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Combining Liver- and Blood-Stage Malaria Viral-Vectored Vaccines: Investigating Mechanisms of CD8+ T Cell Interference

Emily K. Forbes, Sumi Biswas, Katharine A. Collins, Sarah C. Gilbert, Adrian V. S. Hill, and Simon J. Draper

Replication-deficient adenovirus and modified vaccinia virus Ankara (MVA) vectors expressing single pre-erythrocytic or blood-stage Plasmodium falciparum Ags have entered clinical testing using a heterologous prime-boost immunization approach. In this study, we investigated the utility of the same immunization regimen when combining viral vectored vaccines expressing the 42-kDa C terminus of the blood-stage Ag merozoite surface protein 1 and the pre-erythrocytic Ag circumsporozoite protein in the Plasmodium yoelii mouse model. We find that vaccine coadministration leads to maintained Ab responses and efficacy against blood-stage infection, but reduced secondary CD8+ T cell responses against both Ags and efficacy against liver-stage infection. CD8+ T cell interference can be minimized by coadministering the MVA vaccines at separate sites, resulting in enhanced liver-stage efficacy in mice immunized against both Ags compared with just one. CD8+ T cell interference (following MVA coadministration as a mixture) may be caused partly by a lack of physiologic space for high-magnitude responses against multiple Ags, but is not caused by competition for presentation of Ag on MHC class I molecules, nor is it due to restricted T cell access to APCs administered with either RTS,S or the 42-kDa C terminus of MSP1 (12, 25), NYVAC- (26), and L3SEPTL polyprotein (27).

Abbreviations used in this article: Ad, adenovirus; AdHu5, human adenovirus serotype 5; AMA1, apical membrane Ag 1; CSP, circumsporozoite protein; DC, dendritic cell; ICS, intracellular cytokine staining; i.d., intradermal; MSP1, merozoite surface protein 1; MVA, modified vaccinia virus Ankara; pRBC, parasitized RBC; PyCSP, Plasmodium yoelii CSP; PyMSP1, P. yoelii MSP1; RT, room temperature; vp, viral particle.

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Malariaremaisarepresentingsignificantglobalhealthproblem.Plasmodiumsp.,infected~240millionpeopleandcaused~860,000deathsworldwidein2008(1).Itiswidelyrecognizedthatahighlyeffectivevacinationagainstmalaria
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clinicalstudiesinmice,rabbits,andrhesusmacaques(4–10).
PhaseI/IIaclinicaltrialsusingthisstrategyarecurrentlyun-
comprisechimpanzeeadenovirus63andtheorthopoxvirusMVA,
administered8wkapart,expressingthePlasmodiumfalciparum

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The online version of this article contains supplemental material.


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Similarly, studies in the field of HIV-1 vaccines have used mixtures of 3-4 DNA and/or poxvirus or human adenovirus serotype 5 (AdHu5) vaccines, but any potential effects of immune interference between the individual components and their encoded Ags have not been reported (28, 29). Two studies have been performed in which individual components in a multiantigen virosporous and viral-vectored malaria vaccine have been assessed directly in humans when administered alone and in combination (30, 31). In both cases, total IgG responses were not affected by coadministration of Ags; however, T cell responses were not assessed directly. To our knowledge, no study has directly assessed the effects of coadministration of malaria Ags on T cell responses in humans. These data clearly highlight the importance of investigating the individual antigenic components within a multiantigen vaccine and their effect on vaccine immunogenicity and efficacy in both preclinical and clinical studies.

Crucially, a combination vaccine strategy has not been assessed previously with the clinically relevant Ad-MVA prime-boost vectored vaccine platform. In this study, we have evaluated in mice the coadministration of vectors expressing the P. yoelii Ags CSP (PyCSP) and MSP142 (PyMSP142) in an AdHu5-MVA prime-boost regimen. CSP is expressed on the surface of sporozoites, and Abs against this Ag are thought to prevent sporozoite invasion, whereas CSP-specific CD8+ T cell responses [induced by recombinant human adenoviral vectors in mice (32–34)] and CD4+ T cell responses in humans (35), have been associated with protective outcome. MSP142 is the 42-kDa C terminus of MSP1 and is expressed during the late liver-stage and on the surface of merozoites during blood-stage infection. During merozoite invasion of RBCs, MSP142 is cleaved into a 33-kDa (MSP133) and a 19-kDa (MSP119) fragment (36). MSP119 remains associated with the merozoite surface, and Abs against this Ag are capable of providing blood-stage protection. We have shown that vectors expressing PyMSP142 can provide partial liver-stage efficacy because of CD8+ T cells targeting PyMSP119 (4), and protective blood-stage efficacy owing to Ab responses against PyMSP119 (4, 5). In these studies, efficacy was further enhanced if the PyMSP142 vaccine construct included the core domain from the murine α-chain of complement C4 binding protein (mC4bp/IMX108) fused to the C terminus (5, 37).

In this study using an AdHu5-MVA vectored vaccine platform for PyCSP and PyMSP142, we demonstrate an absence of interference for Ag-specific Abs following administration of the vaccine mixture; however, significant CD8+ T cell interference occurs after the MVA boost, resulting in reduced efficacy against sporozoite challenge. This effect is associated with enhanced killing of infected cells in vivo at the time of the boosting immunization in mice that are previously primed against two Ags rather than one. CD8+ T cell interference can be minimized by separate site immunization, which leads to enhanced efficacy of the dual-antigen vaccine regimen. These data have important implications for the development of multicomponent viral vectored vaccines against numerous difficult infectious diseases.

**Materials and Methods**

**Recombinant AdHu5 and MVA vaccines**

The construction of AdHu5 and MVA vectors expressing PyCSP and PyMSP142-mC4bp (IMX108), referred to throughout as PyMSP142, has been described previously (4, 5, 37). Control vectors were made using similar methods. AdHu5-control included the transgene promoter and polyA tail, but no antigenic insert. MVA-control contained GFP.

**Animals and immunizations**

Procedures were performed according to the U.K. Animals (Scientific Procedures) Act 1986 and were approved by the University of Oxford Animal Care and Ethical Review Committee. Six- to 8-week-old female BALB/c (H-2b) and C57BL/6 (H-2d) mice were obtained from Harlan, UK and housed in specific pathogen-free conditions. Immunizations were performed as described in Table I, unless stated otherwise. All vaccines were formulated in endotoxin-free PBS and given intradermally (i.d.) in a total volume of 50 μl (25 μl per ear). There was an 8-wk interval between priming and boosting immunizations in all cases. When only a single malaria Ag vaccine was given, control vaccines were included to equalize viral load.

**Isolation of splenocytes**

Spleens were dissected into PBS, mechanically homogenized, and passed through a 70-μm cell strainer. Erythrocytes were removed by resuspension of cells in ACK lysis buffer (0.15 M NH4Cl + 1 mM KHCO3 + Na2EDTA dissolved in 1L dH2O and adjusted to pH 7.2–7.4 with 1 M HCl; all from Sigma) for 5 min before the addition of excess PBS. Splenocytes were pelleted by centrifugation and resuspended in complete MEM (MEM α-modification, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1-galactamine [4 mM] and 2-ME [50 μM]).

**Isolation of PBMCs from blood**

Seven to ten drops of blood were collected from the tail vein into 200 μl of 10 mM EDTA in PBS. Erythrocytes were removed by incubation with ACK lysis buffer and cells were resuspended in complete MEM.

**Isolation of liver mononuclear cells**

Livers were perfused with PBS and mechanically homogenized. Cells were incubated for 1 h in complete MEM containing 0.7% mg/ml collagenase (Sigma) and 0.03 mg/ml DNase (Sigma) then passed through a 70-μm cell strainer. Cells were washed in PBS and underlaid with Ficoll-Paque PLUS (GE Healthcare) before spinning at 2000 rpm without the brake for 45 min at 21°C to separate cells. Cells at the interphase were carefully removed, washed twice with PBS, and resuspended in complete MEM.

**Pepitides**

PyCSP-specific responses were measured using a peptide against the dominant CD8+ epitope, PyCSP50–288 (38), the subdominant CD8+ epitope, PyCSP50–67 (39), or the CD4+ epitope, PyCSP280–288 (38). PyMSP1-specific T cell responses were measured after stimulation with a pool of peptides spanning the entire length of PyMSP142; this consisted of 52 15mers overlapping by 10 aa (43). Individual peptides are described in Table II.

**Intracellular cytokine staining**

Cellular immune responses in the blood, spleen, and liver were measured by ICS as described previously (4). Cells were restimulated for 5 h at 37°C in the presence of GolgiPlug (BD Biosciences) and peptides specific for PyMSP1, PyCSP, or MVA vector at a final concentration of 1 μg/ml for each peptide, before storage at 4°C overnight.

Cells were then incubated for 15 min in the presence of anti-mouse CD16/CD32 (Fc-block) before surface staining for 30 min with anti-mouse CD8α-PerCP-Cy5.5 (clone 53-6.7) and CD4-Pacific Blue (clone GK1.5). Permeabilization was performed using Cytofix/Cytoperm solution (BD Biosciences) according to the manufacturer’s instructions. Cells were stained intracellularly for 30 min with anti-mouse IFN-γ-allophycocyanin (clone XMG1.2), IL-2–PE (clone MP6-20H4) and TNF-α–FITC (clone MP6-X22). All incubations were performed at 4°C with Abs from eBioscience. Cells were resuspended in 1% formalin solution and responses measured using a CyAn ADP Flow Cytometer (Dako, Ely, U.K.) or LSRII (BD Biosciences). Data were analyzed using Flowjo version 8.7 (Tree Star). For some experiments, analysis of distributions was performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov/spice (43). Results show the stimulated response with background responses from the unstimulated control subtracted (typically <0.05%).

**ELISA**

Blood was collected by tail bleed into microvette tubes, stored at 4°C overnight, and spun at 13,000 rpm for 4 min. Serum was removed and stored at −20°C until use; 2 μg/ml GST-PyMSP1-19 protein (5) or

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a branched PyCSP peptide (MAP4–Py1T-Py3) (44) in PBS was adsorbed to 96-well Nunc-ImmuNoMaxisorp plates (Fisher Scientific) by overnight incubation at room temperature (RT). Plates were washed six times in PBS 0.05% Tween 20 (PBS/T) and blocked for 1 h in PBS/T containing 10% skimmed milk powder. Preboost samples sera were diluted 1:100 and postboost 1:1000 before serial dilution down the plate and incubation for 2 h at RT. Plates were then washed in PBS/T and bound Abs detected by incubation for 1 h with alkaline phosphatase conjugated goat anti-mouse IgG (Sigma). Alternatively bound IgG isotypes were detected using biotin-conjugated rat anti-mouse IgG1 or IgG2a (BD Biosciences) at a concentration of 1 μg/ml in PBS/T for 1 h, before washing and adding ExtrAvidin (Sigma) conjugated to alkaline phosphatase and incubation for 30 min. Plates were then washed and developed using 1 mg/ml p-nitrophenylphosphate substrate pNPP (Sigma) in diethanolamine buffer (Fisher Scientific UK Ltd). OD was read at 405 nm. Endpoint titers were taken as the x-axis intercept of the dilution curve at an absorbance value 3 SD greater than the OD405 for naive mouse serum. All samples were tested in duplicate, and a high-titer reference control sample was included in all experiments.

P. yoelii sporozoite and parasitized RBC challenge

P. yoelii YM sporozoite and parasitized RBC (pRBC) challenges were performed as described previously (4). Sporozoites were obtained by mechanical disruption of the salivary glands from infected Anopheles stephensi mosquitoes in RPMI 1640 medium (Sigma). Infected erythrocytes (i.e., pRBCs) were stored by cryopreservation and passed in a donor mouse prior to use. Mice were infected by i.v. injection with 50, 250, or 1000 sporozoites or 10⁴ pRBCs, and blood-stage parasitemia was monitored after challenge by Giemsa-stained thin blood smear from day 2 for pRBC challenge and day 4 or 5 for sporozoite challenge. Parasitemia was calculated as a percentage of infected RBCs. Mice were considered uninfected if no parasites were observed in 50 fields of view containing ~500 RBCs per field. The lower limit of detection was therefore 0.004%. Mice were culled if they reached ≥80% infected RBCs.

P. yoelii sporozoite immunofluorescence Ab test

P. yoelii YM sporozoites were isolated from the salivary glands of infected mosquitoes and were dissected in PBS containing aziade. Samples were diluted at 10⁵ sporozoites/ml and 10 μl well pipetted onto an eight-well microscope slide, such that each well contained ~3000 sporozoites. Slides were air-dried, wrapped in foil and stored with desiccant at −20°C until use. For the immunofluorescence Ab test, all incubation steps were performed at RT in a dark, humidified chamber, and slides were washed three times in PBS between each step. Wells were blocked for 2 h using 1% BSA in PBS. After washing, serum samples were added at a dilution of 1:100 in PBS. Slides were blocked with 1% BSA and biotin-conjugated goat anti-mouse IgG secondary Ab (Invitrogen) was added at a dilution of 1:200 PBS (in 1% BSA). Slides were incubated for 30 min, washed, and mounted with Mowiol and a coverslip. Slides were dried at RT overnight in the dark. Slides were viewed under a Leica DMI3000 microscope.

Isolation and peptide-pulsing of CD11c⁺ cells

Splenoocytes from naive BALB/c mice were chopped, resuspended in complete MEM, and rotated at RT for 30 min in the presence of 0.3 mg/ml collagenase-dispase (Life Technologies) and 0.02 mg/ml DNase I (Sigma), before the addition of 0.1% EDTA for an additional 5 min to inactivate collagenase and disrupt dendritic cell (DC)–T cell interactions. Splenocytes were then homogenized and passed through a 70-μm cell strainer, and erythrocytes were lysed by addition of ACK lysis buffer for 5 min at 4°C. Cells were then washed twice with cold MEM and rotated at 4°C for 30 min. Cells were then washed and resuspended at 2 × 10⁶ cells/ml in MACS buffer (PBS 7.2, 0.5% BSA, 2 mM EDTA) at a concentration of 2.5 × 10⁶ cells/ml. Cells were then incubated for 10 min at 4°C with 100 μl CD11c MicroBeads per 10⁶ cells and incubation for an additional 15 min at 4°C. Cells were then washed and resuspended at 2 × 10⁶ cells/ml in MACS buffer. CD11c⁺ cells were isolated by passing splenoocytes through an LS MACS column according to the manufacturer’s instructions (Miltenyi Biotech). After positive selection, CD11c⁺ cells were washed thoroughly, resuspended in complete MEM, and pulsed with 10 μg/ml peptide for 16 h. Cells were pulsed with the seven PyMSP119 peptides, the two PyCSP peptides, or a mixture of all nine peptides. The concentration of DMSO was controlled across all wells at 0.24%. After pulsing, cells were washed thoroughly in PBS. Cells were then immediately injected in a volume of 2 × 10⁶ CD11c⁺ cells in a volume of 100 μl (50 μl per ear). In mice that received cells that had been pulsed separately, cells pulsed with each Ag were combined in a 1:1 ratio immediately before injection (2 × 10⁶ total cells).

In vivo CTL assay

Naive BALB/c splenoocytes were prepared by mechanical disruption (as described) as targets for in vivo killing. Cells were washed and resuspended at 5 × 10⁶ cells/ml in warm MEM (without additions, 37°C) and split into two populations. One population of cells was labeled with a high concentration of CFSE (5 μM) and the other with a low concentration of CFSE (0.5 μM; Invitrogen). After the addition of CFSE, cells were immediately incubated for 10 min at 37°C, with regular mixing by inversion. The reaction was stopped by the addition of cold complete MEM. Cells were then washed twice in complete MEM and resuspended at 1 × 10⁶ cells/ml. Cells labeled with a high concentration of CFSE were split into subpopulations and incubated at 37°C for 1 h with PyMSP119 peptide pool, PyCSP, or both, with or without PyMSP119 peptide pool and PyCSP, respectively. All peptides were used at 200 ng/ml. Cells were then washed twice in PBS. For i.v. injection, a 1:1 ratio of high and low CFSE-labeled cells were then combined such that each mouse received 1 × 10⁷ cells labeled with a low concentration of CFSE and were not peptide pulsed, and 1 × 10⁷ cells labeled with a high concentration of CFSE and were pulsed with the indicated peptides. These cells were then injected in a volume of 200 μl in PBS into BALB/c mice that had been previously primed with AdHu5 vaccines as indicated. Immunized mice were culled 5 h later, and a single-cell suspension of splenocytes was prepared. The CFSE intensity of each population was analyzed by flow cytometry. Specific killing was calculated as follows: ratio = (percentage CFSE low / percentage CFSE high); and percentage specific killing = (1 - [mean ratio control primed / ratio primed]) × 100%. In one experiment, splenocytes were labeled with CD11c-allophycocyanin prior to flow cytometry to allow an assessment of the killing of CD11c⁺ cells.

Statistics

Data were analyzed using GraphPad Prism v5.01. The normality of the data was determined using the Kolmogorov-Smirnov one-sample test. For nonparametric data, two groups were compared using a Mann–Whitney U test and multiple groups were analyzed by Kruskal-Wallis test. Data are presented as median ± range or as individual data points. ELISA data were logarithmically (log10) transformed to normalize the data and allow parametric analysis. Parametric data were analyzed using an unequal t test for two groups and are presented as mean ± SD. In all cases, a p value ≤0.05 was considered significant (**p ≤ 0.05, ***p ≤ 0.01, and ****p ≤ 0.001).

Results

Ab responses to PyMSP142 and PyCSP vaccines

BALB/c mice were immunized against the PyMSP142 or PyCSP Ags, or both, either individually or as a mixture, using a previously described AdHu5-MVA prime-boost immunization regimen (4, 5). Details of the specific immunizations regimes are previously described AdHu5-MVA prime-boost immunization regimens (4, 5). The kinetics of the total IgG Ab responses against the PyMSP142 and PyCSP Ags were measured by ELISA over time (Fig. 1A, 1B). Ab responses to each Ag were specific, with no cross-reactivity observed. Responses to both Ags were also detectable 2 wk after the Ad prime and were not significantly affected after coadministration of the two AdHu5 vaccines as a mixture. Following the MVA immunization at day 56, responses to both Ags were significantly boosted (p < 0.0001, by paired t test for both Ags) and no significant differences in PyMSP142- or PyCSP-specific IgG titers were observed, either at week 10 or 18, between mice that had received vaccines targeting a single Ag and those that had received the vaccine mixture targeting both Ags. Furthermore, administration of the vaccine mixture did not affect the titer of the PyMSP142-specific or PyCSP-specific IgG1 or IgG2a 2 wk after the MVA boost (Fig. 1C, 1D). Total IgG titers against each Ag were also measured 2 wk after MVA in C57BL/6 mice that had been similarly immunized (Table I). PyCSP-specific IgG was unaffected following vaccine mixture coadministration, but the titer of PyMSP142-specific IgG was marginally and significantly higher in mice receiving the two-antigen regimen (Fig. 1E). The ability of Ad-MVA vaccination to induce functional PyMSP142-specific IgG Ab responses that inhibit blood-stage parasite growth is well established (4, 5). The ability of
Abs induced by Ad-MVA PyCSP to bind *P. yoelii* sporozoites was also confirmed by an immunofluorescence Ab test (Supplemental Fig. 1).

**T cell responses to PyMSP142 and PyCSP vaccines**

BALB/c mice were immunized as before (Table I) and PBMCs were isolated from the blood 2 and 8 wk after the Ad prime and 2 and 9 wk after the MVA boost to assess the affect of mixing the Ad and MVA vaccines on transgene-specific T cell responses following peptide stimulation (Table II). Production of IFN-γ from CD8+ cells was assessed by ICS following restimulation with a pool of overlapping peptides against PyMSP133 (Fig. 2A) or PyCSP280–288 peptide (Fig. 2B). T cell responses could not be measured against full-length PyCSP because of a lack of availability of peptides, and they were not measured against PyMSP119 because they were previously found to be undetectable (4). Following Ad priming, there was comparable production of Ag-specific IFN-γ from CD8+ T cells in mice immunized with either a single Ag or both Ags. However, 2 wk after the MVA boost, the two-antigen vaccine regimen resulted in significantly lower Ag-specific CD8+ IFN-γ+ responses to both PyMSP1 and PyCSP in comparison with the single-Ag regimens. CD8+ IFN-γ+ responses were also measured in the spleen 2 wk after MVA administration (Fig. 2C). These data confirmed the responses observed in the blood, with significantly lower responses to the PyMSP133 peptide pool and PyCSP280–288 in the two-Ag regimen than if either Ag had been given alone. Because of the availability of cells, responses were also measured against the subdominant PyCSP epitope, PyCSP58–67 (39). These data confirmed that responses to both reported class I H-2d epitopes within PyCSP are lower in mice receiving the vaccine mixture compared with Ad-MVA PyCSP given alone. These data also indicated that the total magnitude of the CD8+ IFN-γ+ T cell response in the two-Ag regimen is no greater than when the Ad-MVA PyCSP vaccines are given alone. In addition to CD8+ T cell responses, CD4+ T cell responses were assessed in the spleen 2 wk after the boost (Fig. 2D). Cells were stimulated with PyMSP133, the published PyCSP CD4+ T cell epitope PyCSP59–79, and the overlapping epitope PyCSP58–67. Responses to each peptide are shown, but overall were extremely low at <0.1% CD4+ T cells; although they trend toward being lower in the dual-antigen regimen, they were not significantly lower. Given the low level of these responses, they

**FIGURE 1.** Ag-specific IgG is maintained in a multistage vaccine regimen. BALB/c mice were immunized with the AdHu5-MVA vaccine regimens described in Table I. Serum was collected 2 and 8 wk after priming and 2 and 9 wk after boost (weeks 10 and 17). Total IgG responses at all time points were measured against PyMSP1α-GST protein (A) and PyCSP-specific MAP4-Py1T-Py3 branched peptide (B) by ELISA. Data are mean ± SD for six mice per group. These data are representative of two independent experiments. IgG1 and IgG2a responses were measured 2 wk after boost against PyMSP119 (C) and PyCSP (D). C57BL/6 mice were also immunized with the vaccines described in Table I. Serum was collected 2 wk after boost and total IgG responses measured against both Ags (E). Bars indicate median responses. The dashed line indicates the limit of detection. *p = 0.018 by Student *t* test.

### Table I. Standard immunization regimes

<table>
<thead>
<tr>
<th>Ags</th>
<th>AdHu5 Prime (Mixed)</th>
<th>MVA Boost (Mixed)</th>
</tr>
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<tbody>
<tr>
<td>MSP1 + CSP</td>
<td>1 × 1010 vp AdHu5-PyMSP142</td>
<td>1 × 107 PFU MVA-PyMSP142</td>
</tr>
<tr>
<td></td>
<td>+ 1 × 1010 vp AdHu5-PyCSP</td>
<td>+ 1 × 107 PFU MVA-PyCSP</td>
</tr>
<tr>
<td>MSP1 + control</td>
<td>1 × 1010 vp AdHu5-PyMSP142</td>
<td>1 × 107 PFU MVA-PyMSP142</td>
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<tr>
<td></td>
<td>+ 1 × 1010 vp AdHu5-Control</td>
<td>+ 1 × 107 PFU MVA-Control</td>
</tr>
<tr>
<td>CSP + control</td>
<td>1 × 1010 vp AdHu5-Control</td>
<td>1 × 107 PFU MVA-Control</td>
</tr>
<tr>
<td></td>
<td>+ 1 × 1010 vp AdHu5-PyCSP</td>
<td>+ 1 × 107 PFU MVA-PyCSP</td>
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In all experiments, mice were vaccinated with the indicated regimes, unless stated otherwise. The viruses used were mixed, and then mice were vaccinated i.d. in the ear in a total volume of 50 µl, with 25 µl given in each ear. There was an 8-wk interval between prime and boost. When only a single malaria Ag was given, control vaccines were included to control for viral load.

vc, dose in viral particles.
were not investigated further in this study. CD8+ T cell responses were also assessed in C57BL/6 mice. Two weeks after the MV A boost, responses were measured in the blood by ICS against the pool of PyMSP133 peptides (Fig. 2E). Responses tended toward being lower in the mice immunized with PyMSP142 + PyCSP, although this did not reach statistical significance. No responses were detected after stimulation with PyCSP280–288 (data not shown), and peptides were not available against full-length PyCSP.

It has been shown previously that CD4+ T cells simultaneously producing IFN-γ, TNF-α, and IL-2 are associated with protection against Leishmania major in a murine challenge model (45), and that IFN-γ and TNF-α are important components of the non-cytolytic pathways underlying CD8+ T cell-mediated efficacy against intracellular liver-stage malaria infection in mice (7, 46). Therefore, in addition to IFN-γ, production of TNF-α and IL-2 was measured and the multifunctionality of the CD8+ T cells was assessed. The quality of the Ag-specific cells was analyzed in terms of production of all three cytokines in blood, spleen, and liver 2 wk after the MVA boost immunization (Fig. 3). Responses in the blood and spleen were measured against both the PyMSP133 peptide pool (Fig. 3A) and PyCSP280–288 peptide (Fig. 3B). As seen previously for IFN-γ, total production of Ag-specific TNF-α and IL-2 was significantly reduced in mice immunized against both Ags in comparison with a single Ag. However, within each compartment the proportion of cells (Fig. 3A,3B, pie charts) that were producing all three cytokines (IFN-γ + TNF-α + IL-2), two cytokines (predominantly IFN-γ + TNF-α), or a single cytokine (predominantly IFN-γ) was largely unaffected by immunizing against both Ags. Because of the relatively small number of cells isolated, mononuclear cells could not be stimulated with each Ag separately. Instead, they were stimulated with a single peptide pool containing PyMSP133 peptide pool, PyCSP280–288, and PyCSP58–67 (Fig. 3C). Although conclusions about the contribution of each individual Ag cannot be made from these data, it was clear that the total cytokine production from liver mononuclear cells of mice that had received the MSP1 + CSP vaccine regimen was no higher than in mice that had received the PyCSP vaccines.
alone—in agreement with data from the spleen and blood. Interestingly, CD8+ T cells expressing IL-2 after restimulation were undetectable in the blood, but were observed in both the spleen and liver.

Protection against *P. yoelii* sporozoite challenge

CD8+ T cell responses, induced by viral vectored vaccines encoding either PyMSP142 or PyCSP, have been reported previously to provide partial or sterilizing liver-stage protection against *P. yoelii* sporozoite challenge (4, 32–34). To assess whether the reduced CD8+ T cell responses after the boost in the MSP1 plus CSP vaccine regimen had an effect on efficacy against sporozoite challenge, mice were challenged intravenously 2 or 10 wk after the MVA boost with either 1000 or 50 sporozoites respectively (Table III). Fifty sporozoites are routinely used in the *P. yoelii* challenge model (4, 5, 47); however, we had already reported that the Ad-MVA PyMSP142 vaccine regimen could protect against a high-dose challenge of 250 sporozoites (48) 2 wk after the MVA boost immunization (4). Therefore, to maximize any protective differences between immunization groups, an even

![Image of Figure 3](http://www.jimmunol.org/)

Table III. Efficacy of the vaccine mixture against *P. yoelii* sporozoite challenge

<table>
<thead>
<tr>
<th>Immunization Regime</th>
<th>No. Survived/ No. Challenged (% Survival)</th>
<th>No. Sterilely Protected/ No. Challenged (% Sterile)</th>
<th>Geometric Mean (Range) Peak Parasitemia of Surviving Mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 Sporozoites, 2 wk after Boost</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP1 + CSP</td>
<td>5/6 (83)</td>
<td>1/6 (16)</td>
<td>4.62 (0–28)</td>
</tr>
<tr>
<td>MSP1 + control</td>
<td>6/6 (100)</td>
<td>1/6 (16)</td>
<td>1.80 (0–7.9)</td>
</tr>
<tr>
<td>CSP + control</td>
<td>4/6 (66)</td>
<td>4/6 (66)</td>
<td>N/A</td>
</tr>
<tr>
<td>Naive, not immunized</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>N/A</td>
</tr>
<tr>
<td>50 Sporozoites, 10 wk after Boost</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP1 + CSP</td>
<td>5/6 (83)</td>
<td>2/6 (33)</td>
<td>4.53 (0–11.8)</td>
</tr>
<tr>
<td>MSP1 + control</td>
<td>6/6 (100)</td>
<td>1/6 (16)</td>
<td>0.20 (0–17.6)</td>
</tr>
<tr>
<td>CSP + control</td>
<td>3/6 (50)</td>
<td>3/6 (50)</td>
<td>N/A</td>
</tr>
<tr>
<td>Naive, not immunized</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

BALB/c mice were immunized with the regimens described in Table I and challenged i.v. with sporozoites at the indicated time points. Parasitemia was monitored as described.
A higher challenge dose of 1000 sporozoites was used at the 2-wk postboost challenge time point (Fig. 4, Table III). In both experiments, fewer mice were steriley protected following immunization with the MSP1 plus CSP regimen in comparison with the CSP alone regimen. Following challenge 2 wk after the MVA boost, only 1 in 6 mice was steriley protected in the MSP1 plus CSP group, compared with 4 in 6 in the CSP alone group, and a similar trend was observed at the late 10-wk challenge time point. Sterilizing liver-stage efficacy had therefore been reduced following coadministration of the MSP1 plus CSP vaccines, indicating that a mixed population of CD8+ T cells against PyMSP133 and PyCSP is less protective in this model than a similar quantity of cells targeting PyCSP alone. However, despite this reduced efficacy at the liver-stage and in agreement with the PyMSP119 Ab data (Fig. 1A), overall survival in the mice vaccinated with MSP1 plus CSP remained high after the onset of blood-stage parasitemia, with 5 in 6 (83%) surviving in each challenge experiment compared with 100% survival following immunization with the MSP1 vaccine alone. For mice challenged 10 wk after the boost, sera were collected and peak parasitemia were correlated significantly with prechallenge total PyMSP119-specific IgG ELISA titer (Fig. 4). These data suggest that, following the onset of blood-stage parasitemia, survival in this model remains dependent on PyMSP119 Ab titers. These data also confirm that the Ad-MVA PyMSP142 vaccine regimen by itself maintains 100% survival following a high-dose challenge with 1000 sporozoites at day 70 and following a challenge with 50 sporozoites 10 wk after the boost.

CD8+ T cell interference is not completely overcome by lowering vaccine dose

One possible explanation for the observed CD8+ T cell interference following the coadministration of the MVA PyMSP142 and PyCSP booster vaccines (but not AdHu5 priming vaccines), was that it was a systemic or spatial effect. Given the relatively high overall magnitude of the responses induced against each Ag individually, it may not be physiologically possible to expand both Ag-specific populations to the same extent when both vaccines were administered together. T cell responses induced by vectored vaccines follow a dose-response curve (49); therefore, it was hypothesized that lowering the dose of the vaccines might reduce the overall magnitude of the response induced and thus prevent the observed CD8+ T cell interference. BALB/c mice were thus immunized as described in Table I, but primed with 10^9 viral particles (vp) of each AdHu5 vaccine (rather than 10^10 vp) and boosted with 10^6 PFU of each MVA vaccine (rather than 10^7 PFU). Two weeks after the MVA boost, mice were culled and splenocytes were restimulated with the PyMSP133 peptide pool or PyCSP260–288 before assaying responses by ICS (Fig. 5). In this case, PyCSP-specific responses were significantly lower (p = 0.0087 by Mann–Whitney U test) than those observed previously, with a median response of 13.7% of CD8+ T cells producing IFN-γ after CSP only vaccination compared with 25.4% observed in the previous experiment (Fig. 2C), and significant CD8+ T cell interference was not observed for this Ag. However, despite the reduced magnitude of the PyCSP CD8+ T cell population, significant interference was still observed after vaccine coadministration for the relatively weaker responses against PyMSP133. Given that overall responses of greater total magnitude are more likely to exhibit interference, a reduction in the magnitude of responses might be required for complete interference to be overcome by lowering vaccine dose.

FIGURE 5. CD8+ T cell competition is not completely overcome by lowering doses of vaccines. BALB/c mice were immunized with the same regimes as described in Table I, but using lower doses of vaccines (10^6 vp of each AdHu5 and 10^6 PFU of each MVA). Ag-specific CD8+ IFN-γ+ responses were measured as before in the spleen 2 wk after boost by ICS. **p < 0.01 by Mann–Whitney U test, excluding negative controls.
magnitude were previously observed in the spleen (Fig. 2C), these data indicate that physiologic space is not the only contributing factor to CD8\(^+\) T cell interference in this model.

**CD8\(^+\) T cell interference can be minimized by immunization at separate sites**

A second possibility was that CD8\(^+\) T cell interference was a local effect, occurring at the draining lymph node sites of T cell boosting, and could be overcome by immunizing with the vectored vaccines at physically separated sites. To investigate this possibility, BALB/c mice were primed as usual with a mixture of 10\(^{10}\) vp of each AdHu5 vaccine and then boosted 8 wk later with 10\(^7\) PFU of each MVA vaccine, except that the two different MVA vaccines were given into separate ears. Two weeks after the MVA boost, PBMCs were isolated and restimulated as before with the indicated peptides before analysis by ICS (Fig. 6A). Comparable PyCSP-specific CD8\(^+\) IFN-\(\gamma\) T cell responses were observed in mice immunized against MSP1 plus CSP or against CSP alone. Importantly, these PyCSP-specific responses were maintained at a magnitude similar to those seen previously when the vaccines had been given as a mixture, rather than at the lower magnitude observed after reduced dose immunization. PyMSP142-specific CD8\(^+\) IFN-\(\gamma\) T cell responses were, however, still lower in mice immunized against MSP1 plus CSP compared with MSP1 alone. Similar results were observed for TNF-\(\alpha\) and IL-2, and the multifunctionality of the cells was unaffected by immunizing at separate sites, in agreement with previous observations (data not shown). Furthermore, total IgG responses against both Ags remained unaffected by immunization against both Ags at separate sites and were maintained at the same level as when the two MVAs had been given as a mixture (Fig. 6B, 6C).

**Immunization at separate sites improves efficacy against P. yoelii sporozoite challenge**

Two weeks after immunization with the MVA vaccines at separate sites, mice were challenged with 1000 sporozoites by i.v. injection. In this instance, on day 5 after the challenge, parasitemia was sites, mice were challenged with 1000 sporozoites by i.v. injection. Two weeks after immunization with the MV A vaccines at separate sites, in agreement with previous observations (data not shown). Furthermore, total IgG responses against both Ags remained unaffected by immunization against both Ags at separate sites and were maintained at the same level as when the two MVAs had been given as a mixture (Fig. 6B, 6C).

**Immunoassay**

**FIGURE 6.** BALB/c mice were primed with a mixture of AdHu5 vaccines as described in Table I, but 8 wk later the two MVA vaccines were coadministered at separate sites—one given in each ear. Twelve days after immunization with MVA, the production of Ag-specific IFN-\(\gamma\) from CD8\(^+\) PBMCs was measured by ICS (A). Bars indicate median responses. These data are representative of two independent experiments. Total IgG against both PyMSP119 (B) and PyCSP (C) were also measured. The dashed line indicates the limit of detection and bars indicate mean response. *\(p < 0.05\) by Mann–Whitney \(U\) test, excluding negative controls.
T cells compete for access to APCs when both Ags are presented on the same APC surface (50). The exact cause of the competition is not known, but it has been suggested it may be due to limited space around the APC, perhaps limiting access to Ag, costimulatory molecules, or survival factors. To investigate the phenomenon in this study, BALB/c mice were immunized with a mixture containing $1 \times 10^{10}$ vp each of AdHu5-PyMSP142 and AdHu5-PyCSP. Eight weeks later, rather than boosting with MVA, the responses were boosted with $2 \times 10^{7}$ peptide-pulsed CD11c+ DCs as reported previously (51). The DCs were pulsed with PyMSP133 and PyCSP peptides, either mixed together or pulsed separately and with cells mixed immediately before i.v. injection. Two weeks later, CD8+ IFN-γ+ responses were measured against the two Ags in the blood by ICS (Fig. 9). In the majority of mice, both PyMSP133- and PyCSP-specific responses were boosted by injecting peptide-pulsed CD11c+ DCs compared with nonpulsed DCs, but the level of boosting did not reach statistical significance because of at least one nonresponder in each group. Nevertheless it demonstrated that responses were not different, regardless of whether the DCs had been pulsed with peptides specific for both Ags either separately or as a mixture. These data suggest that T cell interference is not occurring because of CD8+ T cells competing when both Ags are presented on the same APC.

**Cells presenting two Ags are better targets for killing at the time of MVA boost immunization**

We hypothesized that secondary CD8+ T cell responses may be lower when the two recombinant MVA vaccines expressing both Ags are given together at the same site because of dual infection of cells at the vaccination site. Infection of a single cell with more than one MVA has been demonstrated in vitro (52), and it is likely to occur in vivo given the dose of MVA used. These cells may be better targets for killing by pre-existing transgene-specific cytotoxic CD8+ T cells specific for both PyMSP133 and PyCSP, allowing less time for Ag expression, processing, and cross-presentation to APCs, compared with when the two vaccines are given separately. To investigate this possibility, an in vivo CTL killing assay was performed. In the first experiment, BALB/c mice were primed by i.d. immunization with $2 \times 10^{10}$ vp AdPyMSP142 plus AdPyCSP, AdPyMSP142 plus AdControl, or AdControl only and then left to rest for 8 wk. At the time the MVA-booster immunization would normally be given, the mice were injected with CFSE-labeled, peptide-pulsed splenocytes as described. The mice were culled 5 h after the cells were injected, and Ag-specific killing was calculated by analyzing CFSE-fluorescence intensity (Fig. 10A). This data showed that if cells were pulsed with PyMSP133 and PyCSP peptides mixed together, and therefore presenting both Ags, then ∼40% of the cells were killed within 5 h in mice primed against both Ags. This finding was in contrast with ∼20% killing seen when mice had been primed only against MSP142, or when cells pulsed with only MSP133 peptides were transferred into mice primed against either MSP142 alone or both Ags (Fig. 10B). These data imply that priming mice against two malaria Ags could lead to a greater level of cellular killing following coadministration of two recombinant MVA booster vaccines as mixture, and this is associated with reduced boosting of CD8+ T cell responses against both Ags. However, in a second experiment, similar observations were made when mice primed against both Ags were injected with splenocytes that had been pulsed with the PyMSP133 and PyCSP peptides separately (Fig. 10C). This outcome was the same, regardless of whether the total splenocyte population or only CD11c+ cells were analyzed (data not shown). Although enhanced killing is observed when mice are previously primed against both Ags, the observations using this approach are limited due to the small size of the splenocyte population.
model assay failed to account for the improved CD8+ T cell immunogenicity seen against the PyCSP Ag after coadministration of the two MVA vaccines at separate sites.

Discussion

In this study, we demonstrate the feasibility of a multistage and multiantigen vaccine regimen using a clinically relevant viral vector vaccine delivery platform. In the *P. yoelii* mouse model, we show that coadministration of a mixture of viral vector vaccines expressing different two malaria Ags in an AdHu5-MVA prime-boost regimen results in strong Ab responses against both Ags. However, CD8+ T cell responses were significantly reduced after the MVA boost immunization if the two vaccines were given as a mixture, but this effect was minimized if the two MAVA boost vaccines were administered at separate sites. Importantly, maintaining high-level CD8+ T cell and Ab responses against both the PyCSP and PyMSP142 Ags was associated with the highest levels of efficacy against a stringent high-dose challenge with *P. yoelii* sporozoites.

An important finding from these experiments was that Ab responses were maintained against both Ags after Ad-MVA vaccination in two strains of mice. This finding was confirmed by sporozoite challenge in which no significant differences were observed in survival between mice immunized against PyMSP142 alone, compared with mice immunized against both PyMSP142 and PyCSP. These results are in agreement with a study combining an Ag-specific phenomenon or related to specific vaccine delivery platforms.

In this study we found that, following an Ad-MVA vaccination regimen, the magnitude of the total CD8+ IFN-γ+ T cell response was the same whether mice were immunized with the PyCSP vaccines alone or with both the PyMSP142 and PyCSP vaccines. Despite this finding, after sporozoite challenge the liver-stage protection was reduced in mice receiving the two-antigen regimen, showing that in this case it was more beneficial to have a high number of T cells against PyCSP than the same number of T cells targeting PyCSP and PyMSP133. Given the potential for antigenic interference, this finding highlights the importance of careful consideration of the Ags used in a multiantigen vaccine. It also suggests that, in the *P. yoelii* mouse model, CD8+ T cells against PyCSP are more efficient at killing the pre-erythrocytic parasites than those against PyMSP133. This increased efficiency may be the result of CSP being expressed throughout the sporozoite and early pre-erythrocytic stages of the parasite lifecycle (53), whereas MSP1 is expressed only during the late-liver stage (54).

CD8+ T cell interference may occur for a number of reasons. First, we hypothesized that it could be a systemic or spatial effect, because of the large magnitude of the CD8+ T cell responses induced in BALB/c mice against these two Ags. To address this question we lowered the doses of vaccines, giving 100-fold less AdHu5 and 10-fold less MVA. This reduced the overall magnitude of the Ag-specific immune responses, in agreement with other dosing studies in mice for similar viral vector vaccines (49), and PyCSP-specific CD8+ T cell interference was no longer observed, although the PyMSP133-specific response was still reduced by more than 50% in the two-antigen regimen, indicating that while the magnitude may have been contributing to competition, it is not the sole cause. In addition, altering the ratio of the vaccines, such that mice were boosted with 10-fold more MVA-PyMSP142 than MVA-PyCSP in the two-antigen regimen, could not overcome the PyMSP133-specific CD8+ T cell competition (data not shown).

It has been found that coadministering vaccines at separate sites can overcome CD8+ T cell interference (16). In agreement with that finding, we found that administration of the two recombinant MVA vectors at separate sites eliminated PyCSP-specific CD8+ T cell interference. PyMSP133-specific competition still occurred, but in this case it was minimized, with 70% of the response maintained in the mice immunized against both Ags, whereas in previous experiments the response had been reduced by >50%. Furthermore, when these mice were challenged with *P. yoelii* sporozoites, those immunized at separate sites demonstrated enhanced liver-stage efficacy, as determined by day 5 blood-stage parasitemias.

A number of studies have found that CD8+ T cell interference occurs when CD8+ T cells compete for antigenic epitopes presented on the same APC surface (50). The cause is not known, but it may be limited space around the APC, perhaps limiting access to costimulatory molecules or survival factors. It has been shown that peptide-pulsed DCs can boost MVA primed immune responses (51). To investigate whether this was the case, we boosted mice primed with the mixture of Ad-PyCSP and Ad-PyMSP142 with peptide-pulsed CD11c+ DCs that had either been pulsed with peptides from both Ags together or separately. We found that a modest increase in the production of IFN-γ from CD8+ T cells was observed when Ad-primed responses were
boosted with peptide-pulsed DCs, but there was no significant difference in the magnitude of the CD8+ IFN-γ response between those that had been pulsed with peptide separately or together. This finding is in contrast to those of Kedl et al. (50), who show that when mice are immunized with DCs pulsed with two different peptides, in the presence of high-affinity T cells specific for one of the peptides, then responses to both peptides are impaired if they are presented on the same APC, but only to one peptide if they are presented on separate APC.

We subsequently hypothesized that the killing of MVA-infected cells at the time of the boost by cytotoxic CD8+ T cells, previously primed by the AdHu5 vaccines, may also be a crucial factor in determining the magnitude of secondary CD8+ T cell response. A study of secondary influenza infection showed that CD8+ T cells secreting perforin rapidly terminate Ag presentation in this model (55). Furthermore, studies with MVA and vaccinia virus have shown that during secondary responses the timing of Ag presentation is crucial, with responses detected only to Ags expressed early during the viral life cycle. The development of secondary response to Ags expressed early in the life cycle was dependent on the presence of primed T cells, although a mechanism was not identified (56). Our own results using an in vivo killing assay showed that following injection of peptide-pulsed splenocytes, cells pulsed with both PyMSP133 and PyCSP peptides together were almost twice as likely to be killed within 5 h as those that had been pulsed with peptides corresponding to a single Ag. These data suggest that the more rapid clearance of MVA coinfecting cells expressing both the PyMSP142 and PyCSP Ags results in a reduced period of Ag expression and presentation, and consequently a reduced secondary CD8+ T cell response. We also observed that enhanced killing occurred when cells were pulsed with peptides separately and the cells were mixed immediately before injection. This finding does not appear to be in agreement with the immunogenicity data and may reflect differences in systemic compared with local CTL activity. Otherwise, alternative mechanisms may yet explain the reasons for improved immunogenicity following separate site immunization with two recombinant MVAs.

The outcomes from this study imply that immunizing at separate sites may be necessary to prevent antigenic interference. However,
this practice may be neither practical nor financially viable. A more feasible approach is the development of multivalent MVA vectors, which express a number of malaria Ags from a single virus. Establishing whether CD8+ T cell interference occurs in humans, and the mechanism of interference, will be crucial in deciding which is the more effective approach to developing a multitargeted or multistage malaria vaccine. Phase I/IIa clinical trials are currently underway at Oxford University to assess the efficacy of AdCh63-MVA vectored vaccines expressing P. falciparum MSP1, AMA1, and ME-TRAP alone and in combination (2). Based on the preclinical work presented in this study, vaccines are being administered at separate sites.

In summary, we show that Ad-MVA viral vectored vaccines expressing the Ags PyCSP and PyMSP142 can induce enhanced protection against P. yoelii sporozoite challenge compared with either Ag given alone, provided that MVA vaccinations are given at separate sites. When MVA vaccines are given as a mixture, reduced CD8+ T cell responses are observed. This CD8+ T cell interference is not due to peptide epitope competition for presentation on the same MHC class I molecules, nor is it at the level of the preclinical work presented in this study, vaccines are being administered at separate sites. When MVA vaccines are given as a mixture, reduced CD8+ T cell responses are observed. This CD8+ T cell interference is not due to peptide epitope competition for presentation on the same MHC class I molecules, nor is it at the level of the preclinical work presented in this study, vaccines are being administered at separate sites. 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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1

Visible and fluorescence images of *P. yoelii* sporozoites immunostained with PyCSP antibodies raised in mice. *P. yoelii* sporozoites were killed with azide and air dried onto slides. Mice were immunized with $1 \times 10^{10}$ vp AdHu5-PyCSP and boosted with $1 \times 10^{7}$ pfu MVA- PyCSP. Sera were collected two weeks later and were used at a dilution of 1:100. Sera from naïve (non-immunized) mice were also tested. Bound antibodies were detected using goat anti-mouse IgG conjugated to Alexa Fluor™ 488.