Are Dispensable for Sterile Protection

To distinguish between the principal adoptive immune mechanisms that induce protection against WT parasite challenge infection, we tested whether immunization with *uis3*(-) parasites induces parasite-specific antibodies and whether B cells are essential for protection. After immunization of immunocompetent C57BL/6 mice with three doses of *uis3*(-) at 14-day intervals, all mice harbored antibodies specific for *P. berghei* (Figure 2A) and also for the major sporozoite surface protein of *P. berghei*, ie, the CSP (Figure 2B). In contrast, sporozoite- and

Figure 2. CSP-specific antibodies are produced after *uis3*(-) immunization, but B cells are dispensable for *uis3*(-)-induced protection against WT sporozoite infection. **A** and **B**: *Rag1*^{-/-}, B cell^{-/-}, and WT C57BL/6 mice were immunized i.v. with three doses of 10,000 *uis3*(-) sporozoites or were left nonimmunized. Two weeks after the last immunization, serum was obtained from both immunized and nonimmunized mice and anti-*P. berghei*-specific antibody titers were determined by an immunofluorescence assay (**A**), or CSP-specific antibodies were determined by ELISA (**B**). **C**: B cell^{-/-} and WT C57BL/6 mice were i.v. immunized with three doses of 10,000 *uis3*(-) sporozoites or were left nonimmunized. Two weeks after the last immunization, both immunized and nonimmunized mice were i.v. challenged with 50,000 WT sporozoites. After infection with WT sporozoites, parasitemia was monitored microscopically of Giemsa-stained blood smears at daily intervals for 28 days after injection. **D**: IFN- γ ^{-/-} and WT C57BL/6 mice were i.v. immunized with three doses of 10,000 *uis3*(-) sporozoites or WT mice were left nonimmunized. Two weeks after the last immunization, both immunized and nonimmunized mice were challenged i.v. with 50,000 WT sporozoites. At day 7 after WT sporozoite infection, serum was obtained, and anti-*P. berghei*-specific antibody titers were determined by an immunofluorescence assay. In **A** to **D**, each circle represents one mouse. In **C**, horizontal bars show the mean values.

CSP-specific antibodies were not detected in B-cell-deficient and *rag1*^{-/-} control mice (Figure 2, A and B).

To test whether B cells play a decisive role for protection induced by *uis3*(-) sporozoites, B-cell-deficient (μ MT) and isogenic immunocompetent mice were immunized with three doses of 10,000 *uis3*(-) parasites and challenged with 50,000 WT sporozoites 14 days after the last booster immunization. All immunized B-cell-deficient and WT mice were protected against the WT challenge infection and did not develop parasitemia (Figure 2C). In contrast, *uis3*(-)-immunized IFN- γ ^{-/-} mice challenged with WT sporozoites had a strong anti-parasitic B-cell response (Figure 2D) but inevitably succumbed to WT sporozoite infection as shown in Figure 1B. These findings show that although parasite-specific antibodies are induced by GAP immunization, B cells are neither an essential component of protection against WT sporozoite challenge infection nor protective in the absence of IFN- γ .

T-Cell Depletion Indicates a Major Role of CD8 T Cells

We next determined whether T cells confer protection on immunization with GAP parasites. We immunized C57BL/6 mice with three doses of 10,000 *uis3*(-) parasites at 14-day intervals. Beginning 3 days before challenge with 50,000 WT sporozoites, we depleted total CD4 and/or CD8 T cells by intraperitoneal injection of the respective antibodies. Four groups of immunized mice were studied: 1) those receiving rat IgG as a negative control antibody or those who were depleted of 2) CD4 and CD8 T cells, 3) CD4 T cells, or 4) CD8 T cells (Figure 3). After WT sporozoite challenge, which was performed 14 days after the final booster immunization, all animals treated with rat IgG remained blood stage-negative, whereas all mice depleted of CD4 plus CD8 T cells became patent with prepatent periods similar to naïve animals. This observation indicates that protection is largely mediated by T-cell-dependent mechanisms. Notably, protection was also completely abolished in mice that were depleted of CD8 T cells only, whereas 9 of 11 CD4 T-cell-depleted animals remained protected (Figure 3). Therefore, CD8 T cells seem to play a predominant role in protection against sporozoite challenge.

Adoptive Transfer of Isolated CD8 T Cells Confers Protection to Recipient Animals

To confirm that T-cell-mediated immunity directly protects against sporozoite infection, we performed adoptive transfer experiments. In these experiments, isolation of cells from immunized mice was performed 14 days after the third immunization with *uis3*(-) sporozoites. First, we isolated whole spleen cells from C57BL/6 mice that were either naïve or immunized with three doses of *uis3*(-) parasites. We transferred 2×10^7 spleen cells i.v. into naïve recipient C57BL/6 mice and challenged these animals with 50,000 WT sporozoites 1 day after adoptive

Figure 3. Depletion of CD8 T cells abolishes the protective effect of *uis3*(-) immunization. WT mice were immunized i.v. with three doses of 10,000 *uis3*(-) sporozoites. Two weeks after the last immunization, mice were treated with anti-CD4, anti-CD8, anti-CD4 plus anti-CD8, or rat IgG control antibodies. Thereafter, mice were challenged i.v. with 50,000 WT sporozoites, and antibody treatment was continued. After infection with WT sporozoites, parasitemia was monitored daily by microscopy of Giemsa-stained blood smears for 28 days after injection. Each experimental group included at least five mice.

transfer (Figure 4A). Only animals having received spleen cells from *uis3*(-)-immunized animals were protected against WT sporozoite challenge.

Next, we performed adoptive transfer experiments with magnetic cell sorting-purified splenocytes from *uis3*(-)-immunized animals. An adoptive transfer of 4×10^6 CD4/CD8 T cells conferred protection to all recipient animals (Figure 4B). However, a twofold reduction of the T-cell population resulted in blood stage parasitemia in only two of four recipient animals. Notably, CD4 T cells constitute a larger proportion of this mixed T-cell population (55% CD4⁺ and 39% CD8⁺ T cells).

To identify further the most potent T-cell subset, we selectively purified CD4 or CD8 T cells (Figure 4B). Transfer of 2×10^6 CD4 T cells resulted in parasitemia of two infected animals, while three animals remained protected. Although parasitemia of the patent animals was delayed by 2 days compared with naïve animals, these results suggested that CD4 T cells are unlikely to be the major effector T-cell population that confers consistent sterile protection against GAP parasites. In contrast, an adoptive transfer of 2×10^6 purified CD8 T cells to naïve recipient animals resulted in 100% protection against sporozoite-induced malaria (Figure 3B), which is in good agreement with our depletion experiments. These findings further support the notion that sterile protection is primarily mediated by CD8 T cells.

*Persistence of *uis3*(-) Parasites and Inflammatory T Cells in the Liver after Immunization*

To analyze further the protective effect of *uis3*(-) immunization on sporozoite induced malaria, we performed a histopathological analysis of the liver. Remarkably, at day 14 after the third *uis3*(-) immunization, small foci of inflammatory infiltrates, which were either located periporally or scattered throughout the liver parenchyma, were

Figure 4. Adoptive transfer of CD8 T cells from *uis3*(-) immunized mice protects naïve recipient mice against WT sporozoite infection. **A:** Leukocytes were isolated from spleens of *uis3*(-) immunized (isolation at day 14 after the final booster immunization) and nonimmunized mice (three mice each). Leukocytes of each group were pooled and 2×10^7 cells were injected i.v. into three naïve mice. **B:** CD4 plus CD8 T cells, CD4, or CD8 T cells were isolated from spleens of *uis3*(-) immunized mice, and cells were transferred i.v. to naïve recipient mice in numbers indicated. Each group of recipient mice included four or five animals. In **A** and **B**, mice were infected i.v. with 50,000 WT sporozoites 24 hours after adoptive transfer. Parasitemia was monitored daily by microscopy of Giemsa-stained blood smears for 28 days after injection.

still present (Figure 5A). In contrast, nonimmunized animals harbored reduced numbers of intrahepatic CD45⁺ leukocytes (Figure 5B). Notably, leukocytes consisted mainly of CD4 and CD8 T cells and macrophages. We failed to detect Ly-6G⁺ granulocytes, suggesting a specific response rather than a general inflammatory reaction. In addition, flow cytometry revealed that numbers of intrahepatic CD4 and CD8 T cells were increased in *uis3*(-) immunized animals, in good agreement with refractoriness to natural malaria transmission (Figure 5C).

We finally addressed whether sterile protection depends on parasite persistence. Previous studies showed that targeted elimination of irradiation-attenuated liver stages by treatment with the causally prophylactic drug PQ resulted in loss of protection against challenge infection over time.^{16,24} To test whether elimination of *uis3*(-) parasites by PQ treatment also abolishes protection induced by GAPs, we treated *uis3*(-) immunized mice with

Figure 5. Persistence of inflammatory infiltrates in the liver of immunized mice. **A:** A small intrahepatic CD45⁺ leukocyte infiltrate (arrow) at day 14 after the last immunization with *uis3*(-) sporozoites. **B:** In contrast to immunized mice, only single CD45⁺ leukocytes (arrow) are present in the hepatic parenchyma of a normal mouse. **A** and **B:** Labeling with anti-CD45 antibodies; original magnification, $\times 200$. **C:** Intrahepatic leukocytes were isolated from three *uis3*(-) immunized mice 2 weeks after the last immunization dose and from nonimmunized mice. Isolated leukocytes were stained with anti-CD4-phycoerythrin and anti-CD8-fluorescein isothiocyanate and analyzed by flow cytometry, and the total number of CD4 and CD8 T cells per liver is shown.

Table 1. PQ Treatment of Attenuated Liver Stages Ablates Protection

Mice	PQ treatment [†]	Sporozoite challenge [‡]	No. protected/ no. challenged (prepatency) [§]
Naïve	—	+	0/2 (day 4)
<i>Uis3</i> (–) immunization*	—	+	2/2 (no infection)
	+	Day 14	0/4 (day 6)
	+	Day 32	0/4 (day 3.5)

*Immunization was by three doses of 25,000 *uis3*(–) sporozoites.

[†]PQ treatment was by daily s.c. injection of 60 mg/kg PQ for three consecutive days at day 17 or 32 after the last booster immunization.

[‡]WT sporozoite challenge was by i.v. injection of 50,000 sporozoites at the indicated days after PQ treatment (+, control challenge).

[§]The prepatent period is defined as the time until the first appearance of a single erythrocytic stage in Giemsa-stained blood smears.

PQ 7 days after the final boost and challenged these animals with 50,000 WT sporozoites 14 or 32 days later (Table 1). All PQ-treated mice developed parasitemia between day 6 (for early challenge) and day 3 (for later challenge) after WT challenge infection. These findings strongly indicate that the persistence of *uis3*(–) parasites is a prerequisite for their protective capacity.

Discussion

This study is the first characterization of the basic immune effector mechanisms that mediate sterile protection in genetically attenuated *Plasmodium* parasites. Importantly, T and B cell-deficient *rag1*^{–/–} as well as IFN- γ ^{–/–} mice survived an infection with *uis3*(–) sporozoites without developing parasitemia. These findings clearly illustrate that the developmental arrest of *uis3*(–) sporozoites is independent of lymphocytes and IFN- γ . In addition, *uis3*(–) sporozoites did not cause a breakthrough infection in individuals unable to develop a pathogen-specific T and B-cell response or those deficient in IFN- γ production, which is of considerable importance for their potential use in humans. The safety profile of *uis3*(–) sporozoites is further supported by the fact that experiments were performed in C57BL/6 mice, the most susceptible mouse strain to infection with *P. berghei*.²⁵

The observation that, in contrast to naive animals, *rag1*^{–/–} mice infected with three doses of *uis3*(–) sporozoites did not prevent parasitemia after WT sporozoite challenge infection indicates that the protective effect of *uis3*(–) immunization is mediated by pathogen-specific lymphocytes. The delay of 2 days in parasitemia of *rag1*^{–/–} mice compared with naive WT mice after infection with WT sporozoites (Figure 1A) may be explained by a strong activation of innate immune responses, eg, by natural killer cells, in *rag1*^{–/–} mice. Several lines of evidence indicate that CD8 T cells are the major player in protection against WT *P. berghei* challenge infection. First, depletion of CD8 T cells resulted in parasitemia in 100% of WT sporozoite-challenged mice. Second, adoptive transfer of CD8 T cells protected all mice against parasitemia after WT sporozoite infection. These findings are consistent with data from irradiated sporozoites. In

the latter model, protection against parasitemia is also mediated by CD8 T cells, which rapidly secrete IFN- γ after WT sporozoite infection.^{8–13,24} Because in our experiments both CD8 T cells and IFN- γ were critically important to prevent parasitemia of *uis3*(–)-immunized mice after WT sporozoite challenge infection, it seems likely that IFN- γ production of CD8 T cells plays also an important role in our model.

Previous studies in BALB/c mice have identified an H-2K^d-restricted major histocompatibility complex class I epitope, derived from the CSP, in both *P. yoelii* and *P. berghei*.^{26,27} To identify potential H-2^b-restricted CSP-derived major histocompatibility complex class I or II epitopes permitting the performance of more detailed studies on the parasite-specific T-cell response in C57BL/6 mice, we generated a PepSpot library consisting of 12-amino-acid peptides spanning the whole CSP including the signal peptide. This technique has previously been used successfully to identify novel CD4 and CD8 T-cell epitopes of pathogens, including *Listeria monocytogenes*.²⁹ We tested splenocytes of C57BL/6 mice immunized with three doses with *uis3*(–) sporozoites with this PepSpot library in a standard IFN- γ enzyme-linked immunosorbent spot assay^{28,29} but failed to detect any CSP-specific T cells (data not shown). This observation, if confirmed by other techniques, may imply that different *Plasmodium* antigens serve as protective antigens in genetically diverse individuals. This would also be a strong argument for the use of whole organism vaccines, especially GAPs, since they include the most complete range of protective antigens that may be recognized by genetically diverse individuals.^{20,30}

In contrast to CD8 T cells, both depletion as well as adoptive transfer experiments reveal a minor role for CD4 T cells, which again is in agreement with studies from irradiated sporozoites.⁹ In our experiments, approximately 80% of *uis3*(–)-immunized mice depleted of CD4 T cells remained protected against parasitemia after WT challenge (Figure 2). In addition, only 50% of mice were protected against WT challenge after adoptive transfer of CD4 T cells derived from *uis3*(–)-immunized mice (Figure 3).

Furthermore, *uis3*(–) sporozoites induced an antibody response against sporozoites, including the major surface antigen of *P. berghei*, ie, CSP. However, the complete protection of *uis3*(–)-immunized B cell-deficient mice against high-dose WT challenge infection illustrates that antibodies are not crucial for protection (Figure 2) which is in accordance with data from the model of irradiated sporozoites.^{14,15,31} In addition, *uis3*(–)-immunized IFN- γ ^{–/–} mice developed sporozoite-specific antibodies but were not protected against a WT sporozoite challenge infection, illustrating that antibodies cannot compensate for IFN- γ deficiency. However, parasite-specific antibodies might reduce the spread of WT sporozoites from the site of inoculation to the liver by neutralization,⁸ and thus the functional role of B cells in the control of sporozoites before liver infection may vary depending on the experimental model system and may become more relevant on continuous low-dose natural exposure in endemic areas. In addition, a very recent elegant study

addressed the contribution of CSP in protective immunity using an engineered transgenic *P. yoelii* CSP-tolerant mouse system.³² In this model, PyCSP-specific immune responses, including neutralizing antibodies, were an important yet dispensable determinant for sterile protection. Based on these findings in combination with our failure to identify a H-2^D-restricted epitope in CSP, we propose that GAPs may provide an important tool to identify immune correlates of protection against pre-erythrocytic stages of *Plasmodia*.

Previous experiments in the model of irradiated sporozoites have illustrated that the protection against WT sporozoite infection depends on the persistence of irradiated sporozoites in the liver.¹⁶ Accordingly, targeted elimination of irradiated sporozoites by liver stage-specific compounds, eg, PQ, results in a loss of protection against challenge infection.^{16,24} In our model of GAP-induced protection, PQ treatment resulted in a loss of protection against WT sporozoite infection. This argues for a critical role of persisting GAPs for protection against WT challenge infection. Importantly, protection against WT challenge infection disappeared rapidly within 17 days after PQ treatment. This is in contrast to irradiated sporozoites. In the latter model, immunity wanes over many months, and even 6 months after PQ treatment, some mice were still be protected, which is compatible with a gradual decline of parasite-specific T cells over time.²⁴ In our model, the rapid decline of protection may indicate that, in addition to parasite-specific CD8 and, to a lesser extent, CD4 T cells, the local hepatic inflammatory milieu maintained by persisting parasites plays an important role for the long lasting protection induced by *uis3*(-) immunization. In fact, we have observed that mice are protected for more than 1 year after three doses of *uis3*(-) sporozoites against WT challenge infection, if not treated with PQ after immunization (A.-K.M. and K.M., unpublished data).

In conclusion, the present study provides the basis to explore further the GAP-induced mechanisms that protect the host from natural malaria transmission and supports the follow-up of GAPs as the most potent vaccines against malaria.

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