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MAJOR ARTICLE

Genetically Attenuated *Plasmodium berghei* Liver Stages Induce Sterile Protracted Protection That Is Mediated by Major Histocompatibility Complex Class I–Dependent Interferon-γ–Producing CD8$^+$ T Cells

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(See the article by Tarun et al., on pages 608–16.)

At present, radiation-attenuated plasmodia sporozoites (γ-spz) is the only vaccine that induces sterile and lasting protection in malaria-naive humans and laboratory rodents. However, γ-spz are not without risks. For example, the heterogeneity of the γ-spz could explain occasional breakthrough infections. To avoid this possibility, we constructed a double-knockout *P. berghei* parasite by removing 2 genes, *UIS3* and *UIS4*, that are up-regulated in infective spz. We evaluated the double-knockout *Pbuis3(−)/4(−)* parasites for protective efficacy and the contribution of CD8$^+$ T cells to protection. *Pbuis3(−)/4(−)* spz induced sterile and protracted protection in C57BL/6 mice. Protection was linked to CD8$^+$ T cells, given that mice deficient in β_2m were not protected. *Pbuis3(−)/4(−)* spz–immune CD8$^+$ T cells consisted of effector/memory phenotypes and produced interferon-γ. On the basis of these observations, we propose that the development of genetically attenuated *P. falciparum* parasites is warranted for tests in clinical trials as a pre-erythrocytic stage vaccine candidate.

Malaria claims millions of lives annually [1], and a malaria vaccine is urgently needed [2]. Although pre-erythrocytic stage subunit vaccines are promising, it is unclear whether they will engage the required components of the innate and adaptive immune responses to confer long-term protection. Following the report [3] that immunization with x-irradiated *Plasmodium gallinaceum* sporozoites (spz) confers protective immunity, the use of radiation-attenuated *P. falciparum* spz (*Pf* γ-spz)—nonreplicating, live parasites—as an effective vaccine has been demonstrated in humans [4].

At present, *Pf* γ-spz are the only vaccine inducing lasting and sterile protection in malaria-naive subjects of diverse HLA backgrounds [5]. The use of radiation for spz attenuation is, however, not without risk, because it yields heterogeneously attenuated spz. This process is also radiation dose sensitive [6], and underirradiated spz remain infectious, whereas overirradiated spz are not sufficiently immunogenic to prevent infection [6, 7]. These problems prompted a search for other forms of attenuation that would render the parasite a more reliable vaccine.

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Recently, we described the development of 2 genetically attenuated *P. berghei* parasites, each with a targeted disruption of a single but different gene up-regulated in infective spz and thus designated *UIS3* [8] and *UIS4* [9]. Both genes are essential for the development of liver-stage parasites. Although the protein encoded by the *UIS4* gene localizes to the parasitophorous vacuole (PV) membrane [9], *UIS3* interacts with liver–fatty acid–binding protein within hepatocytes [10]. Immunizations of C57BL/6 mice with either *P. berghei UIS3 (Pbuis3[−])* or *UIS4 (Pbuis4[−])* mutants conferred protection against wild-type (WT) homologous challenge [8, 9].

A single-gene knockout parasite might not be suitable as a vaccine for humans, because it could give rise to breakthrough infections. To safeguard against this possibility, we developed a double-knockout parasite, *Pbuis3(−)/4(−)* parasite in which both genes, *UIS3* and *UIS4,* were deleted (K.M., data not shown). The question remained, however, whether the double-knockout parasite is sufficiently immunogenic to induce protection. The mechanism of induction and maintenance of protection by the genetically attenuated plasmodia is unexplored, although it likely stems from the inability of arrested early liver-stage parasites to develop into mature liver-stage schizonts [8, 9]. We and others [11, 12] have shown that treatment with primaquine, a drug that disrupts liver-stage parasites and, hence, prevents the expression of protein antigens, results in the loss of γ-spz–induced long-term protection in rodents and a shorter time to reinfection in humans [13]. It is believed, therefore, that proteins from the arrested liver-stage parasites provide the key antigens required for the induction of effector $CD8^+$ T cells and, possibly, for the maintenance of protection by memory $CD8^+$ T cells [14].

The evidence that $CD8^+$ T cells are the sine qua non effectors against liver-stage infection comes from the observations that adoptively transferred γ-spz–immune $CD8^+$ T cells confer protection [15] and that γ-spz do not protect mice deficient in $CD8^+$ T cells, either as a result of in vivo depletion of these cells [16] or β_2m [17] or MHC class I (K^bD^b) disruption [14]. In the *Pf*γ-spz model, both $CD8^+$ cytotoxic T lymphocyte [18] and interferon (IFN)–γ responses are critical for protection against liver-stage parasites, and most of the current focus has shifted toward cytokine-producing $CD8^+$ T cells. We have demonstrated that *Pb*γ-spz–induced long-lasting, protective immunity is major histocompatibility complex (MHC) class I dependent [17] and is accompanied by the presence of $CD8^+$ effector memory (T_{EM}) and central memory (T_{CM}) cells in the liver [12]. Whereas $CD8^+$ T_{EM} cells produce IFN-γ in response to infectious challenge, $CD8^+$ T_{CM} cells undergo homeostatic proliferation (U.K., personal observations) and thereby form the reservoir of memory T cells [14].

In the present study, we tested the protective efficacy of *Pbuis3(−)/4(−)* spz against *P. berghei* spz challenge. We also determined whether protection induced by *Pbuis3(−)/4(−)* spz involves MHC class I molecules and $CD8^+$ T cells. The results demonstrate, to our knowledge for the first time, that, similar to *Pb*γ-spz, *Pbuis3(−)/4(−)* spz induced intrahepatic $CD8^+$ T_{EM} and T_{CM} cells and that *Pbuis3(−)/4(−)* spz induced IFN-γ–producing $CD8^+$ T cells that were recalled even after rechallenge at 6 months. This and other studies [8, 9, 19] showing that genetically attenuated plasmodia spz are efficient at inducing and maintaining protective immunity support our efforts to develop genetically attenuated *P. falciparum* spz as a pre–erythrocytic-stage vaccine for human use.

MATERIALS AND METHODS

Mice. Female C57BL/6 and $\beta_2m^{-/-}$ (6–8 weeks old) were purchased from Jackson Laboratory and were housed at Walter Reed Army Institute of Research (WRAIR) and Seattle Biomedical Research Institute animal facilities and handled according to institutional guidelines. All procedures were reviewed and approved by the Animal Care and Use Committees of both institutes and were performed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Generation and propagation of spz. We generated a *Pbuis3(−)/4(−)* double-knockout strain by targeting the UIS4 locus in the *uis*3(−) mutant parasite line [8] with a second selectable marker (human dihydrofolate reductase). Details of the double gene disruption will be published elsewhere (K.M., data not shown). The phenotypic analysis of the *Pbuis3(−)/4(−)* parasite revealed no impairment of blood-stage development, sporogeny, salivary gland invasion, or hepatocyte invasion. Liver-stage development was completely arrested, as shown elsewhere [8, 9].

For *P. berghei* WT or *Pbuis3(−)/4(−)* spz production, *Anopheles stephensi* mosquitoes were fed on gametocyte-infected mice. Then, spz were dissected [12] from the salivary glands of mosquitoes 16–21 days after the blood meal and were used either immediately or after attenuation with gamma radiation (15,000 rad; Cesium-137 source Mark 1 series or Cobalt-60 Model 109; JL Shepard).

Immunizations. Mice were primed (iv) with 75,000 of either *Pb*γ-spz [12] or *Pbuis3(−)/4(−)* spz followed by 2 boost immunizations of 20,000 homologous spz 1 week apart and were challenged with 10,000 infectious spz 1 week later. In some experiments, mice were rechallenged 6 months after challenge. In addition, various regimens of immunization and challenge were performed with *Pbuis3(−)/4(−)* spz. These included 3 immunizations of 10,000 *Pbuis3(−)/4(−)* spz given 1 week apart followed by a challenge of 10,000 infectious spz on day 7 or 118 after the last immunization.

Table 1. Protection of C57BL/6 *Pbuis3(−)/4(−)* sporozoite (spz)–immunized mice against challenge with wild-type (WT) *Plasmodium berghei* spz.

Priming[a]	Boosts[a]	Challenge dose (time point)[b]	No. protected/ no. challenged[c]
75,000	20,000 (7)/20,000 (14)	10,000 (7 and 180)	27/27 (7); 6/6 (180)
10,000	10,000 (14)/10,000 (28)	10,000 (7)	14/14
10,000	10,000 (14)/10,000 (28)	10,000 (118)	14/14

NOTE. An age-matched naive control group was included in each experiment, and these mice all became blood-stage patent at day 5–7 after challenge (data not shown).

[a] Data are no. of spz used for priming and the first and second boost immunizations (day of boost).

[b] Mice were challenged with infectious *P. berghei* WT spz. Time points in parentheses indicate the day of challenge after the final boost.

[c] Data are results from representative experiments; however, >100 mice were immunized with different doses of *Pbuis3(−)/4(−)* spz and remained protected against challenge with infectious *P. berghei* spz.

Thin blood smears were prepared from individual mice starting on day 2 after challenge, and parasitemia was determined microscopically using Giemsa stain. Mice were considered protected if parasites were not detected in 40 fields by day 14 after challenge.

Cell preparation. At various time points after immunization, mice were euthanized by CO_2 inhalation. Livers were perfused with 10 mL of PBS, removed, and pressed through a 70-μm nylon cell strainer (BD Labware), and the cell suspension was processed as described elsewhere [12]. Briefly, cells were resuspended in PBS that contained 35% Percoll (Amersham Pharmacia Biotech) and centrifuged at 800 *g* for 20 min. Erythrocytes were lysed with lysis buffer (Sigma), and the remaining hepatic mononuclear cells (HMCs) were resuspended in complete RPMI 1640 medium. Spleens were removed aseptically, and single-cell suspensions were prepared as described above. For isolation of peripheral blood mononuclear cells (PBMCs), venous blood was collected into microtone tubes that contained K_2 EDTA (BD Biosciences). Erythrocytes were lysed, and the remaining PBMCs were washed in PBS and resuspended in complete RPMI 1640 medium.

Flow cytometry. Four-color staining of HMCs, spleen cells, or PBMCs was performed using a combination of the following monoclonal antibodies (MAbs): fluorescein isothiocyanate (FITC)–conjugated anti-CD45RB (16A), phycoerythrin (PE)–conjugated anti-CD44 (IM7), peridinin-chlorophyll-protein–conjugated anti-CD8a (Ly-2), and allophycocyanin (APC)–conjugated anti-CD44 (IM7) (BD Biosciences). Briefly, 2–10 $\times 10^5$ cells were resuspended in cold assay buffer (PBS containing 1% bovine serum albumin [BSA; Sigma] and 0.01% sodium azide) and incubated with anti-FcR 24G2 (BD Biosciences) and 0.5 μg of the relevant MAb for 30 min at 4°C. Cells were washed and resuspended in cold assay buffer. Flow cytometry was performed on a FACSCalibur (BD Biosciences), and data analysis was performed using CellQuest (version 3.3; BD Biosciences) or FlowJo software (version 8.1.0; Tree Star).

IFN-γ secretion assay. IFN-γ–producing $CD8^+$ T cells were detected as described elsewhere [12] using a secretion assay in accordance with the manufacturer's instructions (Miltenyi Biotec). Briefly, 1×10^6 cells were resuspended in 90 μL cold assay buffer (PBS with 2 mmol/L EDTA and 0.5% BSA) that contained 10 μL of mouse IFN-γ capture reagent and incubated on ice for 5 min. Cells were resuspended in 10 mL of RPMI and incubated for 45 min at 37°C in 5% CO_2 under continuous shaking. After washing, the cells were resuspended in 90 μL of cold buffer that contained 10 μL of PE-labeled IFN-γ detection reagent, 1 μL of anti-CD3 FITC, and 1 μL of anti-CD8 APC and were incubated on ice for 10 min. After washing, the cells were resuspended in 500 μL of cold buffer. Then, 10 μL of 7-amino–actomycin D (25 μg/mL) was added to the cell suspension, and the sample was immediately analyzed by flow cytometry.

Antibody determinations. Serum from *Pbuis3(−)/4(−)* spz-immune WT and $\beta_2m^{-/-}$ mice was tested for circumsporozoite (CS) protein–specific antibodies by ELISA. Briefly, 2-fold serial dilutions of serum were dispensed into duplicate wells that had been previously coated with *P. berghei* glutathione S-transferase–CS protein (provided by Dr. E. Angov, WRAIR). The plates were incubated for 1 h at 22°C. After washes, 100 μL of anti-mouse IgG alkaline phosphatase conjugate (1 μg/mL) was added and incubated for 1 h at 22°C. After washes, 100 μL of alkaline phosphatase substrate was added and incubated for 1 h at 22°C. The reaction was stopped with 10% SDS/H_2O, and the plates were read on an ELISA plate reader (SPECTRAmax M2; Molecular Devices) using SoftmaxPro software (version 4.7.1; Molecular Devices) at 450 nm.

Statistical analysis. Data are presented as the means ± SDs, and the differences among groups were analyzed by the

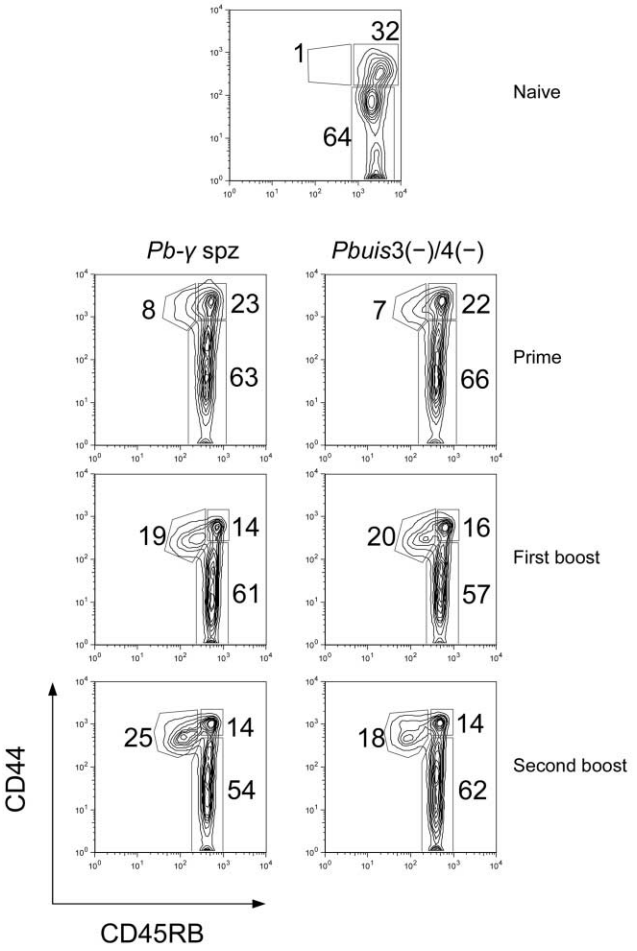

Figure 1. Induction by radiation-attenuated *Plasmodium berghei* sporozoites (Pbγ-spz) and *Pbuis3(−)/4(−)* spz of similar populations of phenotypically distinct subsets of $CD8^+$ T cells. *A,* Hepatic mononuclear cells isolated from livers of 3 individual naive or 3 immune mice 6 days after the indicated immunization and analyzed by flow cytometry. Lymphocytes labeled with anti-CD8 monoclonal antibodies (MAbs) were gated on a forward/side-scatter plot, and gates were applied to identify $CD8^+$ T cells. $CD8^+$ T cell subsets were revealed using anti-CD45RB and anti-CD44 MAbs, and percentages of subsets are shown as a dot plot from a representative mouse. The experiments were performed 3 times with 3 mice/group, and cells from individual mice were assayed. Data in the text are the mean ± SD of responses observed in 9 individual mice.

Mann-Whitney *U* test using Graphpad Prism software (version 4.0c). $P < .05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Sterile protection after immunization with* Pbuis3(−)/4(−) *spz. *P. berghei* parasites deficient in a single gene induce protection in mice [8, 9, 19]. However, single-knockout parasites may not be sufficiently attenuated and may compensate for loss of a single gene, resulting in an occasional breakthrough infections [9, 19]. To preclude this possibility, we constructed a novel double-knockout *Pbuis3(−)/4(−)* strain and tested its ability to confer sterile protection against homologous WT spz challenge in C57BL/6 mice. Mice were immunized with 75,000, 20,000, and 20,000 *Pb*γ-spz or *Pbuis3(−)/4(−)* spz 1 week apart and challenged with 10,000 spz after the last immunization. In some cases, mice were rechallenged 6 months after the primary challenge. Whereas naive mice became parasitemic within 5–7 days after challenge, both *Pb*γ-spz- and *Pbuis3(−)/4(−)* spz–immune mice were fully protected against primary and secondary (day 180) challenges (table 1). A further investigation showed that 3 immunizations with 10,000 *Pbuis3(−)/4−)* spz also conferred full protection at challenge 118 days later (table 1).

These data show for the first time that multiple immunizations with double-knockout *Pbuis3(−)/4(−)* spz confer sterile and long-lasting protection against *P. berghei* liver-stage infection. Collectively, >100 mice remained solidly (100%) protected. Experiments are in progress to assess the duration of protective immunity beyond the initial 6-month period.

Hepatic CD8⁺ T_{EM} cells induced by* Pbuis3(−)/4(−) *spz. *Pb*γ-spz enter hepatocytes, where they undergo aborted development into liver-stage parasites that induce sterile immunity characterized by the presence of activated/memory hepatic $CD8^+$ T cells. Because the knockout parasites also colonize hepatocytes [8, 9], we sought to determine whether *Pbuis3(−)/4(−)* spz–induced protective immunity against *P. berghei* liver-stage infection is also associated with the presence of activated/memory hepatic $CD8^+$ T cells.

Hepatic and splenic $CD8^+$ T cells isolated from *Pb*γ-spz– and *Pbuis3(−)/4(−)* spz–immune mice were analyzed for the expression of the activation-related surface markers, CD44 and CD45RB, at different time points after priming and boost immunizations. Consistent with our previous observations with *Pb*γ-spz [12], *Pbuis3(−)/4(−)* spz–induced hepatic $CD8^+$ T cells that consisted of 2 distinct populations: $CD8^+$ T_{CM} cells ($CD44^{hi}CD45RB^{hi}$) and $CD8^+$ T_{EM} cells ($CD44^{hi}CD45RB^{lo}$) (figure 1). Naive liver $CD8^+$ T cells contained a negligible percentage and number of $CD8^+$ T_{EM} cells, but ~30% already exhibited a T_{CM} cell phenotype. After priming, the *Pbuis3(−)/4(−)* spz–induced $CD8^+$ T_{EM} cells represented 10% ± 2% of the hepatic $CD8^+$ T cells, and after the last boost immunization they increased to 28% ± 5% (figure 1), whereas $CD8^+$ T_{CM} cells concomitantly decreased from 25% ± 2% after priming to 17% ± 2% after boost immunization. The percentage of each $CD8^+$ T cell subset at each time point examined was remarkably similar between the *Pb*γ-spz– and *Pbuis3(−)/4(−)* spz–immune mice (figure 1). As previously reported for *Pb*γ-spz [12, 20], only a small percentage (< 10%) of $CD8^+$ T_{EM} cells was found in the spleens of *Pbuis3(−)/4(−)* spz–immune mice (data not shown), thus confirming the enrichment of $CD8^+$ T_{EM} cells in the liver, a nonlymphoid organ, which is the site of liver-stage malaria infection.

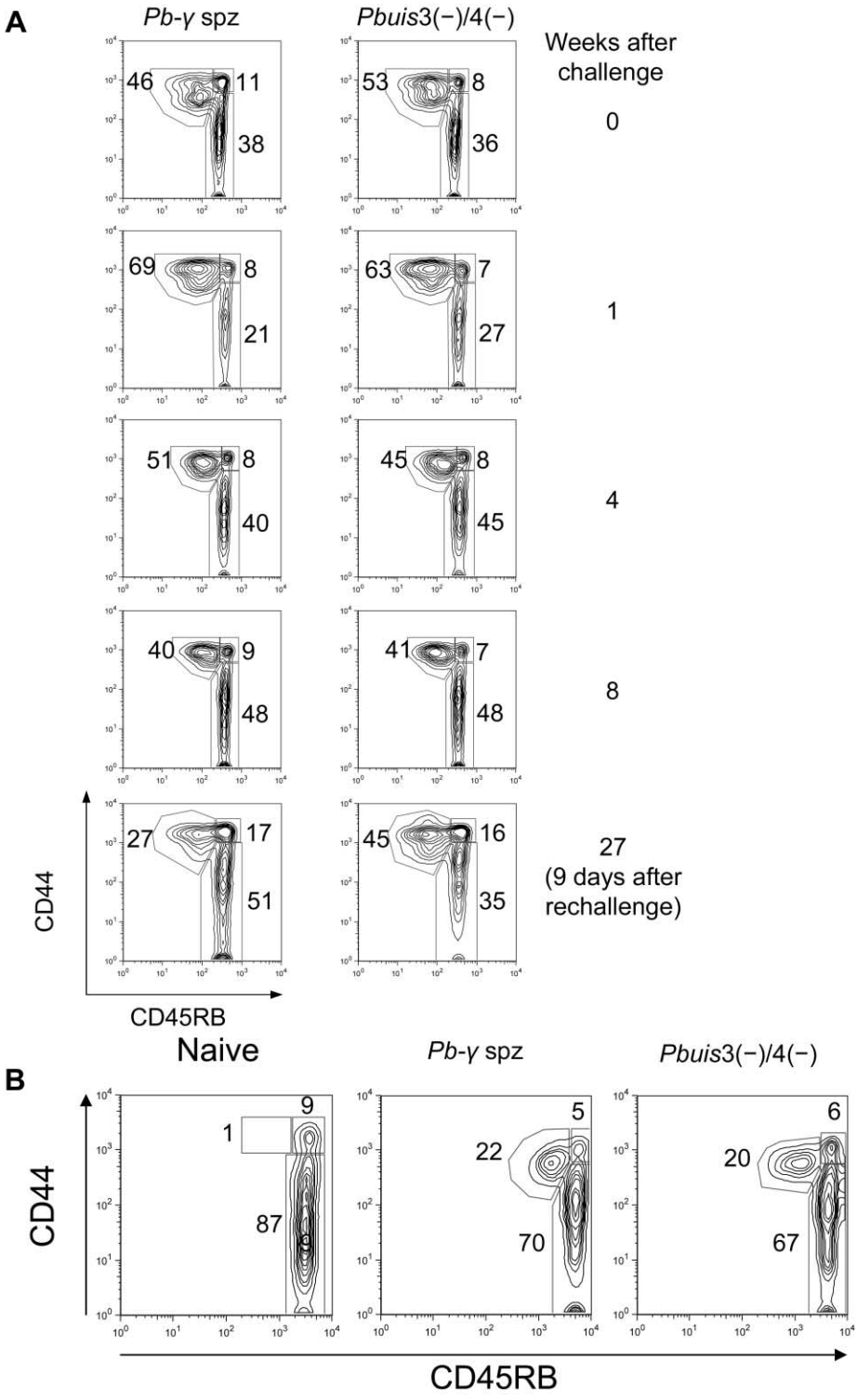

Figure 2. CD8+ T effector memory cells maintained after challenge and rechallenge of radiation-attenuated *Plasmodium berghei* sporozoites (Pbγ-spz) and *Pbuis3(−)/4(−)* spz–immunized mice. *A,* Hepatic mononuclear cells isolated from livers of individual mice at the indicated time points after immunization and challenge and analyzed as described in figure 1. The percentages of CD8+ T cell subsets are shown as a dot plot from a representative mouse. The experiments were performed 3 times with 3 mice/group, and cells from individual mice were assayed. The data in the text are shown as the mean ± SD of responses observed in 9 individual mice. *B,* Mononuclear cells isolated from peripheral blood of naive or immune mice 6 days after the second boost with *Pb*γ-spz or *Pbuis3(−)/4(−)* spz, and analyzed as described for panel A. Results are from 1 of 3 representative experiments.

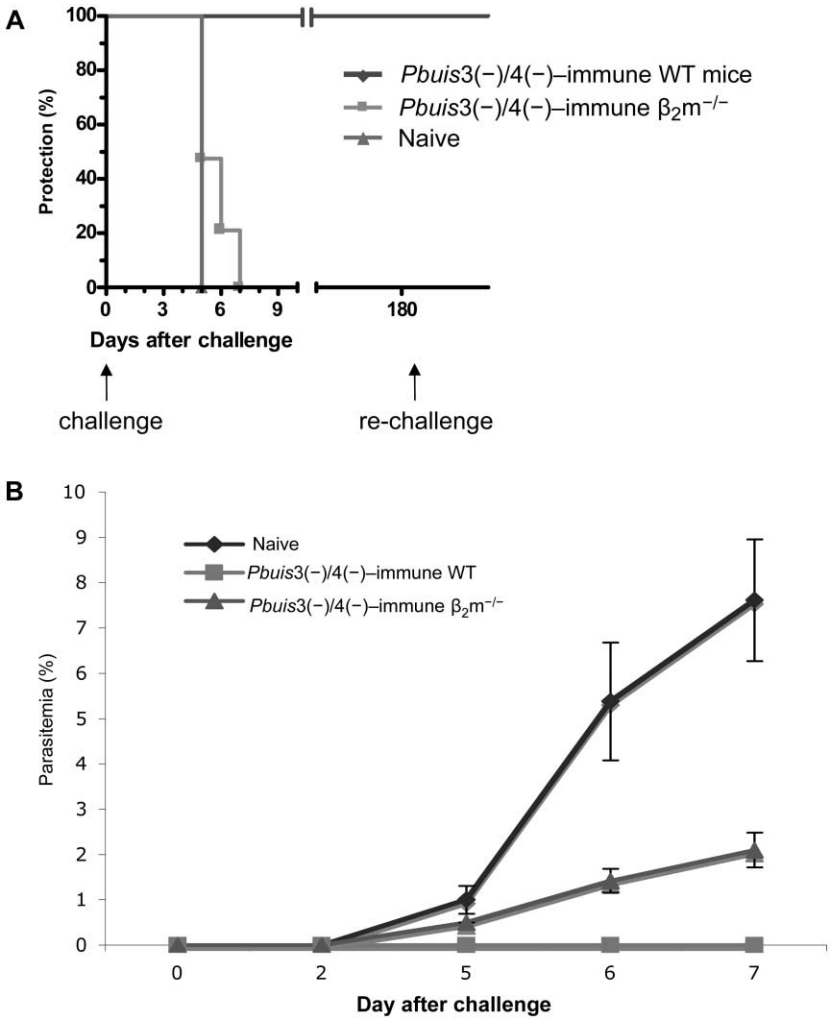

Figure 3. No induction of protective immunity by genetically attenuated *Plasmodium berghei* in $\beta_2m^{-/-}$ mice. Wild-type (WT; $n = 5$) and $\beta_2m^{-/-}$ mice ($n = 19$) were immunized with 75,000, 20,000, and 20,000 *Pbuis3(−)/4(−)* sporozoites (spz) administered 1 week apart. One week after the final immunization, mice were challenged with 10,000 infectious *P. berghei* spz. Naive mice ($n = 5$) were used as infectivity controls. WT mice were rechallenged with 10,000 infectious *P. berghei* spz 6 months after the first challenge. Parasitemia was monitored by microscopy of Giemsa-stained blood smears every other day, starting 2 days after challenge. *A,* Percentage of mice remaining parasite free at the indicated time points. *B,* Average ± SE percentage of parasitized red blood cells per mouse. Immunized WT mice are indicated in by squares, immunized $\beta_2m^{-/-}$ mice by triangles, and naive infectivity controls by diamonds.

Protracted protection induced by* Pbuis3(−)/4(−) *spz and associated with the persistence of hepatic CD8$^+$ T_{EM} cells. We asked whether similar to *Pb*γ-spz–induced immunity [12], the *Pbuis3(−)/4(−)* spz–induced hepatic CD8$^+$ T_{EM} cells are maintained during protracted protection. In a longitudinal study, we measured the levels of CD8$^+$ T_{EM} and T_{CM} cells at the indicated time points after the challenge and rechallenge (figure 2*A*). One week after challenge, the accumulation of CD8$^+$ T_{EM} cells peaked (~60%) in both groups of mice, owing to either recruitment from the CD8$^+$ T_{CM} cells, to influx of extrahepatic cells into the liver, or to both. Numbers of hepatic CD8$^+$ T_{EM} cells in both groups decreased after the first week, presumably due to attrition, as observed during infection [21]. At 8 weeks after challenge, percentages of CD8$^+$ T_{EM} cells remained at ~40% in both groups (figure 2*A*). To examine the recall of memory responses, mice were rechallenged at 6 months, and CD8$^+$ T cells were analyzed for subset distribution. In the *Pb*γ-spz–protected mice, 25% ± 6% of hepatic CD8$^+$ T cells were CD8$^+$ T_{EM} cells, whereas, in *Pbuis3(−)/4(−)* spz–protected mice, CD8$^+$ T_{EM} cells represented 42% ± 5% of the total liver CD8$^+$ T cells. The differences, however, were not statistically significant.

We also detected ~20% of circulating CD8$^+$ T_{EM} cells in blood 6 days after spz challenge of both *Pbuis3(−)/4(−)* spz– and *Pb*γ-spz–immune mice but not in the blood of naive mice (figure 2*B*). These observations demonstrate the feasibility of

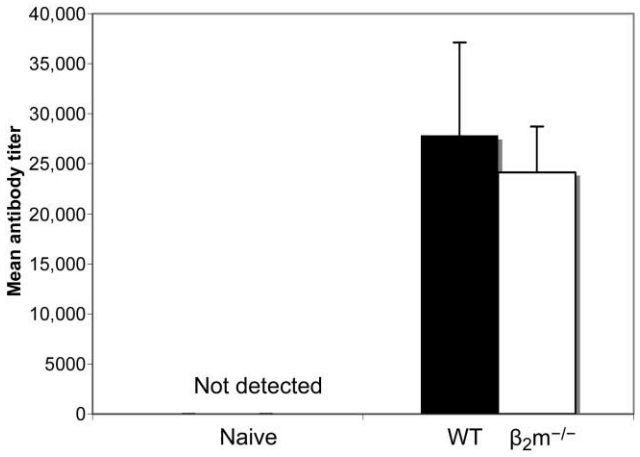

Figure 4. Similar levels of anti–*Plasmodium berghei* circumsporozoite (CS) protein antibodies in $\beta_2m^{-/-}$ and wild-type (WT) mice. $\beta_2m^{-/-}$ ($n = 5$) and WT ($n = 5$) mice were immunized with 75,000, 20,000, and 20,000 *Pbuis3(−)/4(−)* sporozoites administered 1 week apart. Serum samples were obtained 6 days after tertiary immunization and assayed for anti–*P. berghei* CS protein antibodies by ELISA. Serum samples from naive mice served as negative controls. *Black bar,* WT mice; *white bar,* $\beta_2m^{-/-}$ mice

using peripheral blood $CD8^+$ T cell subsets as a surrogate indicator of hepatic $CD8^+$ T cells in human subjects participating in malaria vaccine trials, including those planned with the double-knockout *P. falciparum* spz.

Mediation by CD8⁺ T cells of protection against liver-stage infection in* Pbuis3(−)/4(−) *spz–immunized mice. The relevance of the $CD8^+$ T cells to *Pbuis3(−)/4(−)*spz–induced protection is not known; therefore, we investigated their contribution using the $CD8^+$ T cell–deficient $\beta_2m^{-/-}$ mouse model. *Pbuis3(−)/4(−)* spz–immunized WT and $\beta_2m^{-/-}$ mice were challenged with 10,000 spz 1 week after the last immunization. As expected, *Pbuis3(−)/4(−)* spz–immune WT mice remained sterilely protected. However, consistent with our observations with *Pb*γ-spz [17], all of the *Pbuis3(−)/4(−)* spz–immunized $\beta_2m^{-/-}$ mice became parasitemic by day 7 after challenge (figure 3*A*). In contrast to naive WT mice, which developed parasitemia by day 5, the *Pbuis3(−)/4(−)* spz–immunized $\beta_2m^{-/-}$ mice had a slight delay in the onset of parasitemia, and by day 5 only 50% of mice were parasitemic. In addition, although the level of parasitemia in both groups was 1% at day 5, it increased in the WT naive mice to 8% on day 7, whereas it remained at 2% in the $\beta_2m^{-/-}$ mice (figure 3*B*).

Although the failure to achieve sterile protection in $\beta_2m^{-/-}$ mice likely stems from the absence of surface expression of MHC class I molecules and, hence, $CD8^+$ T cells, we wanted to rule out a possible defect in the antibody response as a contributor to this failure. It has been well established that *Pb*γ-spz–induced protection is multifactorial [22] and that $CD4\ T^+$ helper cells [22] and B cells [23] play a significant role in mediating protective immunity. *P. berghei* CS protein–specific antibody titers of *Pbuis3(−)/4(−)* spz–immunized $\beta_2m^{-/-}$ and WT mice were 24,150 and 27,875, respectively, and these differences were not statistically significant (figure 4). We presume that the delayed onset and lower level of parasitemia seen in *Pbuis3(−)/4(−)* spz–immunized $\beta_2m^{-/-}$ mice were controlled in part by the CS protein–specific antibodies. This observation confirms previous findings from the *Pb*γ-spz [24] and *Py*-spz

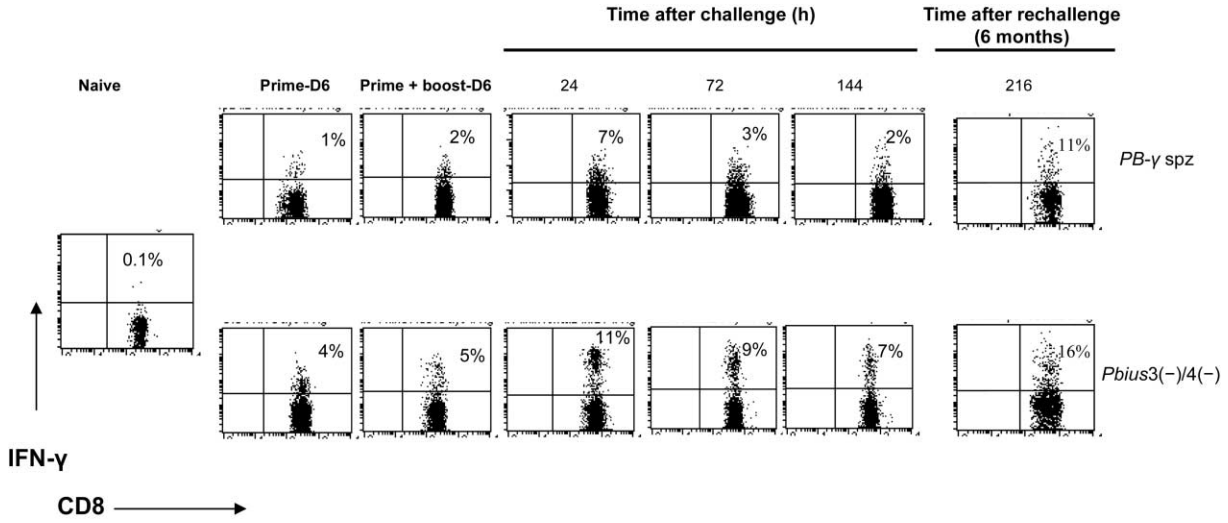

Figure 5. Higher frequency of interferon (IFN)–γ–producing $CD8^+$ T cells in the liver of *Plasmodium berghei Pbuis3(−)/4(−)* sporozoites (spz)–immunized vs. Pbγ-spz–immunized mice. Hepatic mononuclear cells (HMCs) were isolated 6 days after both prime and prime-boost immunizations and at 24, 72, 144, and 216 h after challenge from livers of *Pb*γ-spz– or *Pbuis3(−)/4(−)* spz–immunized mice. IFN-γ–secreting $CD8^+$ T cells was identified by fluorescent labeling using an IFN-γ secretion assay (see Materials and Methods). The percentage of IFN-γ–secreting T cells in the gated $CD3^+CD8^+$ T cell populations was determined by flow cytometry and is indicated in the upper quadrants. Dots plots are representative of 3 mice/group.

[25] models that, although essential, antibody responses alone cannot mediate protection.

Efficient IFN-γ production by* Pbuis3(−)/4(−) *spz–immune CD8⁺ T cells. The exact mechanism by which $CD8^+$ T cells confer protection is still not understood, although it is known that IFN-γ can mediate the destruction of liver-stage infection [16, 26]. We [12] and others [27] have demonstrated that IFN-γ–producing liver $CD8^+$ T cells are linked to both induction and persistence of protective immunity. To determine whether *Pbuis3(−)/4(−)* spz–induced $CD8^+$ T cells functioned similarly, we analyzed IFN-γ–producing $CD8^+$ T cells after prime-boost immunizations with *Pbuis3(−)/4(−)* spz and after infectious challenge. Immunizations with either *Pb*γ-spz or *Pbuis3(−)/4(−)* spz induced IFN-γ–producing hepatic $CD8^+$ T cells (figure 5). However, priming with *Pbuis3(−)/4(−)* spz induced a 4-fold higher response than that induced by *Pb*γ-spz (4% ± 1% vs. 1% ± 0.2%), and, after boost immunization, a 2.5-fold increase (5% ± 1% vs. 2% ± 1%) was still evident. The peak response in both groups occurred at 24 h after challenge, although the percentage of IFN-γ–producing $CD8^+$ T cells at this time was 1.6-fold higher in the *Pbuis3(−)/4(−)* spz– than in the *Pb*γ-spz–immune–challenged mice (11% ± 2% vs. 7% ± 2%). The *Pbuis3(−)/4(−)* spz–immune–challenged mice continued to exhibit a more robust response than the *Pb*γ-spz–immune–challenged mice at 72 and 144 h after challenge with an ~3-fold higher percentage of IFN-γ–producing $CD8^+$ T cells at both time points (9% ± 0.5% vs. 3% ± 0.6% and 7% ± 0.2% vs. 3% ± 0.5% at 72 and 144 h, respectively). The fluorescence data also revealed a higher intensity of IFN-γ production by the *Pbuis3(−)/4(−)* spz–induced $CD8^+$ T cells than in *Pb*γ-spz–immune $CD8^+$ T cells. Likewise, the attrition or contraction of the IFN-γ–producing $CD8^+$ T cells was lower in *Pbuis3(−)/4(−)* spz–immune than in *Pb*γ-spz–immune mice. To determine the recall of memory responses, mice in both groups were rechallenged at 6 months, and $CD8^+$ T cells were analyzed for IFNγ production. In *Pbuis3(−)/4(−)* spz–immune mice, 19% ± 2% of the hepatic $CD8^+$ T cells produced IFN-γ, whereas, in *Pb*γ-spz–immune mice, IFN-γ–producing $CD8^+$ T cells represented 14% ± 6% of liver $CD8^+$ T cells. The differences, however, were not statistically significant. IFN-γ–producing hepatic $CD8^+$ T cells were not detected in the *Pbuis3(−)/4(−)* spz–immune–challenged $\beta_2 m^{-/-}$ mice (data not shown).

The data presented here show for the first time that genetically attenuated *P. berghei* spz with double gene deletions induced protective immunity that was long-lived and linked to MHC class I–dependent, IFN-γ–producing hepatic memory $CD8^+$ T cells. Although both *Pb*γ-spz and *Pbuis3(−)/4(−)* spz promoted differentiation of $CD8^+$ T cells into phenotypically similar T_{EM} and T_{CM} cell subsets, collectively, the data suggest that *Pbuis3(−)/4(−)* spz might be superior for the induction of protection. Although the mechanisms for this improved efficacy remain to be investigated, a number of scenarios could be considered. First, the *Pbuis3(−)/4(−)* spz might be more immunogenic than *Pb*γ-spz, owing to differences in the state of the genes between the 2 parasite strains. Genetic attenuation disrupts specific genes, creating homogeneously arrested parasites, whereas attenuation by gamma radiation may damage genes randomly, leading to the loss of highly antigenic proteins. In addition, it is possible that genetic arrest is at a stage that best reflects the repertoire of protective antigens against subsequent transmission. By contrast, only a subpopulation of γ-spz might arrest at this point. Hence, relative to *Pb*γ-spz, the *Pbuis3(−)/4(−)* spz could produce a broader spectrum of parasite antigens, some with high binding affinities to MHC class I molecules, which might be reflected in the robust IFN-γ response.

The *Pbuis3(−)/4(−)* spz–derived proteins might engage antigen processing and presentation pathways more efficiently than proteins derived from *Pb*γ-spz. It is relevant that the *UIS4*-encoded protein is associated with the PV membrane [9] that surrounds the parasite subsequent to its invasion of hepatocytes. Although Py*uis4(−)* parasites form a PV membrane (S.H.I.K., data not shown), it is likely that the PV membrane has compromised function and may not protect the parasite from the host's intracellular proteolytic enzymes; instead, it might allow for a provision of a broader universe of antigenic proteins. Both the *Pyuis3(−)* and *Pyuis4(−)* parasites disappear by 40 h after invasion (S.H.I.K., data not shown), which might be partly explained by the leaky PV membranes surrounding the *Pbuis3(−)/4(−)* parasites. In turn, it is also possible that the leaky membranes might trigger early apoptosis of the invaded hepatocytes, thus leading to efficient cross-presentation by dendritic cells (DCs), of a wide spectrum of these liver-stage antigens [19]. Alternatively, some of these antigens may also be exported by the hepatocyte, possibly in the form of exosomes and subsequently taken up by DCs for presentation to $CD8^+$ T cells [28]. It should be also noted that long-term retention of parasite antigens, possibly in the form of antigen-antibody complexes bound to follicular DCs could account for protracted recruitment of $CD8^+$ T cells subsequent to the demise/disappearance of the parasite.

Three doses of 10,000 *Pbuis3(−)/4(−)* can protect against an infectious spz challenge given 3 months after the last boost immunization without an intermittent infectious spz challenge. In conclusion, these are compelling data in support of the genetically attenuated plasmodia organisms as a pre-erythrocytic vaccine candidate and, thus, the development of genetically attenuated *P. falciparum* parasites for the use in phase Ia trials in humans is fully warranted.

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