

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, Pfs230, and Pfs48/45 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-poxviral 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 pHlsec 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* DH5 α (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(His₆) 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 glycosylphosphatidylinositol 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 (ChAd63) encoding different transgenes at a dose of 10⁸ infectious units (IU) and 8 weeks later boosted with vaccinia virus-modified virus strain Ankara (MVA) encoding the relevant transgene at a concentration of 10⁷ 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 vaccinations were administered intramuscularly as a 50- μ l dose containing sterile PBS and 15 μ g of protein formulated with 5 μ l of Abisco 100 adjuvant (provided by the Jenner Institute adjuvant bank). For the viral vector and protein mixture group, a 50- μ l vaccine dose comprised PBS, MVA expressing the relevant transgene, and 15 μ g of the recombinant protein formulated with 5 μ l 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 Immuno Maxisorp Plates were coated with protein diluted in PBS to a concentration of 1 μ g/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 immunospot (ELISPOT) assays, respectively, at a final individual peptide concentration of 5 μ g/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- μ m-pore-size cell strainer, and cells were pelleted by centrifugation at 500 \times *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 \times *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, nitrocellulose-bottomed 96-well Multiscreen HA filtration plates were coated with anti-mouse IFN- γ monoclonal antibody (MAb) overnight at 4°C. PBMCs were isolated from peripheral blood collected from tail vein bleeds into 200 μ l 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 μ l of culture was plated into duplicate wells. A 50- μ l 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 anti-mouse-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- μ m-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 \times *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 500 μ l 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 (GK1.5) and anti-IFN-γ APC (XMG1.2) (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-Cy 7, anti-CD8 eFluor 450 (BD Biosciences). The liver-resident invariant natural killer T cells (iNKT cells) were stained with CD1d tetramer conjugated to PE. Nonspecific binding of antibodies was prevented by incubating with anti-CD16/CD32 Fcγ III/II 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) were sterile filtered and diluted in sterile PBS. Normal rat IgG (nRatIgG) was purchased from Sigma and purified by the same method. For depletion of CD4⁺ or CD8⁺ 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. The *in vivo* depletion of liver-resident Kupffer cells was accomplished by intravenous administration of liposome formulated clodronate 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). Naive 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. The purity of Fab fragments was verified by nonreducing SDS-PAGE.

Transgenic (tg) *P. berghei* expressing firefly luciferase. The tg *P. berghei* parasites (PbGFP-Luccon) used in these studies expressed fusion GFP (mutant 3) and firefly luciferase (LucIAV) gene under the control of constitutive EF1a promoter (46). The parasites were generated by a stable double-crossover homologous integration of the transgene into the P230p locus of the *P. berghei* reference line ANKA clone cl15cy1. The tg parasite was kindly provided by Oliver Billker from Wellcome Trust Sanger Institute, Hinxton, United Kingdom.

Parasite preparation. Female *A. stephensi* mosquitoes were fed on phenylhydrazine (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 D-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 with Dunn's multiple-comparison posttest, whereas normally distributed data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison posttest. The effect of two variables was explored using two-way ANOVA with Bonferroni's multiple-comparison 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

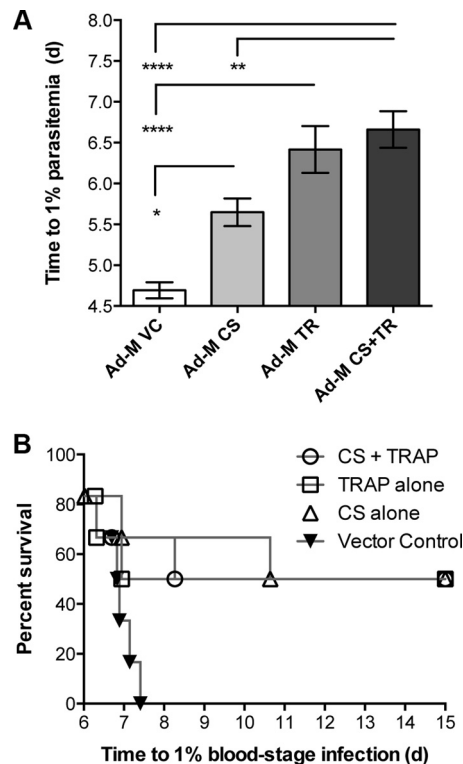


FIG 1 C57BL/6 mice and CD1 outbred mice were vaccinated using a prime-boost strategy. Viral vectors containing either PbTRAP or PbCSP were intramuscularly injected into the thigh muscles of the mice, with 10^8 IU of ChAd63, followed by MVA at 10^7 PFU/ml 8 weeks later. Mice vaccinated using the combination 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 1% blood-stage parasitemia in C57BL/6 mice ($n = 6$ per group). (B) Time to 1% blood-stage parasitemia in outbred CD1 mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (determined by one-way ANOVA with Bonferroni's multiple-comparison posttest).

the aim of providing enhanced immunity in mice to rodent malaria *Plasmodium berghei*. Virus-vectored vaccines, adenovirus prime and MVA boost (Ad-M) PbCS and Ad-M PbTR, were trialed both separately and together as a combination vaccine on inbred C57BL/6 and outbred CD1 mice. A heterologous prime-boost regimen using replication-deficient ChAd63 (Ad) and modified vaccinia virus strain Ankara (M) with an 8-week interval was used to deliver the antigens. This regime was previously found to optimize immune responses (27, 41).

The combination vaccine (Ad-M CS + Ad-M TR [Ad-M CS+TR]) produced better protection against sporozoite challenge than either component trialed alone, but this improvement was not significantly better than the protection achieved by Ad-M TR, according to a prediction of time taken to reach 1% blood-stage parasitemia in C57BL/6 after challenge with 2,000 sporozoites (spz) per mouse (Fig. 1A). The Ad-M CS+TR achieved a delay to 1% parasitemia of 6.7 days, while the Ad-M CS and Ad-M TR vaccines yielded delays of 5.6 and 6.4 days, respectively; all three vaccination groups took significantly longer to reach 1% parasitemia than the 4.7 days taken in mice vaccinated with an Ad-M

vehicle control ($P < 0.0001$) (Fig. 1A). The levels of sterile protection achieved in CD1 mice showed a similar effect, with 50% of animals vaccinated with Ad-M CS, Ad-M TR, and Ad-M CS+TR vaccines exhibiting sterile protection compared to no sterile protection seen with animals injected with Ad-M vehicle control (Fig. 1B). Since all C57BL/6 showed blood-stage parasitemia, a bar graph was judged the clearest way to present these results, whereas for mixed outcome with CD1, a Kaplan-Meier curve was the most clear.

Humoral and cellular immune responses to the combination vaccine were compared to those of mice vaccinated with the Ad-M CS and Ad-M TR components. Figure 2Ai shows that the combination vaccine elicits significantly lower antibody titers against the CS protein than does the Ad-M CS vaccine used alone; the same is true of anti-TRAP antibody titers when the combination is compared to Ad-M TRAP (Fig. 2Aii). This evidence of antigenic interference could explain why the combination vaccine did not work significantly better than the Ad-M TR vaccine.

Taken together these data suggested that the combination vaccine had some potential but required further optimization. To this end, experiments were performed to elucidate the mechanism of protection of the combination vaccine, as detailed below.

In the Ad-M CS+TR combination vaccine, protection is mediated by antibodies against PbCS and a $CD8^+$ response against PbTRAP. ICS showed that high $CD8^+$ IFN- γ^+ T-cell responses were seen only in response to PbTRAP, whereas no significant $CD4^+$ responses were seen except for a small but detectable response with PbTRAP using the Ad-M CS+TR regime (Fig. 2Bi and Bii). In an experiment examining the mechanisms of protection of the Ad-M CS, Ad-M TR, and Ad-M CS+TR vaccines, either $CD8^+$ or $CD4^+$ T cells were depleted in vaccinated and control C57BL/6 mice. Neither $CD8^+$ nor $CD4^+$ T-cell depletion affected liver-stage burden in Ad-M CS-vaccinated mice compared to the control (Fig. 3Ai), and there was a strong correlation between the endpoint CSP Ab titer and the time to 1% blood-stage infection (Fig. 3Bi), suggesting that antibodies help mediate protection in this Ad-M CS regimen. A correlation was not seen between CSP T-cell SFU and time to 1% blood-stage infection (Fig. 3Bii). $CD8^+$ but not $CD4^+$ T-cell depletion abrogated the protective effect of Ad-M TR (Fig. 3Aii) and Ad-M CS+TR (Fig. 3Aiii) vaccinations, suggesting that $CD8^+$ T cells contribute to protection in these regimens; flow cytometry confirmed successful depletion of $CD4^+$ and $CD8^+$ T cells (Fig. 3C).

High levels of anti-PbCS Ab are required for protection against *P. berghei* challenge in C57BL/6 mice. 3D11 is an MAb against the repeat region of PbCS (32). It was used to determine the antibody titers required to achieve sterile protection against *P. berghei* challenge. Administration of 3D11 in three intravenous injections of 300 μ g each, producing antibody levels of >5 ELISA units, was sufficient to produce sterile protection against 2,000 sporozoites in all challenged animals (Fig. 4A and B). This demonstrated that high levels of antibody production by a PbCS vaccine would be required to achieve sterile protection of vaccinated mice.

Trialed with Ad-M TR in a combination regime, sterile protection was achieved with much lower dosages of 3D11 than required with 3D11 alone (Fig. 4C). Sterile protection of all mice using the 3D11-TR combination vaccine was achieved with three injections of 150 μ g of 3D11 MAb each. This experiment demonstrated that an Ad-M CS+TR vaccine is able to achieve better protection

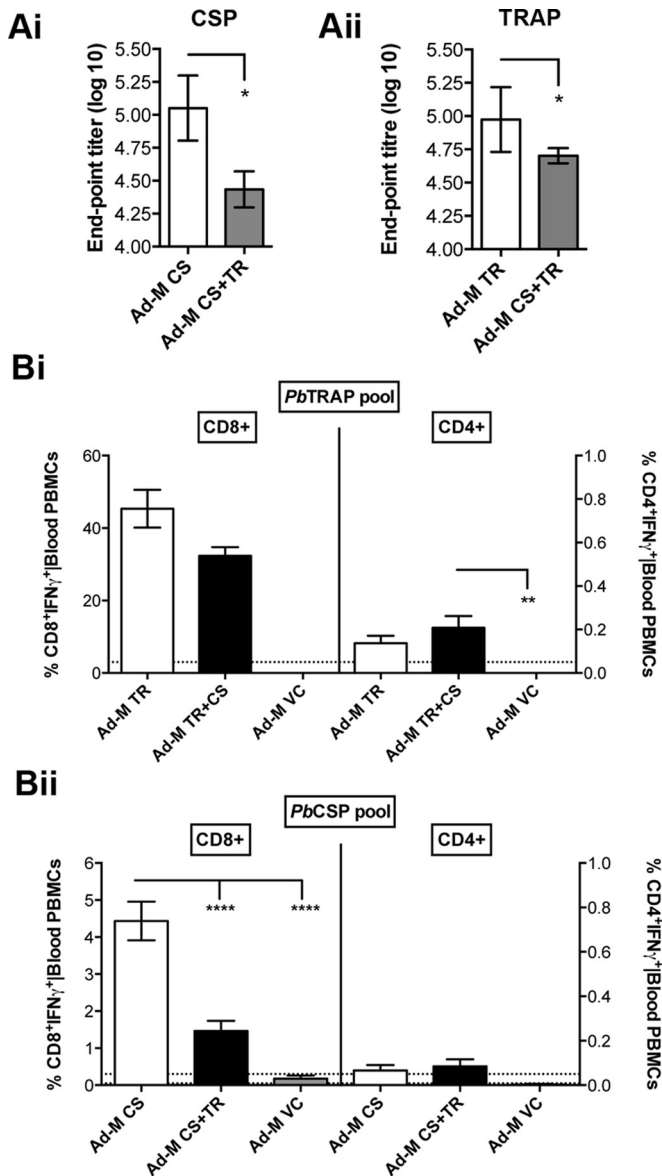


FIG 2 Humoral and cellular responses following Ad-M vaccinations using PbCSP and PbTRAP alone and in combination. C57BL/6 mice were vaccinated as described in Fig. 1. Two weeks after boost administration and 1 day before challenge, the humoral and cellular responses to vaccination were assessed using endpoint ELISA and ICS respectively. (A) Whole IgG Ab titers were measured against PbCSP (i) and PbTRAP (ii) recombinant proteins. No response was seen in this or subsequent ELISAs to serum from vehicle control groups. (B) Whole-blood PBMCs were restimulated with single peptide pools representing the full length of PbTRAP (i) and PbCSP (ii) antigens. The frequencies of CD8⁺ IFN-γ⁺ (left y axis) or CD4⁺ IFN-γ⁺ (right y axis) T-cell subsets were quantified. *, *P* < 0.05; **, *P* < 0.01; ****, *P* < 0.0001 (as determined by one-way ANOVA with Bonferroni's multiple-comparison posttest).

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),

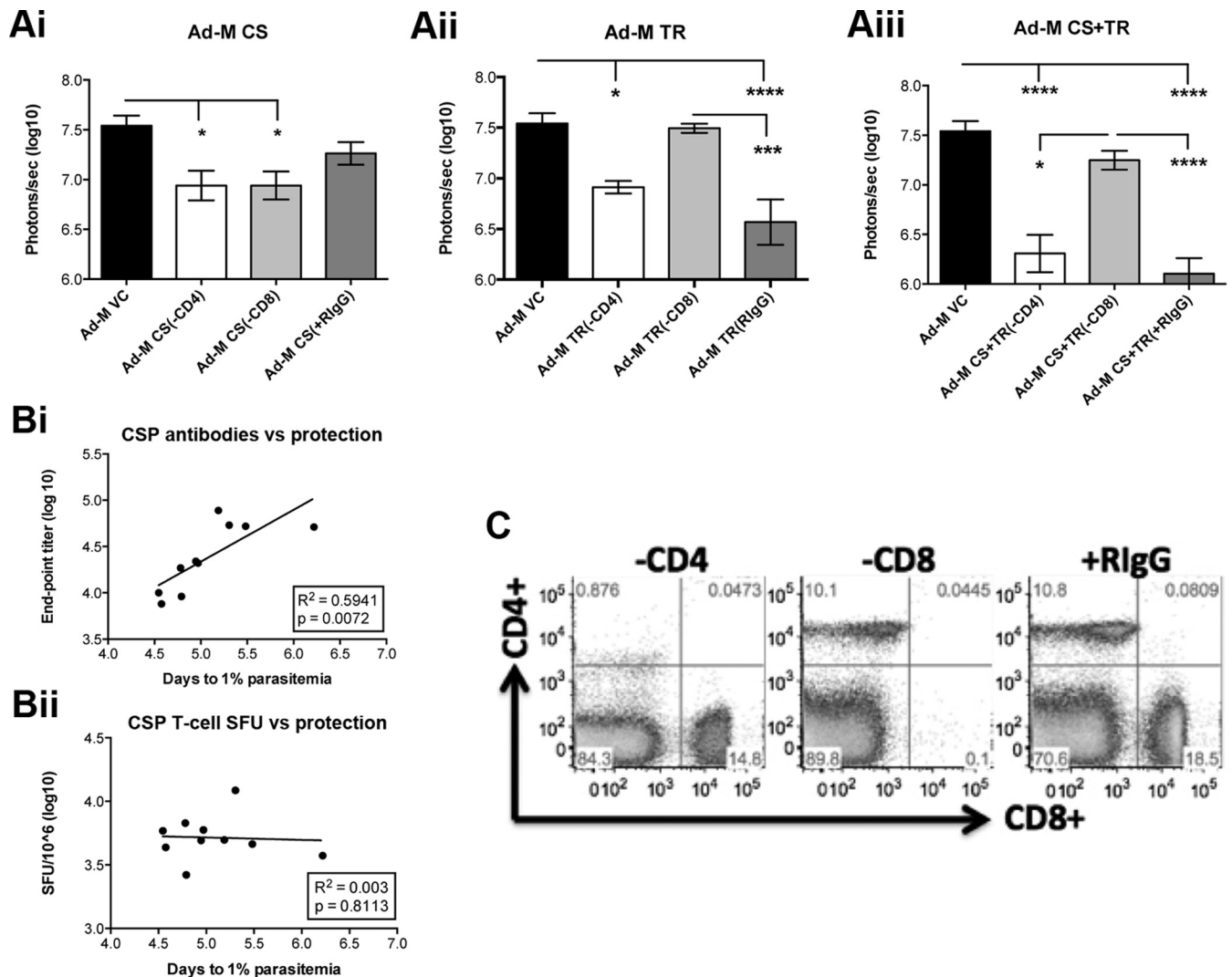


FIG 3 Depletion of CD4⁺ or CD8⁺ T cells in mice vaccinated with Ad-M PbCSP or Ad-M PbTR vaccines. (A) C57BL/6 mice ($n = 20$ per group) were vaccinated with Ad-M CSP (i), Ad-M TR (ii), or a combination of both (iii). A vector control group (Ad-M VC) ($n = 10$) was included. Two weeks after the boost, a selection of animals from each group ($n = 6$ per group) were depleted of CD4⁺ or CD8⁺ T cells, using three 200- μ g intraperitoneal injections of rat GK1.5 or 2.43 MAb, respectively, on days -2, -1, and 0 relative to challenge with 5,000 *P. berghei* GFP/Luc spz. Control rat IgG1 (+RlgG) antibodies were used as an injection control ($n = 8$ per group). At 44 h after challenge, the BLS emitted from infected animals was quantified. Means with the standard errors of the mean (SEM) are shown. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ (as determined by one-way ANOVA with Bonferroni's multiple-comparison posttest). (B) A strong correlation between time to reach 1% parasitemia (Tt01) and anti-PbCSP Ab titers (i), but not *ex vivo* IFN- γ ELISPOT responses (ii), suggests that protection is mainly mediated by Abs in Ad-M CSP vaccination. (C) Representative FlowJo plots demonstrate the depletion of CD4⁺ or CD8⁺ T cells compared to vaccinated animals that received control rat IgG1.

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+Ad-M TR confers sterile protection by PbCSP-specific antibodies reducing the number of sporozoites reaching the liver and CD8⁺ T cells destroying 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 chal-

lenge. 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-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.

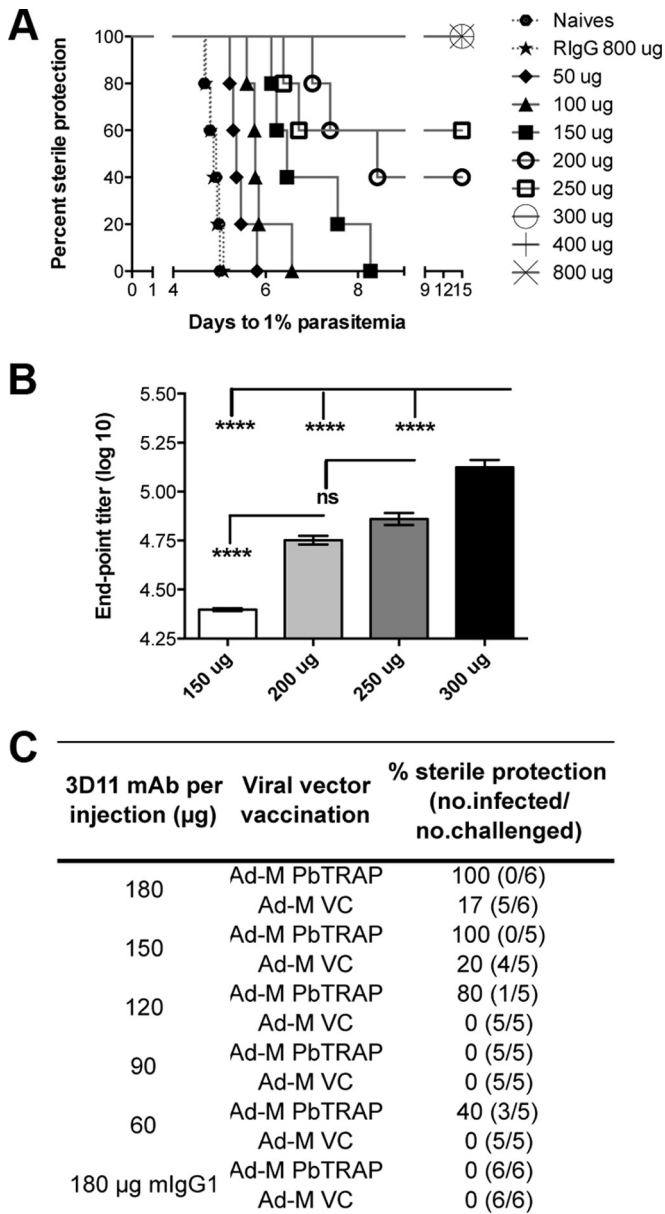


FIG 4 Enhanced protective effect of 3D11 MAb with the Ad-M PbTR vaccine. Naive C57BL/6 mice ($n = 5$ to 6 per group) were injected i.v. with three doses of anti-PbCSP mouse MAb 3D11 on days -3 , -2 , and -1 relative to the challenge. The mice were then challenged with 2,000 *P. berghei* spz each. A variety of 3D11 dosage sizes were used; these are labeled on each graph. (A) Linear regression modeling was used to predict Tt01 for parasitized mice. The Tt01 values were used for Kaplan-Meier analysis. Mice that didn't develop blood-stage infection by day 9 still demonstrated sterile protection by day 15 postchallenge. Blood-stage parasitemia was monitored using Giemsa-stained blood films. (B) 3D11 MAb titers from the serum of treated mice were measured by endpoint ELISA, immediately before challenge. (C) C57BL/6 mice ($n = 5$ to 6 per group) were vaccinated using a heterologous prime-boost regime using ChAd63 and MVA viral vectors expressing PbTR. The dosage of 3D11 was varied, in combination with either Ad-M TR or Ad-M VC, and challenged as above. ****, $P < 0.0001$ (as determined by one-way ANOVA with Bonferroni's multiple-comparison posttest). Means with the SEM are shown.

Initially a heterologous chimpanzee adenovirus 63 (Ad) and modified vaccinia virus strain Ankara (MVA) prime-boost strategy (Ad-M) was used with both PbCS and PbTR antigens. The Ad-M regimen has been demonstrated to induce strong humoral

and cellular immune responses in mice (29, 43), rabbits (40), rhesus macaques (52), and humans, showing an excellent safety profile in the latter (53, 54) using different malarial antigens. The combination of these antigens failed to induce significantly superior protection compared to the most protective single antigen immunization, however.

The degree of protection observed with Ad-M CS+TR combination vaccine in C57BL/6 mice was substantially lower than that seen in a study by Schneider et al. (55), where DNA/MVA prime-boost vaccination employing the combination of the same PbCS 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.

A previous study using human adenovirus serotype 5 combined with WR vaccinia virus encoding *P. yoelii* CSP (25) detected higher levels of protection than found here using an Ad-M CSP regime with *P. berghei*, probably because of the greater immunogenicity of WR vaccinia virus. In a direct comparison, chimpanzee adenoviruses have been shown to be as immunogenic or more immunogenic than human adenovirus vaccine vectors (56, 57).

Since a trend toward increased immunity was observed when 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 regime 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

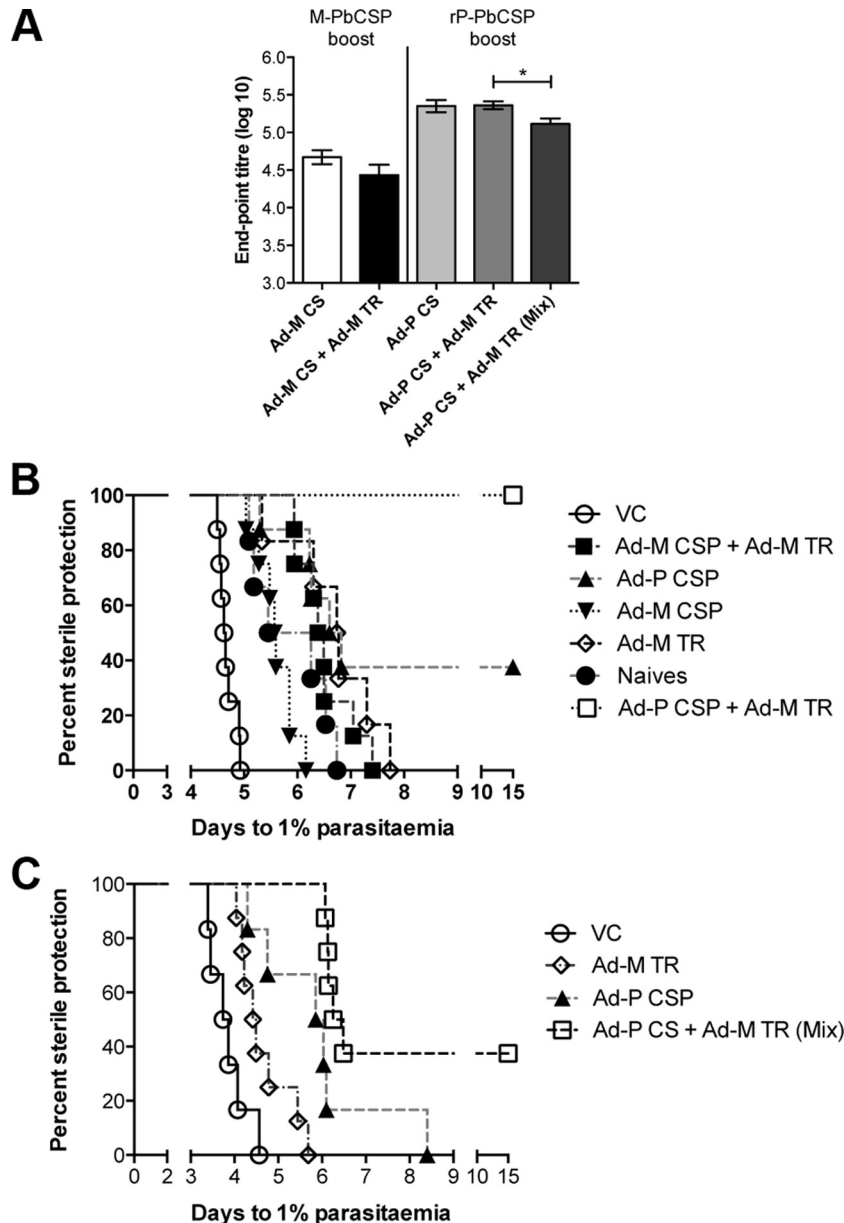


FIG 5 A particulate PbCSP vaccine improves antibody responses and response to challenge in a combination vaccine. C57BL/6 mice ($n = 6$ to 14 per group) were vaccinated with different regimens according to group as follows: naive control group, GFP antigen using ChAd63 prime and MVA boost; Ad-M TR, PbTRAP antigen using ChAd63 prime and MVA with Abisco 100 boost; Ad-P CSP, PbCSP antigen using ChAd63 prime and 15 μ g PbCSP in Abisco 100 boost; and Ad-M PbCSP, PbCSP antigen using ChAd63 prime and MVA boost. In the “Ad-P CS+Ad-M TR(Mix)” combination, the vaccines were mixed before being injected into left and right legs. In the “Ad-M CS+Ad-M TR” combination vaccine, the PbCSP vaccines were injected into the left leg, and the PbTRAP vaccines were injected into the right leg. (A) PbCSP-specific whole IgG serum Ab titers measured 2 weeks after boost by endpoint ELISA. *, $P < 0.05$ (as determined by unpaired t test). Means with the SEM are shown. Two weeks after boost mice were challenged with 2,000 *P. berghei*. (B and C) Kaplan-Meier survival curves based on predicted time to 1% parasitemia.

limited protection to date, though these trials did not use the most protective Ad-M or Ad-P regimens.

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 pro-

tektive 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-

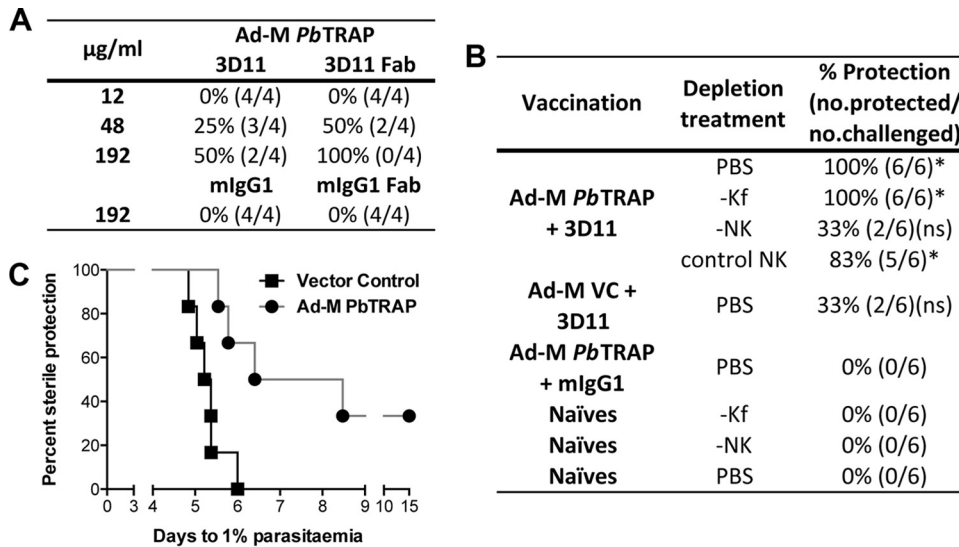


FIG 6 Immunological mechanisms of the 3D11 and Ad-M PbTRAP combination vaccine. C57BL/6 mice ($n = 6$ per group) were vaccinated with a heterologous prime-boost regimen using ChAd63 and MVA viral vectors expressing PbTRAP. A control group ($n = 6$) was vaccinated with empty vector control viruses. (A) The role of the Fc region in protection was investigated using a sporozoite neutralization assay. A total of 50,000 *P. berghei* spz were incubated with either intact or Fab fragments of 3D11 at a range of concentrations. A total of 5,000 sporozoites, each opsonized with either 3D11 or 3D11 Fab, were then i.v. injected into Ad-M PbTRAP-vaccinated animals. The percentages of animals experiencing sterile protection are shown. (B) Two weeks after boost and on days -3 , -2 , and -1 relative to challenge with 2,000 *P. berghei* spz, vaccinated animals were injected with 120 µg of 3D11 MAb each day. Control mIgG1 was administered in an identical manner. Additionally, either NK ($-NK$) or Kupffer ($-Kf$) cells in the vaccinated animals were depleted by multiple i.v. injections of either anti-asialo GM1 or clodronate liposomes, respectively, prior to challenge. An NK depletion control was included. The levels of sterile protection were used to quantify the effects of different treatments on vaccine-induced protection. *, $P < 0.05$; ns, nonsignificant [as determined by Fisher exact test comparing the indicated group to the (Ad-M PbTRAP) + mIgG1 group]. (C) C57BL/6 mice were immunized with Ad-M PbTRAP or empty viral Ad-M VC vaccines ($n = 6$ per group). Two weeks after boost animals were challenged with 200 *P. berghei* spz. Kaplan-Meier survival curves were determined based on the predicted time to 1% parasitemia.

sis, receptor-mediated phagocytosis, and/or perhaps inhibition of sporozoite binding to the liver (66). Touray et al. demonstrated an almost complete lysis *in vitro* of *P. gallinaceum* sporozoites by anti-sporozoite Abs and mammalian serum compared to only 11 to 15% lysis observed using nonimmune serum (67). The serum samples from RTS,S-immunized volunteers contained opsonizing abs and these antibodies significantly enhanced *in vitro* endocytic activity of the FcR(+) THP-1 human monocyte line in protected volunteers compared to those susceptible to sporozoite infection (68). Complement fixation and endocytosis of opsonized sporozoites relies in part on the Fc region of the Ab. However, in the present study no such role for the Fc region was discovered. In fact, the Fc-independent neutralization of *P. berghei* sporozoites is in agreement with a previous observation where comparable levels of neutralization were achieved upon opsonization of *P. berghei* sporozoites with either intact or Fab fragments of 3D11 (32). However, it remains unclear whether Fc-independent phagocytosis was occurring and, if so, what was its contribution to the protection induced by the Ad-M TR+3D11 combination vaccine.

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 ⁺ and NK⁺ IFN- γ ⁺ lymphocyte subsets that was observed and is consistent with publications suggesting that NK-specific IFN- γ responses may be tightly associated with the T-cell responses at 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 *ex vivo* IFN- γ 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 may be because anti-CSP antibodies played the

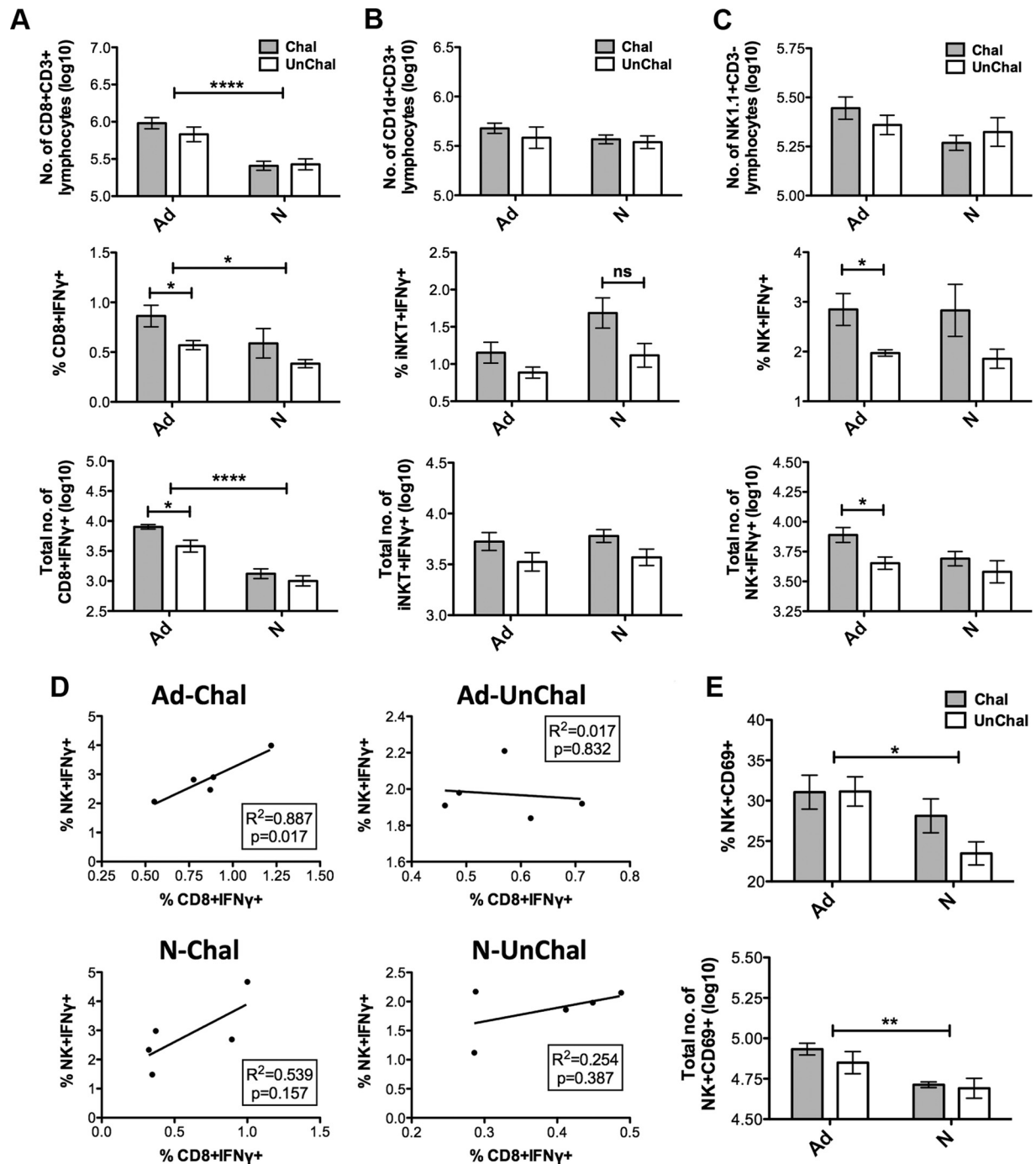


FIG 7 ICS analysis of liver-resident lymphocytes. C57BL/6 mice ($n = 5$ per group) were immunized with a single ChAd63-PbTRAP vaccine (Ad) and 2 weeks later challenged with 3,000 *P. berghei* spz. At 24 h after challenge, the livers from vaccinated challenged (Ad-Chal) and unchallenged (Ad-UnChal) animals were extracted, and lymphocytes were purified and quantified. Livers from naive 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.

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

causing the destruction of the lower number of infected hepatocytes.

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