Tailoring a Combination Preerythrocytic Malaria Vaccine

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The leading malaria vaccine candidate, RTS,S, based on the Plasmodium falciparum circumsporozoite protein (CSP), will likely be the first publicly adopted malaria vaccine. However, this and other subunit vaccines, such as virus-vectored thrombospondin-related adhesive protein (TRAP), provide only intermediate to low levels of protection. In this study, the Plasmodium berghei homologues of antigens CSP and TRAP are combined. TRAP is delivered using adenovirus- and vaccinia virus-based vectors in a prime-boost regime. Initially, CSP is also delivered using these viral vectors; however, a reduction of anti-CSP antibodies is seen when combined with virus-vectored TRAP, and the combination is no more protective than either subunit vaccine alone. Using an adenovirus-CSP prime, protein-CSP boost regime, however, increases anti-CSP antibody titers by an order of magnitude, which is maintained when combined with virus-vectored TRAP. This combination regime using protein CSP provided 100% protection in C57BL/6 mice compared to no protection using virus-vectored TRAP alone and 40% protection using adenovirus-CSP prime and protein-CSP boost alone. This suggests that a combination of CSP and TRAP subunit vaccines could enhance protection against malaria.

There are approximately 3.4 billion people at risk of malaria infection, 207 million cases and 627,000 deaths annually (1). An effective vaccine could have a greater impact than any other intervention (2, 3), and yet such a vaccine remains elusive.

Sterile protection against blood-stage malaria infection in both animal models and humans can be obtained by vaccination with whole radiation-attenuated sporozoites (spz) (4–6) or genetically attenuated parasites (7–11) incapable of developing beyond the liver stage. Difficulties associated with cost, production, and deployment of whole-parasite malaria vaccines to regions where malaria is endemic make it unlikely that such vaccines will play a central role in the control or eradication of malaria in the near future.

Subunit vaccines, consisting of single or multiple antigens from various stages of the malaria parasite, have been a focus of research development. These include the preerythrocytic-stage antigens circumsporozoite (CS) protein (12) and thrombospondin-related adhesive protein (TRAP) (13), the blood-stage antigens MSP-1 (14, 15), AMA-1 (16), and RH-5 (17), and the Plasmodium vivax antigen Duffy binding protein (18, 19); the transmission-blocking antigens PfS25, Pvs25, PfS230, and Pf48/45 have also been investigated as potential subunit vaccines (20–23).

The current leading malaria vaccine candidate, RTS,S, is a subunit vaccine undergoing phase III clinical trials in Africa (12). This vaccine consists of part of the CS protein of Plasmodium falciparum malaria fused to the hepatitis B virus surface antigen (HBsAg) and coexpressed in yeast with HBsAg. The vaccine is administered as a protein-in-adjuvant formulation. The most recent results indicate that administering three doses of RTS,S protects 37% of infants (24) and 47% of children (12) against severe malaria.

Adenoviral-poxxviral prime-boost protocols have been developed to maximize protective efficacy using viral-vectored vaccines (25). Viral vectored vaccines using chimpanzee adenoviral vector (ChAd63) or modified vaccinia strain Ankara (MVA) to deliver antigens show great promise, stimulating high T-cell responses (26–28). Multi-epitope TRAP (ME.TRAP) antigen delivered using virus-vectored vaccines produces very high levels of sterile protection in rodents (29), and in a recent phase IIa clinical trial (27) it was determined that this vaccine in a ChAd63-MVA prime-boost regime induced sterile protection in 21% of human volunteers.

With less than half of human volunteers seeing protective effects in recent trials, there is clearly a requirement for an improved, potent malaria vaccine. One potential improvement could be in combining two subunit vaccines to achieve enhanced protection. This is the approach explored here, using two of the leading malaria vaccine candidates, CSP and TRAP, and a commonly used murine model of malaria using Plasmodium berghei (30); murine models represent an inexpensive and useful way to examine vaccines in a preclinical setting before progression to human trials. CSP is involved in parasite motility and attachment and invasion of the liver of the vertebrate host (31). The first demonstration of anti-CSP antibody (Ab)-mediated protection was in P. berghei (32), and CD8+ T cells also play a role (33). TRAP also facilitates invasion of the liver (34, 35) and is involved in parasite motility (35, 36); P. berghei TRAP-specific CD8+ T cells have been shown to inhibit the liver stage (37).

Either CSP or TRAP used individually in a vaccine provides suboptimal levels of protection. In this study, their combination was tested and optimized.

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**MATERIALS AND METHODS**

**Protein expression and purification.** The mammalian codon optimized *P. berghei* CSP and TRAP genes were cloned into the pHSeC plasmid with the His tag at the 3’ end under the control of CMV enhancer and chick beta-actin promoter (38). DNA constructs were produced in *Escherichia coli* DH5α (Life Technologies) cells and purified using an endotoxin-free Plasmid Mega Kit (Qiagen). HEK-293T cells were transiently transfected using a DNA-polyethylenimine mix. This resulted in secreted proteins with N-terminal ETG and C-terminal GTK(His)6 tags. The conditioned media with secreted proteins were dialyzed against phosphate-buffered saline (PBS), and proteins were purified by immobilized Co2+-affinity chromatography, followed by size exclusion chromatography in 20 mM Tris-HCl (pH 8.0)–300 mM NaCl. Protein size was verified by Western blotting with the Pentahis monoclonal primary antibody (1:1,000 dilution; Qiagen) and goat anti-mouse IgG peroxidase-conjugated secondary antibody (1:2,000; Sigma).

**Animals.** The age-matched, 6-week-old female inbred C57BL/6 (H-2b) and outbred CD1 (ICR) strains of mice used in this study were purchased from Harlan (USA). All animals and procedures were used in accordance with the terms of the UK Home Office Animals Act Project License. Procedures were approved by the University of Oxford Animal Care and Ethical Review Committee.

**Viral vector vaccines.** The PbCSP insert in viral vectored and protein vaccines was full length without modification (GenBank accession no. P23093.1). The PbTRAP insert (GenBank accession no. AAB63302.1) in viral vectors was modified by removal of the transmembrane domain, and a tPA (human plasminogen activator; GenBank accession no. K03021) leader sequence was inserted in the upstream region of the gene, replacing the native endogenous leader sequence, since it has been suggested that this adjustment facilitates antigen expression within the host cells (39). In addition, the transmembrane (TM) domain or glycosylphatidylinositol anchor was removed from PbTRAP by introducing two stop codons in order to maximize protein secretion from any virus-transduced cell (40).

Prior to immunization animals were anesthetized using an inhalation chamber containing a mixture of gases, isoflurane (23.5%) and oxygen (12 L/min). Mice were primed with simian adenoviral vector 63 chamber containing a mixture of gases, isoflurane (23.5%) and oxygen DH5coli using a DNA-polyethylenimine mix. This resulted in secreted proteins using a DNA-polyethylenimine mix. This resulted in secreted proteins with N-terminal ETG and C-terminal GTK(His)6 tags. The conditioned media with secreted proteins were dialyzed against phosphate-buffered saline (PBS), and proteins were purified by immobilized Co2+-affinity chromatography, followed by size exclusion chromatography in 20 mM Tris-HCl (pH 8.0)–300 mM NaCl. Protein size was verified by Western blotting with the Pentahis monoclonal primary antibody (1:1,000 dilution; Qiagen) and goat anti-mouse IgG peroxidase-conjugated secondary antibody (1:2,000; Sigma).

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Prior to immunization animals were anesthetized using an inhalation chamber containing a mixture of gases, isoflurane (23.5%) and oxygen (12 L/min). Mice were primed with simian adenoviral vector 63 (ChAd63) encoding different transgenes at a dose of 107 infectious units (IU) and 8 weeks later boosted with vaccinia virus-modified virus strain Ankara (MVA) encoding the relevant transgene at a concentration of 105 PFU/ml, unless stated otherwise. All viral vector vaccines were administered intramuscularly in endotoxin-free PBS. Vaccinations with two different transgenes in coadministration groups were carried out either with each vaccine injected into the left or right thigh muscle separately or with the mixture of both vaccines administered in a single thigh muscle, as indicated in Results. All recombinant ChAd63 and MVA viral vectors used throughout this study were generated at the Jenner Institute’s vector core facility.

**Protein vaccines.** All recombinant protein vaccines were administered intramuscularly as a 50-μl dose containing sterile PBS and 15 μg of protein formulated with 5 μl of Abisco 100 adjuvant (provided by the Jenner Institute adjuvant bank). For the viral vector and protein mixture group, a 50-μl vaccine dose comprised PBS, MVA expressing the relevant transgene, and 15 μg of the recombinant protein formulated with 5 μl of Abisco 100 adjuvant.

**Whole IgG ELISA.** Enzyme-linked immunosorbent assays (ELISAs) measuring total IgG were carried out as described previously (41). In brief, blood was collected from mice using tail vein bleeds and stored overnight at 4°C. On the following day, the tubes were spun at 13,000 rpm for 4 min, and the serum was collected and stored at 4°C until the assay. Nunc ImmunoMaxisorp Plates were coated with protein diluted in PBS to a concentration of 1 μg/ml, followed by incubation overnight at room temperature. Plates were washed with PBS–0.05% Tween 20 (PBS/T) and blocked with 10% skimmed milk powder in PBS/T. Sera were diluted, typically at a starting concentration of 1:100, added into duplicate wells, and serially diluted 3-fold down the plate. Plates were incubated for 2 h at room temperature and then washed as described before. Goat anti-mouse whole IgG conjugated to alkaline phosphatase was added for 1 h at room temperature. After a final wash, p-nitrophenylphosphate at 1 mg/ml in diethanolamine buffer was used as a developing substrate. The optical density at 405 nm (OD405) was read using a model 550 microplate reader. Serum antibody endpoint titers were taken as the x axis intercept of the dilution curve at an absorbance value three standard deviations greater than the OD405 for serum from a naive mouse. A standard positive serum sample and naive serum sample were added as controls for each assay. Naive mouse serum was negative for antigen-specific responses against all of the recombinant proteins used.

**Peptides.** Crude 20-mer peptides overlapping by 10 amino acids and representing full-length *P. berghei* CSP and TRAP were synthesized by Mimotopes. Peptides were reconstituted in dimethyl sulfoxide at a concentration of 50 mg/ml. The peptides were combined into a single pool or three different subpools for their use in intracellular cytokine staining (ICS) and ex vivo gamma interferon (IFN-γ) enzyme-linked immunosorbent assay (ELISPOT) assays, respectively, at a final individual peptide concentration of 5 μg/ml.

**Splenocyte preparation.** Naive mice were sacrificed by cervical dislocation, and their spleens were removed. The spleens were homogenized in PBS and passed through a 70-μm-pore-size cell strainer, and cells were pelleted by centrifugation at 500 × g for 5 min. The cell pellet was then treated with ammonium chloride-potassium (ACK) lysis buffer for 5 min before being washed with 25 ml of PBS. Splenocytes were then immediately centrifuged at 500 × g for 5 min, and the resulting pellet was resuspended in 10 ml of complete medium (D10) per spleen. Total number of splenocytes was determined by CASY counter and adjusted to 107 cells per ml of complete medium for use in ex vivo IFN-γ ELISPOT assays.

**Ex vivo IFN-γ ELISPOT assay.** Ex vivo IFN-γ ELISPOT assays were carried out using peripheral blood mononuclear cells (PBMCs) isolated from blood as previously described (42). In brief, nitrilcellulose-bottomed 96-well Multiscreen HA filtration plates were coated with antimouse IFN-γ monoclonal antibody (MAB) overnight at 4°C. PBMCs were isolated from peripheral blood collected from tail vein bleeds into 200 μl of 10 mM EDTA/PBS. Erythrocytes were lysed using ACK lysis buffer, and PBMCs were harvested using centrifugation. The cells were washed, resuspended in complete medium, and counted using a CASY counter, and 50 μl of culture was plated into duplicate wells. A 50-μl portion of each of the peptide subpools diluted in medium plus 250,000 naive splenocytes was added to test wells as a source of antigen-presenting cells. Medium and naive splenocytes were also plated onto the negative-control wells. Plates were incubated at 37°C in 5% CO2, for approximately 18 h. The plates were then washed and incubated with biotinylated antinouse IFN-γ MAB (Mabtech), followed by incubation with a streptavidin alkaline phosphatase polymer. Spots were developed by addition of color development buffer and counted using an ELISPOT reader and accompanying software. Results are expressed as spot-forming units (SFU) per million PBMC. Background responses in medium-only wells were subtracted from those measured in wells stimulated by one of three peptide subpools.

**Isolation of liver-resident lymphocytes.** Livers from mice were perfused with PBS and dissected, mashed, and passed through a 100-μm-pore size filter. The liver cells were centrifuged at 1,350 rpm for 7 min, and the pellet was resuspended in 15 ml of 33% isotonic Percoll and then centrifuged at 693 × g for 12 min without brake. Cell debris and the supernatant were removed. The pellet was resuspended in 2 ml of ACK, and the erythrocytes were lysed for 5 min. Then, 25 ml of PBS was added, followed by centrifugation at 1,500 rpm for 5 min. The lymphocytes were washed with complete minimal essential medium (MEM) and resuspended in 300 μl of complete MEM.

**ICS.** For intracellular cytokine staining (ICS), ACK lysis buffer-treated whole-blood PBMCs were incubated for 5.5 to 6 h in the presence of a peptide pool representing the antigen of interest at individual peptide
concentrations of 5 μg/ml. Golgi-Plug (BD Biosciences) (1 μl/ml) and anti-CD107a PE (clone eBio/D4B) at a final dilution of 1:200 were also included. Phenotypic analysis of CD8+ and CD4+ T cells was performed by staining PBMCs using the following antibody clones: anti-CD8 PerCP-Cy5.5 (clone 53-6.7) and eFluor 650-coupled anti-CD4 ( GK.1.5) and anti-IFN-γ APC (XMGL1.2) (BD Biosciences). Also, the surface staining of liver-resident lymphocytes was performed using the following antibodies: anti-NK1.1 FITC, anti-CD3 Alexa Fluor 700, anti-CD69 PE-Cy7, anti-CD8 eFluor 450 (BD Biosciences). The liver-resident invariant natural killer T cells (inK T cells) were stained with CD1d tetramer conjugated to PE. Nonspecific binding of antibodies was prevented by incubating with anti-CD16/CD32 Fcγ receptor prior to staining. Flow cytometric analyses were performed using an LSRII instrument. Data were analyzed using either FACS Diva or FlowJo software. Analysis of multifunctional CD8+ T-cell responses was performed using Boolean analysis in FlowJo software, Pestle, and SPICE 4.0 kindly provided by M. Roederer (National Institutes of Health, Bethesda, MD).

**In vivo CD8+ and CD4+ T-cell depletions.**

The T-cell depletions were performed as described previously (43). Briefly, in vivo-depleting MABs were purified by protein G affinity chromatography columns from hybridoma culture supernatants. Anti-CD4 GK1.5 (rat IgG2a) and anti-CD8 2.43 (rat IgG2a) and anti-CD2.3 (rat IgG2a) were sterile filtered and diluted in sterile PBS. Normal rat IgG (nBalg1G) was purchased from Sigma and purified by the same method. For depletion of CD8+ or CD4+ T cells, mice were injected intraperitoneally with 200 μg of the relevant MAB on days −2 and −1 before and on the day of challenge. Depletion of CD4+ and CD8+ T cells was confirmed by flow cytometry of surface-stained PBMCs from depleted and control mice a day after the challenge.

**In vivo Kupffer and NK cell depletions.**

In the in vivo depletion of liver-resident Kupffer cells was accomplished by intravenous administration of liposome formulated edaravone 48 h prior to challenge at a concentration of 10 μl of suspension per 1 g of mouse (44). Control liposomes containing PBS were also included. To deplete natural killer (NK) cells, mice received a single dose of 200 μl of anti-asialo GM1 antiserum diluted 1:8 in 0.5% PBS on days −2, 0, and +2 relative to challenge, as described previously (45). Naïve rabbit serum was also used as a control (kindly provided by A. Douglas). NK cell depletion was confirmed by flow cytometry of surface-stained PBMCs from depleted and control mice 3 days after the challenge.

**Sporozoite neutralization assay.**

Sporozoite neutralization assay was performed as described previously (32). At room temperature 50,000 salivary gland wild-type P. berghei sporozoites were incubated with either undigested 3D11 or 3D11 Fab in total of 0.5 ml of RPMI 1640. After a 30-min incubation, 500 μl of ice-cold RPMI 1640 was added, and all sample tubes were placed on ice. Immediately thereafter, 100 μl of culture containing 5,000 sporozoites was injected intravenously (i.v.) into each animal.

**Fragmentation of 3D11.**

Fab fragments of 3D11 MAb were generated by incubation with immobilized Ficin in the presence of 25 mM cysteine according to the manufacturer’s instructions (Thermo Scientific Pierce). Fab fragments were purified by passing twice over a protein A column. Fragmentation of 3D11 was performed by incubation with immobilized Ficin in the presence of 25 mM cysteine according to the manufacturer’s instructions (Thermo Scientific Pierce). Fab fragments were purified by passing twice over a protein A column. Fragmentation of 3D11 was performed by incubation with immobilized Ficin in the presence of 25 mM cysteine according to the manufacturer’s instructions (Thermo Scientific Pierce).

**Transgenic (tg) P. berghei expressing firefly luciferase.**

The tg P. berghei parasites (PbGFP-Luccon) were kindly provided by Oliver Billker from Wellcome Trust Sanger Institute, Hinxton, United Kingdom.

**Parasite preparation.**

Female A. stephensi mosquitoes were fed on phencyclidazone (PHZ)-treated female TO mice infected with P. berghei parasites. At 21 days after the feed, the mosquitoes were dissected, the salivary glands were isolated, and the salivary gland sporozoites were extracted and diluted in RPMI 1640. The total number of sporozoites was determined using a hemocytometer, and specified quantities were injected i.v. into mice.

**Thin-blood smears to assess parasitemia.**

Thin-blood smears were prepared on glass slides from a drop of blood obtained from a tail clip. The blood smear was allowed to air dry before fixation with methanol and stained for at least 1 h using 5% Giemsa diluted in distilled H2O. The slides were then removed and allowed to air dry at room temperature.

**Statistical modeling to predict parasitemia.**

To obtain the time taken to reach 1% blood-stage parasitemia, a linear regression model was used, as described previously (29). Briefly, blood parasite counts were obtained for 3 to 5 consecutive days, twice a day, starting on day 4 after challenge. The logarithm to base 10 of the calculated percentage of parasitemia was plotted against time after challenge. The Prism 5 (GraphPad) statistical analysis package was used to generate a linear regression model using the linear part of the blood-stage growth curve. From this, the time taken to reach 1% parasitemia was obtained, expressed in days postchallenge. As expected for vaccines containing only preerythrocytic-stage antigens, all infected mice exhibited similar exponential blood stage growth regardless of treatment group. The time to reach 1% parasitemia was used in survival analyses to assess vaccine efficacy; mice without detectable parasitemia after 15 days were considered to have complete sterile protection. This approach has been used previously; the time to blood-stage parasitemia reflects the number of parasites erupting from the liver, provided there is no blood-stage immunity (47).

**In vivo imaging system (IVIS).**

The bioluminescent luciferase signal (BLS) was detected by imaging whole animals using the in vivo IVIS 200 imaging system, as described previously (48). Briefly, after intravenous injection of tg P. berghei sporozoites, the animals were anesthetized using an isoflurane chamber at a different time points depending on the experiment. Prior to imaging, 100 μl of α-luciferin substrate dissolved in sterile PBS to a concentration of 50 mg/kg was subcutaneously injected into the neck region of mice. Animals were imaged for 60 to 180 s at a binning value of 8 and field of view (FOV) of 12.8 cm, 8 min after the injection of the substrate. Mice remained anesthetized throughout the procedure. Quantification of BLS was performed using Living Image 4.2 image analysis software. Regions of interest were created around the liver area of the mouse body and kept constant for all animals. The measurements were expressed on a log scale as total flux of photons emitted from animals adjusted per second of exposure time.

**Statistics.**

GraphPad Prism 5.0 for Mac OS was used for all statistical analysis unless indicated otherwise. The Kolmogorov-Smirnov test for normality was used to determine whether the values followed a Gaussian distribution prior to statistical analysis when comparing two or more populations. An unpaired t test was used to compare two normally distributed groups, whereas the Mann-Whitney rank test was used to compare two nonparametric groups. If more than two groups were present, the nonparametric data were compared using the Kruskal-Wallis test posttest. Correlation strength was tested using either Pearson’s or Spearman’s tests as indicated in the results section. Kaplan-Meier survival curves were used to represent protective efficacy to challenge with P. berghei. When required, protection was also assessed using IVIS in vivo imaging and data log10 transformed prior to analysis. All ELISA titers were also log10 transformed before analysis. A P value of <0.05 was considered statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

**RESULTS**

Combining virus-vectored CSP and TRAP vaccines fails to improve protective efficacy. CSP and TRAP vaccines, by themselves, elicit immune responses and some degree of protection (12, 24, 26, 27). In the present study, these vaccines were combined with
FIG 1 C57BL/6 mice and CD1 outbred mice were vaccinated using a prime-boost strategy. Viral vectors containing either PbTRAP or PbCSP were intra-muscularly injected into the thigh muscles of the mice, with 10⁶ IU of ChAd63, followed by MVA at 10⁷ PFU/ml 8 weeks later. Mice vaccinated using the combination vaccine were injected with Ad-M TR in one thigh and Ad-M CSP in the other. The vehicle control used empty ChAd63 and MVA-GFP-OVA. Two weeks after boost vaccination mice were challenged by i.v. injection of 2,000 P. berghei sporozoites. The protective efficacy is here presented as time predicted to reach 1% blood-stage parasitemia using a linear regression model. Blood-stage parasitemia was monitored from day 4 postchallenge. (A) Time to predicted to reach 1% blood-stage parasitemia using a linear regression model. (B) Time to 1% blood-stage parasitemia in C57BL/6 after challenge with 2,000 sporozoites in all challenged animals (Fig. 4A and B). This demonstrates that high levels of anti-PbCS Ab are required for protection against P. berghei challenge in C57BL/6 mice. 3D11 is an MAb on July 20, 2018 by guesthttp://iai.asm.org/Downloaded from
against malaria than the components alone, and thus provided proof of concept for a combination vaccine.

An Ad-P CS/Ad-M TR combination vaccine confers sterile protection to C57BL/6 mice. Using an Ad-P CS vaccine with 15 μg of recombinant PbCS in Abisco 100 as a boost produced almost 10-fold-higher titers of anti-PbCS antibody than did the Ad-M CS vaccine (Fig. 5A). This order-of-magnitude increase was maintained when the Ad-P CSP vaccine was combined with the Ad-M TR vaccine. Unlike with the Ad-M CSP+TR combination vaccine, no antigenic interference was seen when Ad-P CSP and Ad-M TR were injected into separate sites. A slight but statistically significant decrease in antibody titers was seen when Ad-P CS and Ad-M TR were mixed and injected into the same site (Fig. 5A).

The Ad-P CS vaccine, despite achieving anti-PbCS Ab titers as high as conferred sterile protection using 3D11 MAb, conferred sterile protection on a nonsignificant number of mice in one experiment (Fig. 5B) and on no mice in another (Fig. 5C) after challenge with 2,000 *P. berghei* spz.

The combination regimen, whether CSP and TRAP vaccines were injected into separate legs (Fig. 5B) or mixed and coinjected (Fig. 5C), consistently conferred superior levels of sterile protection compared to the Ad-P CSP or Ad-M TR vaccines alone. Ad-P CS and Ad-M TR administered as a combination vaccine into separate sites produced sterile protection in 100% of mice, suggesting that the increase in antibody titers caused by Ad-P CS compared to Ad-M CS was sufficient to improve the combination CS-TR vaccine to the desired level of efficacy, as suggested by the results given above (Fig. 4C). Thus, the CS+TR vaccine was successfully tailored to achieve the desired results.

In the 3D11+Ad-M TR combination vaccine, protection is mediated by antibodies in an Fc-independent fashion. NK cells and CD8+ T cells are important mediators of protection. The mechanisms responsible for the effectiveness of the Ad-P CS+Ad-M TR combination vaccine were examined. These mechanisms were investigated using 3D11 MAb instead of Ad-P CS since it was not practical to optimize the Ad-P CS dosage to a level required to maximize sensitivity in these experiments.

Removal of the Fc portion of the 3D11 MAb had no effect on levels of sterile protection compared to intact 3D11 in a sporozoite neutralization assay (Fig. 6A). This suggests that the mechanism of protection of 3D11 in this combination vaccine is not via Fc-dependent phagocytosis by Kupffer cells or other macrophages. It could, however, still be mediated by Fc-independent phagocytosis.

Depleting Kupffer cells had no significant effect on the protection conferred by the Ad-M TR+3D11 vaccine (Fig. 6B). Depleting NK cells, on the other hand, did decrease the protection associated with this vaccine (Fig. 6B), suggesting that NK cells may play an important role in the mechanism of protection.

ICS analysis of three cell types from the livers of vaccinated and unvaccinated and then challenged and unchallenged mice provided further data on the mechanisms of protection. Vaccinated mice were found to have more CD8+ CD3+ T cells than naive mice and that, of these vaccinated mice, challenged mice have a higher proportion and higher absolute numbers of CD8+ IFN-γ+ cells (Fig. 7A). No significant difference in the numbers or proportions of CD1d+ CD3+ iNKT cells were found between vaccinated or challenged mice (Fig. 7B). However, NK cells which are IFN-γ+ were found to be present in higher numbers and in a higher proportion in vaccinated and challenged mice than in vaccinated and unchallenged mice (Fig. 7C). There was found to be a correlation between NK+ IFN-γ+ cell percentage and CD8+ IFN-γ+ cells in vaccinated and challenged mice only and not in other groups (Fig. 7D). From this and the fact that there was found to be a higher proportion and higher absolute numbers of CD69+ NK cells in vaccinated compared to nonvaccinated mice (Fig. 7E),
it appeared that NK cells could be important in the mechanism of protection conferred by Ad-M TR. These data, together with other data presented above (Fig. 3), suggest a model for the mechanism of protection of this combination vaccine whereby 3D11/PbCSP-specific antibodies reduce the number of sporozoites reaching the liver and CD8+/H11001 T cells destroy any hepatocytes infected by the few remaining sporozoites. With the Ad-P CSP+/Ad-M TR regime, protection may also be mediated by CSP-specific CD8+ T cells and IFN-γ, as found in other studies (33, 49–51); however, this was not examined here. A prediction of this model is that Ad-M TR alone should confer sterile protection when fewer sporozoites are used in a challenge. This was found to be the case: in a challenge with 200 sporozoites, Ad-M TR was able to confer sterile protection to 38% of challenged mice (Fig. 6C).

DISCUSSION

Currently, the most protective preerythrocytic-stage malaria vaccine candidates in human clinical trials—RTS,S and Ad-M ME.TRAP—rely on the induction of high Ab titers and powerful T-cell responses, against CS and TRAP proteins, respectively. However, individually both vaccines elicit only suboptimal protection against experimental malaria sporozoite challenge. Here, a P. berghei mouse malaria model was used to assess the efficacy of a vaccine combining both antigens.
Ad-M regimen has been demonstrated to induce strong humoral immunity (Ad-M) was used with both PbCS and PbTR antigens. The modified vaccinia virus strain Ankara (MVA) prime-boost strategy combined with WR vaccinia virus encoding PbCSP and PbTRAP antigens elicited 60 and 100% sterile protection against 1,000 and 200 \( P. \) berghei sporozoite challenges, respectively. However, in the latter case at the time of boosting MVA was administered intravenously, which is a more protective mode of vaccination. Since the intravenous injection of MVA viral vectors is not permitted in the clinical setting, the intramuscular route of administration was chosen.

The degree of protection observed with Ad-M PbTRAP and PbCS antigens were coadministered into separate sites, it seemed likely that this particular combination could offer a high degree of protective efficacy if it was carefully tailored for the concurrent induction of higher Ab and T-cell responses. The MAb 3D11 specific against the central repeat region of PbCS antigen was used to determine anti-PbCS Ab requirements necessary for sterile protection in a C57BL/6 animal model. As expected, a clear dose-dependent effect was observed, and an extremely high concentration of anti-CS 3D11 was required for complete sterile protection. Substantially smaller amounts of 3D11 were sufficient for sterile protection if the MAb vaccinations were performed on previously Ad-M PbTRAP-immunized animals.

Thus, the heterologous Ad prime/protein-in-adjuvant boost (Ad-P) regimen for the administration of PbCS antigen was explored, since reports have previously suggested that Ad-P regimen induces significantly higher Ab responses compared to these regimens administered individually \((52, 58, 59)\). Immunization of C57BL/6 mice using the Ad-P PbCSP and Ad-M PbTRAP combination vaccine conferred an exceptionally high level of sterile protection when each antigen was administered into separate sites.

Interestingly, an Ad-P PbCSP and Ad-M PbTRAP combination vaccine administered as a mixture into a single site at the time of prime and boost was less protective than a separate-site vaccination strategy. The mixing of vaccines prior to single-site administration seemed to increase antigenic interference between PbTRAP and PbCSP immunogens, resulting in diminished immune responses against individual antigens, which, in part, could explain the lower level of protective efficacy \((60)\). However, the challenge against the mixed and coinjected vaccination regimen was more rigorous, as evident from the lower time taken to reach 1% blood-stage parasitemia in naive mice; this, too, could explain the lower level of protective efficacy. In either case, the combination of CSP and TRAP vaccines substantially enhanced protection compared to individual immunizations. Trials combining \( P. \) falciparum CSP and TRAP with other antigens \((61, 62)\) have shown...
limited protection to date, though these trials did not use the most protective Ad-M or Ad-P regimes.

The cell depletion experiments performed on Ad-M PbTRAP- and 3D11 MAb-immunized mice demonstrated that the protection provided by this combination vaccine was mediated by anti-PbCS Abs capable of neutralizing a large proportion of spz, with PbTRAP-specific CD8\(^+\) T cells and NK cells providing an important second line of defense against parasites that manage to establish intrahepatic infection. The protection provided by highly protective irradiated spz vaccination has also been shown to be dependent on cellular and humoral immune responses against various spz-stage antigens (63). However, the results obtained using anti-asialo GM1 depleting polyclonal Abs should be interpreted cautiously, since the specificity of these Abs has been questioned (64, 65), and these antibodies may also reduce CD8\(^+\) T-cell numbers, leading to diminished efficacy.

Anti-CSP Ab-mediated sporozoite neutralization could be accomplished by various mechanisms: complement-dependent ly-
The decreased level of protection obtained after depletion of NK cells is consistent with studies performed by Doolan and Hoffman (69), where protective immunity by irradiated P. yoelii sporozoites or plasmid DNA-vaccinated mice was mediated by effector CD8+ T cells and NK cells and depended on interleukin-12 (IL-12), IFN-γ, and nitric oxide (NO). Moreover, in a follow-up study, the same authors established that perforin, granzyme B, and Fas L (Fas ligand) were not required for the protection induced by irradiated P. yoelii sporozoites (45). These authors suggested that the protective response is initiated by antigen-specific CD8+ T cells that upon recognition of specific peptide-MHC complexes secrete IFN-γ, which, in turn, induces IL-12 secretion from innate immune cells, such as macrophages, dendritic cells, or monocytes. The IL-12 subsequently stimulates the NK cells, which then may directly kill infected hepatocytes or induce antiparasitic activity through the secretion of IFN-γ. The fact that the depletion of NK cells significantly abrogated protection in this study provides some support for this model. This could also explain the strong correlation between CD8+ IFN-γ expression and NK cell LDH release during the preerythrocytic stage (70), as well as the blood stage (71), of infection. The cytotoxic activity of CD8+ T cells has also been demonstrated to be one of the principal mediators of protection against sporozoite challenge (45, 72).

Significantly higher numbers of CD8+ T cells were measured in the livers of mice that were immunized with ChAd63-PbTRAP than in the livers of naive animals. Potent TRAP-specific cellular immune responses were also measured 2 weeks after the ChAd63-PbTRAP prime by ELISPOT assay.

Previous studies have found a role for cytotoxic CD8+ T cells and IFN-γ in mediating protection, including with CSP (33, 49–51). These could play a role in the protection mediated by the vaccine regimens presented here; although depletion of CD4+ and CD8+ T cells did not abrogate the protective effect of the Ad-M CSP vaccine, this might be because anti-CSP antibodies played the role.
principal role in protection, as evident from the strong correlation with time to reach 1% parasitemia (Fig. 3Bi).

In summary, a highly protective vaccination regime was developed by combining two suboptimal subunit vaccines, CSP and TRAP, in a *P. berghei* malaria mouse model. The successful vaccination regime combined Ad-P CS and Ad-M TR. This elicited strong Ab responses against CS and CD8+ T-cell responses against TRAP, reducing the number of sporozoites reaching the liver and liver resident lymphocytes.

**FIG 7** ICS analysis of liver-resident lymphocytes. C57BL/6 mice (*n* = 5 per group) were immunized with a single ChAd63-PbTRAP vaccine (Ad) and 2 weeks later challenged with 3,000 *P. berghei* spz. At 24 h after challenge, the livers from vaccinated challenged (Ad-Chal) and unchallenged (Ad-UnChal) animals were extracted, and lymphocytes were purified and quantified. Livers from naïve challenged (N-Chal) and unchallenged (N-UnChal) animals were also included. ICS was performed, and samples were acquired using a BD LSRII flow cytometry instrument. The total number per mouse liver of CD8+ T cells (A), iNKT cells (B), and NK cells (C) was quantified (top panel), as well as the percentage (middle panel) and total number (bottom panel) of IFN-γ-positive cells within each subset for all treatment groups. (D) A strong correlation was obtained between the proportions of CD8+ IFN-γ+ and NK+ IFN-γ+ cells for the Ad-UnChal group, but not other groups. (E) The percentage (top panel) and total number (bottom panel) of NK+ CD69+ cells per liver for each treatment group were determined. ****, *P* < 0.0001; **, *P* < 0.01; *, *P* < 0.05; ns, nonsignificant (as determined by two-way ANOVA). Means with the SEM are shown.
causing the destruction of the lower number of infected hepatocytes.

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