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Enhancing Blood-Stage Malaria Subunit Vaccine Immunogenicity in Rhesus Macaques by Combining Adenovirus, Poxvirus, and Protein-in-Adjuvant Vaccines

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Protein-in-adjuvant formulations and viral-vectored vaccines encoding blood-stage malaria Ags have shown efficacy in rodent malaria models and in vitro assays against *Plasmodium falciparum*. Abs and CD4$^+$ T cell responses are associated with protective efficacy against blood-stage malaria, whereas CD8$^+$ T cells against some classical blood-stage Ags can also have a protective effect against liver-stage parasites. No subunit vaccine strategy alone has generated demonstrable high-level efficacy against blood-stage infection in clinical trials. The induction of high-level Ab responses, as well as potent T and B cell effector and memory populations, is likely to be essential to achieve immediate and sustained protective efficacy in humans. This study describes in detail the immunogenicity of vaccines against *P. falciparum* apical membrane Ag 1 in rhesus macaques (*Macaca mulatta*), including the chimpanzee adenovirus 63 (AdCh63), the poxvirus modified vaccinia virus Ankara (MVA), and protein vaccines formulated in Alhydrogel or CoVaccine HT adjuvants. AdCh63-MVA heterologous prime-boost immunization induces strong and long-lasting multifunctional CD8$^+$ and CD4$^+$ T cell responses that exhibit a central memory-like phenotype. Three-shot (AdCh63-MVA-protein) or two-shot (AdCh63-protein) regimens induce memory B cells and high-titer functional IgG responses that inhibit the growth of two divergent strains of *P. falciparum* in vitro. Prior immunization with adenoviral vectors of alternative human or simian serotype does not affect the immunogenicity of the AdCh63 apical membrane Ag 1 vaccine. These data encourage the further clinical development and coadministration of protein and viral vector vaccine platforms in an attempt to induce broad cellular and humoral immune responses against blood-stage malaria Ags in humans. ***The Journal of Immunology*****, 2010, 185: 7583–7595.**

Although recent and encouraging evidence suggests that the epidemiology of *Plasmodium falciparum* malaria is changing across certain parts of Africa (1), the worldwide burden of disease from malaria remains a major public health problem (2). The development of a highly effective malaria vaccine remains a top priority to help combat this disease. Given that pre-existing malaria-control measures are likely to be combined with RTS,S or other similarly effective pre-erythrocytic vaccines in the medium term, it is acknowledged that only highly effective blood-stage vaccines could feasibly complement such pre-erythrocytic control strategies in the future (3). However, despite extensive efforts and >30 published clinical trials of blood-stage vaccine candidates, no single approach or vaccine platform has shown a convincing or significant protective effect in human phase IIa/b efficacy trials (4).

Blood-stage malaria vaccine development has classically focused on recombinant protein-in-adjuvant formulations, with the aim of inducing high-titer growth-inhibitory Abs against merozoite Ags involved in erythrocyte invasion, such as the widely studied merozoite surface protein 1 (MSP1) (5) and apical membrane Ag 1 (AMA1) (6). In recent years, viral-vectored blood-stage malaria vaccines have also been developed (7, 8). Heterologous prime-boost immunization regimens, involving replication-defective adenoviruses of human or simian serotype or the orthopoxvirus-modified vaccinia virus Ankara (MVA), have shown particular promise in mice and rabbits (9–11). These vectored vaccines can induce qualitatively different types of immune responses to protein-in-adjuvant vaccines, including high-titer Th1 isotype-skewed Ab responses and, most notably, strong T cell responses (9). Abs induced by such regimens in mice and rabbits against MSP1 and AMA1 were shown to inhibit the growth of *P. falciparum* in vitro (10, 11), and vaccination with an adenovirus-MVA regimen can protect mice against a lethal challenge with blood-stage *P. yoelii* (9). Importantly, CD8$^+$ T cells against MSP1 can also reduce the *P. yoelii* liver-stage parasite burden, given that

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The online version of this article contains supplemental material.

Abbreviations used in this paper: AdCh3, chimpanzee adenovirus 3; AdCh63, chimpanzee adenovirus 63; AdHu5, human adenovirus serotype 5; AdHu6, human adenovirus serotype 6; Ad_M, chimpanzee adenovirus 63-modified vaccinia virus Ankara apical membrane Ag 1; AMA1, apical membrane Ag 1; ASC, Ab-secreting cell; GIA, growth-inhibitory activity; ICS, intracellular cytokine staining; mBC, memory B cell; MSP1, merozoite surface protein 1; MVA, modified vaccinia virus Ankara; NAb, neutralizing Ab; NaSCN, sodium thiocyanate; PBS/T, PBS/Tween; RT, room temperature; SFU, spot-forming unit; Tcm, central memory T cell; Tem, effector memory T cell.

this Ag is expressed by parasites toward the end of hepatic development (12), and another study documented the ability of $CD4^+$ T cells against AMA1 to contribute to blood-stage immunity against *P. chabaudi* rodent malaria, independently of Ab (13).

Blood-stage malaria vaccine development has arguably suffered from a lack of in-depth understanding of the immune effector mechanisms that contribute to protection in vivo in humans. Studies of naturally acquired immunity and experimental immunization in humans and animal models have painted a complex picture (14), with protection associated with Abs against merozoite Ags (15), specific IgG isotypes (16), T cells against blood-stage parasites (17–20), innate effector cell types (21–23), serum cytokines (21, 24), and regulatory T cells (25). Numerous other studies described the immunomodulatory effects of parasite exposure on T and B lymphocyte populations (26–28). It is perhaps not surprising that single-modality vaccine platforms, aiming to induce a single type of immune response, have failed to impact on the blood-stage growth rates of *P. falciparum* in clinical trials. This is further supported by recent studies showing that growth- and invasion-inhibitory Abs are not directly associated with, or do not completely account for, disease outcome in the field (29, 30).

An alternative aim for vaccination is to induce broad functional cellular and humoral immune responses against target Ags. Recently, we combined, in mice, two leading subunit vaccine platforms for *P. falciparum* MSP1 (i.e., viral-vectored vaccines with protein Ag in adjuvant), and we showed that immunization regimens combining these two approaches can achieve simultaneous Ab and T cell responses which equal, or in some cases surpass, the best immune responses achieved by either vaccine technology alone (31). Such broad T cell and Ab responses can be achieved by using three-stage (adenovirus-MVA-protein) vaccination protocols, or an alternative two-stage adenovirus prime–protein-boost protocol can be equally effective for Ab induction with lower levels of T cell immunogenicity. These murine studies also described further possible advantages of viral vectors in achieving consistent Ab priming, enhanced Ab avidity, and Th1-type IgG isotype skew. Data from the HIV-1 (32–36) and liver-stage malaria vaccine fields (37, 38) also encourage efforts to combine adenoviral-vectored vaccines with protein vaccines.

In this article, we describe the use of chimpanzee adenovirus 63 (AdCh63) and MVA in a heterologous prime-boost regimen in rhesus macaques. The viral vectors express two alleles of the AMA1 Ag, and this regimen induces strong growth-inhibitory Ab responses and potent multifunctional $CD4^+$ and $CD8^+$ T cell responses. Subsequent boosting with a biallelic protein AMA1 vaccine, formulated in Alhydrogel or the novel vaccine adjuvant CoVaccine HT (39), in a three-shot AdCh63-MVA-protein regimen or two-shot AdCh63-protein regimen, induces memory B cells (mBCs), the highest levels of functional Ab, and maintains T cell responses.

Materials and Methods

Generation of viral vector and protein vaccines

rAdCh63 and markerless rMVA vectors expressing *P. falciparum* AMA1 were generated and purified using methods that were described previously (11, 31). The vectors express a 3483-bp biallelic AMA1 transgene composed of the human tPA leader sequence (9), followed by aa 25–546 of the ectodomain of AMA1 (strain 3D7) (GenBank Accession #U65407; http://www.ncbi.nlm.nih.gov/genbank/), a glycine-proline linker (GGGPGGG), and then aa 25–622 including the ecto-, transmembrane-, and C-terminal domains of AMA1 (strain FVO) (GenBank Accession #AJ277646 [aa 25–613] and #U84348 [aa 614–622]). A number of amino acid substitutions (8 in 3D7 and 10 in FVO) to prevent potential N-linked glycosylation were included, as described elsewhere (40). The final construct is codon optimized for human expression and was synthesized by GeneArt (Regensburg, Germany). To minimize any chance of homologous recombination between the similar coding sequences for the two alleles of AMA1, codon optimization included minimum homology at the nucleotide sequence level for these two sequences (final base pair sequences are 77.6% similar). Production of protein AMA1 vaccines (3D7 and FVO alleles) is described elsewhere (41). Briefly, both proteins are composed of the prodomain and ectodomain (domains I–III) of the relevant AMA1 allele. A single cleavage within domain II of the 3D7 AMA1 protein was noted during reduced SDS-PAGE analysis, similar to that reported by other investigators (40).

Animals and immunizations

Rhesus macaques (*Macaca mulatta*) were housed at the Institute of Neurobiology and Molecular Medicine (Rome, Italy). During handling, the animals were anesthetized by i.m. injection of 10 mg/kg ketamine hydrochloride. The ethical committee of the Italian Ministry of Health approved this research. Macaques were immunized i.m. in the deltoid muscle of the arm with 5×10^{10} viral particles AdCh63 AMA1, 5×10^8 PFU MVA AMA1, or 50 μg recombinant protein vaccine (1:1 mix of 3D7 and FVO proteins: 25 μg each). Protein vaccines were formulated and rotated until use and injected within 30 min. Total injection volume for all vaccines was 0.5 ml.

Vaccine formulation

The viral-vectored vaccines were diluted in sterile PBS. The protein vaccines were formulated in adjuvant: CoVaccine HT (a novel proprietary vaccine adjuvant of Protherics Medicines Development, a BTG Company, London, U.K.) as a 1:1 mix with protein in PBS (10 mg sucrose fatty acid sulfate ester per dose) or Alhydrogel (Brenntag BioSector, Frederiksund, Denmark) as a 1:1 mix with protein. The aluminum content in the Alhydrogel component was 0.85 mg per dose (equivalent to 0.69% Alhydrogel), and the vaccine was formulated in low-phosphate PBS (<5 mM). Adsorption of the AMA1 protein vaccine to Alhydrogel adjuvant was determined by an AMA1-specific ELISA. The following formulations were prepared, as described earlier, and then centrifuged at 4000 rpm for 10 min: a dose of protein vaccine formulated in Alhydrogel, a dose of protein vaccine formulated in PBS without Alhydrogel, and Alhydrogel without recombinant protein in PBS. Centrifugation should sediment protein adsorbed to Alhydrogel, and AMA1-specific Ab responses in the supernatant, as detected by ELISA, should consequently be lower than in the protein-only control. Ninety-six–well Nunc-Immuno Maxisorp plates (Fisher Scientific, U.K.) were coated with 50 μl the three different supernatants, serially diluted down the plate, and allowed to adsorb at room temperature (RT) overnight. The following day, plates were washed in PBS containing 0.05% Tween 20 (PBS/T) and blocked with PBS/T containing 10% skimmed milk. Serum from AMA1-immunized mice was diluted 1:100, and 50 μl was added to each treatment well. Naive mouse serum was added to duplicate wells for each treatment as a negative control. Bound Abs were detected using alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule) (Sigma-Aldrich, Dorset, U.K.) diluted 1:5000 in PBS/T. Analysis confirmed that >99.9% of the protein vaccine was adsorbed.

PBMCs and serum preparation

Blood samples were obtained from the femoral vein of anesthetized animals into vacutainer blood-collection systems (BD Biosciences, U.K.) and transported overnight to the Jenner Institute using ambient-temperature containers (Laminar Medica, Tring, Hertfordshire, U.K.). Lithium heparin-treated peripheral blood was diluted in HBSS, and PBMCs were isolated by density-gradient centrifugation in Accuspin tubes containing Histopaque-1077 (Sigma-Aldrich). Cells at the interface were collected into R10 medium (RPMI 1640, supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, and 50 μg/ml gentamicin [all from Sigma-Aldrich]), spun down, and treated with ACK lysis buffer for 5 min at RT to lyse erythrocytes. Cells were diluted in HBSS and then washed in R10 medium before use in the assays. PBMCs were resuspended in R10 medium, and live cells were counted by trypan blue staining. Excess cells were frozen in FCS containing 10% DMSO and stored in liquid nitrogen. If required, frozen cells were thawed in a water bath at 37°C and immediately transferred into warm R10 medium. Cells were washed twice in warm R10, resuspended in R10 containing 25 U/ml benzonase (Sigma-Aldrich), and incubated overnight at 37°C, 5% CO_2 in a humidified incubator. The next day, live cells were counted, washed, and resuspended in fresh R10 medium and used in the assay. For serum preparation, untreated blood samples were stored at 4°C overnight and then the clotted blood was centrifuged for 30 min ($1000 \times g$). Serum was stored at −20°C.

Peptides for T cell assays

PEPscreen peptides were purchased from Sigma-Aldrich. The peptides, 15 aa in length and overlapping by 10 aa, covered the entire AMA1 insert present in the viral-vectored vaccines (Supplemental Table I). Peptides were reconstituted in 100% DMSO at 20 mg/ml and combined into various pools for ELISPOT and intracellular cytokine staining (ICS) assays, as described in the text and Supplemental Tables I and II. The human adenovirus serotype 5 (AdHu5) hexon pool (PepTivator AdV5 Hexon) was purchased from Miltenyi Biotec (Surrey, U.K.).

Ex vivo IFN-γ ELISPOT

Fresh PBMCs were used in all ELISPOT assays, with the exception of the assay for the AdHu5 hexon in which frozen cells were used. Anti-monkey IFN-γ mAb GZ4 (Mabtech, Nacka, Sweden) was diluted to 15 μg/ml in PBS. MAIP ELISPOT plates (MAIPS4510; Millipore, Watford, U.K.) were coated with 50 μl/well and stored overnight at 4˚C. The following day, wells were blocked with 100 μl/well R10 medium and incubated at 37˚C for >1 h. PBMCs were resuspended at 5×10^6 cells/ml in R10 medium, and 50 μl/well was added to wells in duplicate. Fifty microliters per well AMA1 peptide pools (final concentration each peptide 5 μg/ml) or AdHu5 hexon pool (final concentration each peptide 2 μg/ml) diluted in R10 medium were also added to test wells, 50 μl/well R10 medium and DMSO control were added to negative unstimulated wells, and 50 μl/well staphylococcal enterotoxin B (final concentration 1 μg/ml) was added to positive control wells. Plates were incubated at 37˚C, 5% CO_2 in a humidified incubator for 18–20 h. Plates were washed six times with PBS, and 50 ng/well biotinylated anti-human IFN-γ mAb 7-B6-1 (Mabtech) in PBS was applied. Plates were incubated for 2 h at RT, washed six times with PBS, and 50 ng/well streptavidin alkaline phosphatase polymer in PBS was applied. Plates were incubated for 1 h at RT and washed again six times in PBS. Spots were developed by addition of 100 μl/well color development solution (Bio-Rad, Hemel-Hempstead, Hertfordshire, U.K.) diluted in water and counted using an ELISPOT counter (Autoimmun Diagnostika, Straßberg, Germany). Results are expressed as IFN-γ spot-forming units (SFU) per million PBMCs. Background responses in media-only wells were almost always <20 spots and were subtracted from those measured in peptide-stimulated wells.

Multiparameter flow cytometry

Cytokine secretion by PBMCs was assayed by ICS. Staining Abs were rat or mouse anti-human IgG mAb purchased from eBioscience (Hatfield, U.K.) or BD Biosciences. PBMCs were restimulated in R10 medium for 6 h at 37˚C with 1 μg/ml staphylococcal enterotoxin B (positive control samples), a pool of all 170 AMA1 peptides (Supplemental Table I) at a final concentration of 1 μg/ml each peptide and 0.85% total DMSO (AMA1-stimulated samples), or 0.85% DMSO final concentration (unstimulated samples). Restimulation was carried out in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich), 0.7 μg/ml monensin (GolgiStop; BD Biosciences), 1 μg/ml mouse anti-human CD49d (BD Biosciences), 10 μl CD107a-PE-Cy5 (clone H4A3), and 10 μl CD28-allophycocyanin (clone CD28.2). Cells were stored at 4˚C overnight and stained the next day in three steps (30 min at 4˚C for each step). Cells were surface stained in FACS buffer (PBS with 0.01% sodium azide and 0.1% BSA) and washed between each step: step 1) CD95-biotin (clone DX2) and 1% heat-inactivated normal macaque serum and step 2) streptavidin-Qdot565 (Invitrogen, Paisley, U.K.), CD4 Qdot605 (clone OKT4), CD14-eFluor450 (clone 61D3), CD20-Pacific Blue (clone 2H7), and ViViD LIVE/DEAD Fixable Blue Dead Cell Stain Kit for ultraviolet excitation (Invitrogen). Cells were then fixed and permeabilized in Cytofix/Cytoperm (BD Biosciences) for 10 min at 4˚C before intracellular staining in Permwash with CD3-Alexa Fluor 700 (clone SP34-2), CD8α allophycocyanin-Alexa Fluor 780 (clone RPA-T8), IFN-γ–FITC (clone B27), TNF-α–PE-Cy7 (clone MAb11), and IL-2–PE (clone MQ1-17H12). Samples were washed, resuspended in PBS, and then analyzed using a LSRII Flow Cytometer (BD Biosciences) and FlowJo v8.8 (Tree Star, Ashland, OR). The Boolean gate platform was used with individual gates to create response combinations. Pestle v1.6 and SPICE v5.0 software (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were used to analyze the T cell response profiles. Background responses in unstimulated control cells were subtracted from the AMA1-stimulated response.

Total IgG ELISA

Serum was analyzed for Abs, as previously described (9), except rAMA1 protein was adsorbed to 96-well Nunc-Immuno Maxisorp plates at a concentration of 2 μg/ml in PBS. 3D7 AMA1 (42) was a kind gift from Dr. Chetan Chitnis (International Center for Genetic Engineering and Biotechnology, New Delhi, India), and FVO AMA1 (43) was a kind gift from Dr. Mike Blackman (National Institute of Medical Research, London, U.K.). Bound Abs were detected using alkaline phosphatase-conjugated rabbit anti-monkey IgG (whole molecule) (Sigma-Aldrich) diluted 1:5000. End point titers were taken as the *x*-axis intercept of the dilution curve at an absorbance value three standard deviations greater than the OD_{405} for pre-immunization serum for each individual macaque (typical cut-off OD_{405} for positive sera = 0.15).

Isotype ELISA

Purified recombinant rhesus IgG1, IgG2, IgG3, and IgG4 (reference Ig) were obtained through the National Institutes of Health Nonhuman Primate Reagent Resource. Wells were coated with 5 μg rhesus reference IgG isotype or whole macaque or human serum diluted 1:100 in PBS. Plates were blocked, washed, and developed as for total IgG. Abs were detected using alkaline phosphatase-conjugated rabbit anti-monkey IgG or goat anti-human IgG (Fab) diluted 1:5000, or for isotypes biotinylated anti-human IgG1 (HP6091), anti-human IgG2 (HP6014 or HP6002), anti-human IgG3 (HP6050), anti-human IgG3-hinge (HP6047), anti-human IgG4 (HP6025), or anti-human IgG4-Fc (HP6023) all at 1:1000 dilution, followed by ExtrAvidin-alkaline phosphatase diluted 1:5000.

Avidity ELISA

Ab avidity was assessed by sodium thiocyanate (NaSCN)-displacement ELISA, as previously described (31). Briefly, sera were diluted to a level calculated to give a titer of 1:300, and then an ascending concentration of the chaotropic agent NaSCN was added down the plate (0–7 M). Plates were incubated for 15 min at RT before washing and development as for total IgG.

Neutralizing Ab assay

Human adenovirus serotype 6 (AdHu6)-, AdCh63-, and chimpanzee adenovirus 3 (AdCh3)-neutralizing Ab (NAb) titers were assayed, as previously described (44). Briefly, 3.5×10^4 HEK293 cells were seeded per well in a 96-well plate for 2 d. Each adenoviral vector, recombinant for secreted alkaline phosphatase and incubated for 1 h at 37˚C alone or with serial dilutions of serum, was added to the 95–100% confluent 293 cells and incubated for 1 h at 37˚C. Supernatant was removed and replaced with 10% FCS in DMEM. Secreted alkaline phosphatase expression was measured 24 ± 2 h later with the chemiluminescent substrate from the Phospha-Light kit (Applied Biosystems, Foster City, CA).

In vitro growth-inhibitory activity assay

Half-volume assays of in vitro growth-inhibitory activity (GIA) using half-area plates were performed as described previously (41). Purified IgG was tested against 3D7 and FCR3 strains of *P. falciparum* at 5 or 10 mg/ml. An internal control sample showed ~80% GIA against both strains and was included in all assays.

mBC ELISPOT

MAIP ELISPOT plates were coated with 5 μg/ml rAMA1 (3D7) protein in sterile PBS (at least nine wells for each sample). PBS alone was added to the Ag blank wells, and macaque serum diluted 1:250 in PBS was used as a positive control. The ELISPOT plates were stored at 4˚C until use. Frozen PBMCs were used in assays and prepared as above. Cells were resuspended in R10 medium at a final concentration of 2×10^6 PBMCs/ml. One hundred microliters were added to 96-well flat-bottom culture plates with 100 μl 1:2500 *Staphylococcus aureus* (Cowan strain bacteria; Calbiochem, Nottingham, U.K.), 1:6000 or 167 ng/ml PWM (Sigma-Aldrich), and 1:40 or 5 μg/ml CpG oligonucleotide ODN-2006 (5′-TCG-TCG-TTT-TGT-CGT-TTT-GTC-GTT-3′; InvivoGen, Nottingham, U.K.). The cells were incubated at 37˚C in 5% CO_2 for 6 d before being resuspended and washed twice in R10 medium. The coated ELISPOT plates were washed with PBS, blocked for 1 h with R10 medium at 37˚C, and the cultured cells were added. Plates were incubated for 5 h at 37˚C in 5% CO_2. Ab-secreting cells (ASCs) were detected with a 1:5000 dilution of alkaline phosphatase-conjugated rabbit anti-monkey IgG in PBS. Plates were developed and counted as for ELISPOT assays described above. Ab-forming spots were relatively large, spherical in size, and with "fuzzy" granular edges. Spots that did not fit this description were not counted and were considered in vitro artifacts. Results are expressed as AMA1 (3D7)-specific cultured ASCs per million original PBMCs.

Statistical analysis

Data were analyzed using GraphPad Prism version 5.03 for Windows (GraphPad, San Diego, CA). ELISA end point titers were normalized by $\log_{10}$ transformation for analysis. Where appropriate, fold changes in

ELISA titer, with 95% CI of the mean, are reported. The Mann–Whitney *U* test was performed as appropriate to compare mean responses between two groups. The Wilcoxon signed-rank test was performed to compare mean responses between paired data across two observations. The Friedman test with posthoc Dunn analysis, as appropriate, was used to compare responses between paired data when there were more than two observations. Correlations were analyzed using the Spearman rank test. A p value ≤ 0.05 was considered significant in all cases.

Results

T cell immunogenicity of viral vector/protein immunization in rhesus macaques

Ag-specific T cell induction by the AdCh63-MVA regimen was investigated in this study for the first time, to our knowledge, in rhesus macaques. Eleven monkeys were immunized i.m. with AdCh63-MVA, AdCh63-MVA-protein, or AdCh63-protein-protein AMA1 vaccines, according to the schedule outlined in Table I. The kinetics and magnitude of the T cell response to AMA1 were assessed over time by ex vivo IFN-γ ELISPOT (Fig. 1*A*), following restimulation of PBMCs with overlapping peptides spanning the entire biallelic AMA1 insert present in the viral vectors (Supplemental Table I). Weak Ag-specific T cell responses were detectable at week 8 following the AdCh63 prime (median, 133; range: 57–530 SFU/million PBMCs; $n = 11$); however, in agreement with previous mouse data, these were boosted significantly by MVA ($p = 0.004$), reaching a median level of 2629 SFU/million PBMCs (range, 738–5335 SFU/million PBMCs; $n = 9$) at week 10, with no change apparent in the two macaques that did not receive MVA (median, 101 SFU/million PBMCs at week 10). The T cell effector response decreased by week 16, but small significant boosting effects were evident following protein vaccine administration in both adjuvants at week 16 ($p = 0.008$; $n = 8$), and the same trend was observed for protein immunization in CoVaccine HT at week 35. In agreement with the mouse data for the MSP1 Ag (31), those monkeys receiving both viral-vectored vaccines (AdCh63-MVA with or without protein) in the immunization regimen maintained stronger ex vivo T cell responses at the late week 31 time point in comparison with those that received only one viral vector (AdCh63-protein).

T cell responses were detected in multiple peptide pools spanning the AMA1 vaccine insert, although these were mainly directed against the ectodomain regions of AMA1 and not the C-terminal tail (Fig. 1*B*, Supplemental Fig. 1). Within the ectodomains, responses were detectable against pools of peptides unique to each of the 3D7 and FVO alleles of AMA1 present in the vaccine, as well as common to the two alleles. Despite the difference in magnitude, the hierarchy of these responses was comparable at weeks 8 and 10 after the AdCh63 and AdCh63-MVA immunizations, respectively, although responses were significantly higher ($p < 0.05$) in the common pool at week 8 and the 3D7 pool at week 10 in comparison with the C-terminal pool following a paired analysis. T cell responses were also measured to a pool of peptides corresponding to the vaccine insert versus a pool containing peptides encoding native AMA1 (Supplemental Table II), because amino acid substitutions were included in the AMA1 viral vaccine sequences to remove sites of potential N-linked glycosylation. Comparable immunogenicity was observed at all time points tested (Fig. 1*C*), with a significant correlation overall (Fig. 1*D*), although there was a small, but significant ($p = 0.02$), reduction in the peak T cell responses at week 10 (Fig. 1*C*).

T cell multifunctionality following viral vector immunization

$CD3^+$ T cell functionality was assayed by ICS at week 10, the peak of the Ag-specific T cell response after AdCh63-MVA AMA1 immunization (Fig. 1*A*). $CD4^+$ T cell responses could be detected in all animals that received the prime-boost regimen (Fig. 2*A*, Supplemental Fig. 2), with TNF-α the dominant cytokine and significantly lower, but similar, levels of IFN-γ and IL-2 and, as expected, no detectable CD107a (a marker of CTL degranulation). $CD8^+$ T cell responses were detected in seven of nine animals that received the prime-boost regimen, with similar levels of IFN-γ, CD107a, and TNF-α and slightly lower levels of IL-2 (but only reaching significance [$p < 0.05$] in comparison with TNF-α). As expected, no responses were detectable by ICS in the two animals that only received AdCh63, with the exception of low-level induction of TNF-α by $CD4^+$ T cells (in agreement with this dominant response from the AdCh63-MVA–immunized animals). Similar to other macaque studies using recombinant human adenovirus vectors (AdHu5, AdHu26, and AdHu48) encoding Ags from HIV-1 (45, 46), distinct populations of T cells expressing 1+, 2+, 3+, or 4+ functional markers/cytokines were clearly evident following a Boolean gate analysis, and these differed in magnitude (as the percentage of parent population) between the $CD4^+$ and $CD8^+$ subsets (Fig. 2*B*). The AMA1 effector T cell responses were also phenotyped according to the memory markers CD95 and CD28 (Fig. 2*C*) (47). The $CD4^+$ T cell response was dominated by a central memory T cell (Tcm) phenotype ($CD28^{hi}CD95^+$), whereas the $CD8^+$ T cell response showed a dominant Tcm population and a smaller effector memory T cell (Tem) ($CD28^-CD95^+$) population. Reassuringly, no effector markers/cytokines were detected from the $CD28^+CD95^-$ naive population; interestingly, although the proportion of cells expressing IFN-γ, CD107a, and TNF-α was comparable between the $CD8^+$ Tcm and Tem populations, this was not the case for IL-2 production, which was observed at higher levels within the Tcm population.

Ab immunogenicity of viral vector/protein immunization

The AdCh63 vaccine was able to prime IgG responses against 3D7 and FVO AMA1 (Fig. 3*A*, 3*B*), with all 11 macaques showing detectable responses above baseline (Fig. 3*C*). These titers were boosted significantly ($p = 0.004$) for 3D7 (210-fold increase in the mean, 95% CI: 89–499) and for FVO (87-fold increase in the mean,

Table I. *Schedule of immunizations and vaccination history*

Group	Week 0 (Day 0)	Week 8 (Day 56)	Week 16 (Day 112)	Week 35 (Day 245)	*n*	Vaccine History
1	AdCh63	MVA	Protein/CoVaccine HT	–	3	*a*, *c*, *c*
2	AdCh63	MVA	Protein/Alhydrogel	–	3	*b*, *b*, *c*
3	AdCh63	MVA	–	–	3	*b*, *a*, *d*
4	AdCh63	–	Protein/CoVaccine HT	Protein/CoVaccine HT	2	*a*, *c*

Macaques were immunized i.m. at each time point in this study with the indicated vaccines, as described in *Materials and Methods*. Prior to the start of this study (day 0 = 28 July 2008), 10/11 macaques had received recombinant adenoviral-vectored vaccines against other diseases, as follows. In 2007, six macaques received three vaccinations against hepatitis C virus: either 2× AdHu6 prime and 1× AdCh3 boost (*a*) or 2× AdCh3 prime and 1× AdHu6 boost (*b*) on April 26, 2007 (week 0), May 22, 2007 (week 4), and October 9, 2007 (week 24) at a dosage of 5×10^{10} viral particles for all immunizations. In early 2003, four macaques received vaccines against HIV-1: two immunizations with AdCh3 (*c*); one macaque was completely adenovirus-immunization naive at the start of this study (*d*).

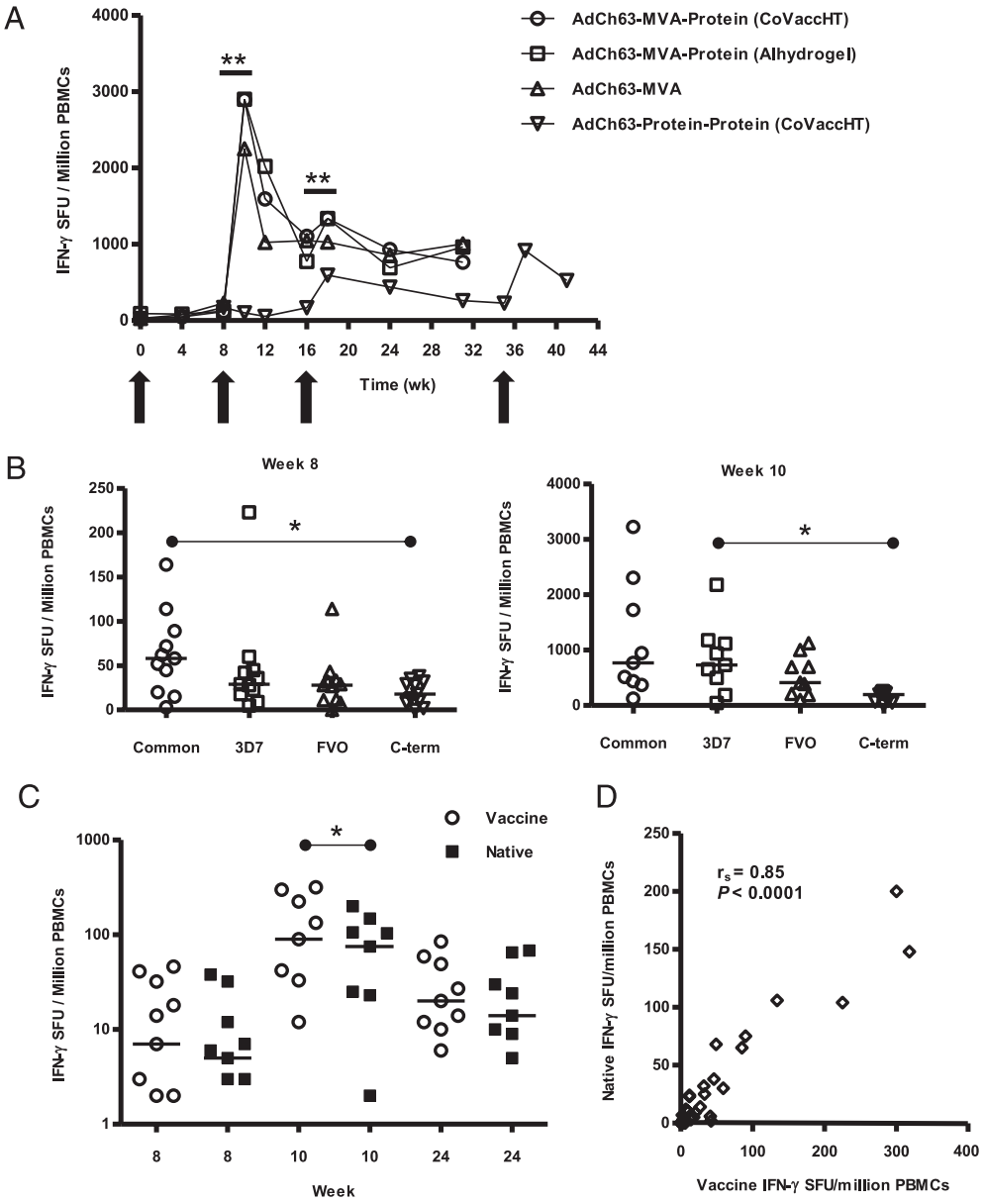

FIGURE 1. Ex vivo IFN-γ ELISPOT T cell immunogenicity of viral vector/protein immunization in rhesus macaques. Eleven rhesus macaques were immunized with AdCh63, MVA, and protein AMA1, as described in Table I. *A*, Mean ex vivo IFN-γ ELISPOT responses to the AMA1 insert (summed response across all of the individual peptide pools) are shown over time in PBMCs for each group. *B*, Breakdown of the total AMA1 response at selected time points into pools of peptides corresponding to peptides common to both alleles of AMA1 in the vaccines or unique to the 3D7 and FVO alleles or the C-terminal region of the Ag. $*p < 0.05$, Friedman paired analysis followed by the Dunn multiple-comparison test. *C*, Responses at selected time points to a pool of peptides containing sequences where amino acids have been substituted in the vaccine insert to remove sites of potential N-linked glycosylation (Vaccine) versus a pool of peptides containing the native AMA1 sequence found in the *P. falciparum* parasite (Native). *D*, Spearman correlation of vaccine versus native responses at the time points shown in *C*. Individual data points and the median are in shown in *B* and *C*. $*p < 0.05$; $**p < 0.01$, by Wilcoxon signed-rank test (*A*, *C*).

95% CI: 56–136) following MVA AMA1 immunization at week 8 (Fig. 3*A*–*C*). IgG titers to both alleles of AMA1 declined significantly ($p = 0.004$) by week 16 (10-fold decrease in the mean for 3D7, 95% CI: 6–19; 5-fold decrease in the mean for FVO, 95% CI: 2–12), but they were significantly boosted ($p = 0.03$) by protein AMA1 immunization at this time point (23-fold increase in the mean for 3D7, 95% CI: 14–38, and 7-fold increase in the mean for FVO, 95% CI: 4–14). Interestingly, comparable and non-significantly different IgG titers were achieved to both alleles of AMA1 following AdCh63-MVA-protein immunization, irrespective of whether the protein AMA1 vaccine was formulated in CoVaccine HT or Alhydrogel (Fig. 3*A*, 3*B*). Overall, these titers were significantly higher than titers achieved following the MVA boost for 3D7 AMA1 ($p = 0.03$) but were only comparable for FVO AMA1 ($p = 0.125$). In agreement with previous mouse data using an alternative adjuvant (31), the highest IgG titers were achieved in the two macaques that received the AdCh63-priming immunization, followed by a single protein immunization in CoVaccine HT at week 16. IgG titers in all groups declined over time, with those monkeys boosted with protein in CoVaccine HT maintaining 3-fold higher median titers for FVO AMA1 4 mo later at week 31 (Fig. 3*A*, 3*B*) in comparison with protein boosting in Alhydrogel ($p = 0.07$ for FVO, but $p = 0.79$ for 3D7). IgG titers to both alleles of AMA1 correlated significantly over the course of the study (Fig. 3*D*). A second immunization at week 35 with protein formulated in CoVaccine HT in the two AdCh63-protein–vaccinated monkeys

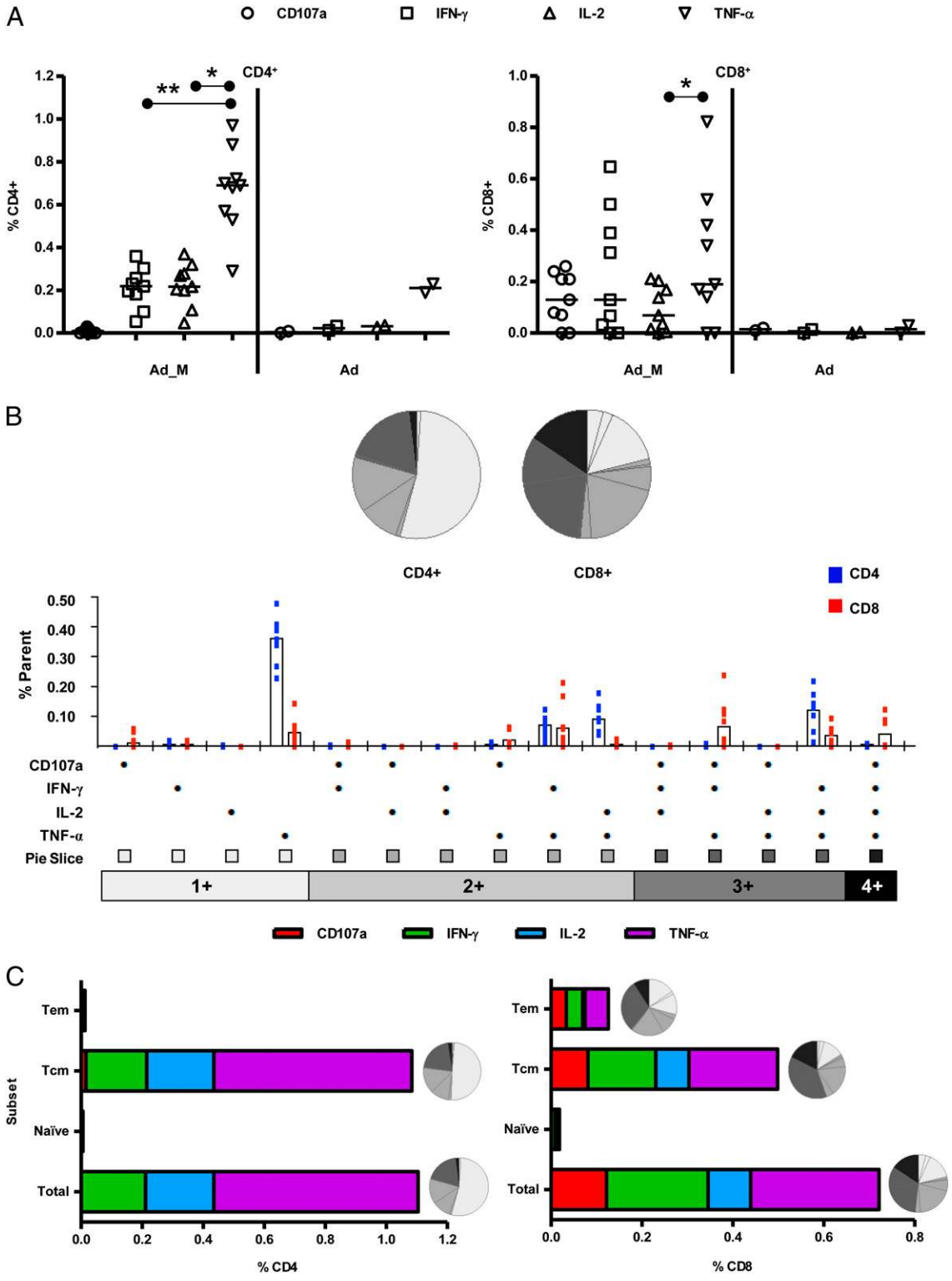

FIGURE 2. T cell multifunctionality following viral vector immunization. The multifunctionality of the $CD3^+$ T cell responses was assessed by polychromatic flow cytometry and ICS at week 10. PBMCs were restimulated with a pool of AMA1 peptides, and cells were stained as described in *Materials and Methods*. Gating strategy and representative plots are shown in Supplemental Fig. 2. *A*, Percentage of $CD4^+$ and $CD8^+$ T cells positive for CD107a, IFN-γ, IL-2, or TNF-α following AdCh63 AMA1 (Ad) or AdCh63-MVA AMA1 (Ad_M) immunization. Individual data points and the median are shown. The $CD107a^+$ population was excluded in the $CD4^+$ analysis. *B*, The multifunctional compositions of the T cell responses following Ad_M immunization. These are grouped and color-coded according to the $CD4^+$ and $CD8^+$ subsets and the number of functions detected for each T cell population. The pie charts summarize the fractions of AMA1-specific $CD4^+$ or $CD8^+$ T cells that are positive for a given number of functions. Individual data points and mean percentage of the parent $CD4^+$ or $CD8^+$ response (open bars) are shown for each of the functional populations indicated on the *x*-axis. *C*, Effector T cells (all cells producing a cytokine or CD107a in response to AMA1 peptide restimulation) were phenotyped by staining for the memory markers CD28 and CD95. Graphs show the breakdown of the total $CD4^+$ or $CD8^+$ T cell response into the mean of the four functions (data taken from *A*), as well as how these are composed from the Tcm ($CD28^{hi}$ $CD95^+$), Tem ($CD28^-CD95^+$), and naive ($CD28^+CD95^-$) phenotypes. Pie charts indicate the multifunctionality of each detectable population, as described in *B*. $*p < 0.05$; $**p < 0.01$, Friedman paired analysis followed by Dunn multiple-comparison test.

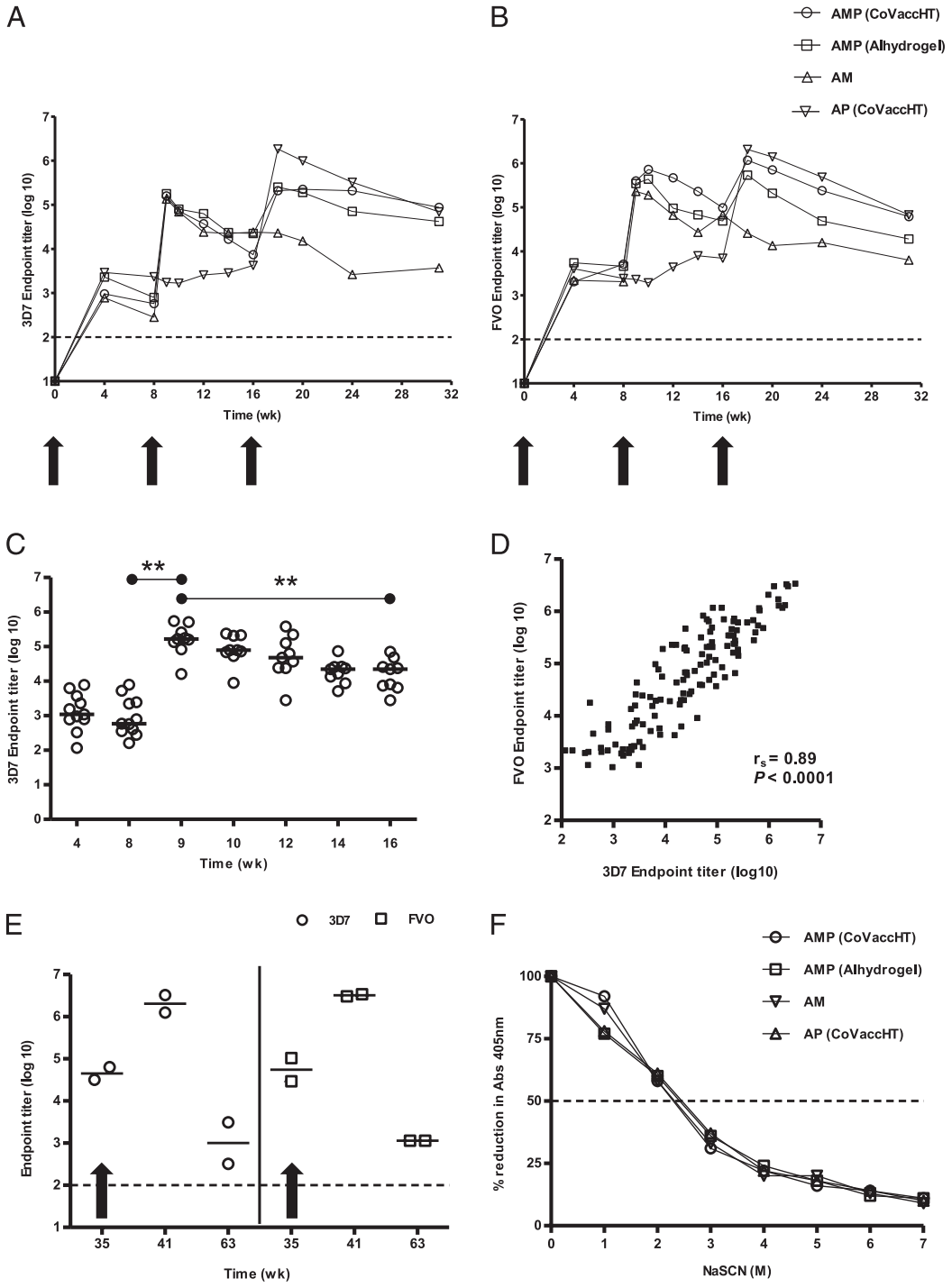

FIGURE 3. Ab immunogenicity of viral vector/protein immunization in rhesus macaques. Eleven rhesus macaques were immunized with AdCh63, MVA, and protein AMA1, as described in Table I. Total IgG ELISA responses against 3D7 AMA1 (*A*) and FVO AMA1 (*B*) as measured in the serum over time. The median response is shown for each group. All sera were negative at day 0 when tested at 1:100 dilution and are plotted below this detection limit. *C*, Individual and median 3D7 AMA1 IgG titers following AdCh63 immunization (weeks 4–8; $n = 11$) and AdCh63-MVA immunization (weeks 9–16; $n = 9$). *D*, Spearman correlation of 3D7 and FVO AMA1 serum IgG ELISA titers over the course of the entire study ($n = 129$). *E*, Individual and median 3D7 and FVO AMA1 IgG titers following AdCh63-protein (CoVaccine HT) immunization (week 35) and AdCh63-protein-protein (CoVaccine HT) immunization (weeks 41 and 63). *F*, NaSCN-displacement ELISA to measure the avidity of FVO AMA1-specific IgG in serum from week 24. Median displacement is shown for each group against an increasing concentration of NaSCN. The level of NaSCN required to displace 50% of the IgG is indicated. $^{**}p < 0.01$, Wilcoxon signed-rank test.

boosted IgG titers back up to the high levels previously achieved. These once again declined with a comparable kinetic to that seen after the first protein immunization and remained at detectable levels at week 63 (Fig. 3*E*).

We previously reported that, in mice, IgG of higher avidity developed against the $MSP1_{19}$ Ag in those animals receiving any immunization regimen containing AdCh63 and MVA, possibly as the result of enhanced levels of $CD4^+$ T cell help for B cell

responses in those groups receiving the prime-boost viral-vectored regimens (31). We also noted a Th1-type isotype skew (IgG2a > IgG1) in those mice receiving the AdCh63-MVA viral-vectored vaccines in comparison with those receiving $MSP1_{19}$ Ag formulated in Montanide ISA720. However, unlike in the mice immunized with MSP1, the IgG induced against AMA1 in the macaques was of comparable avidity at the late week-24 time point, irrespective of whether they received an AdCh63-MVA-protein, AdCh63-MVA, or AdCh63-protein immunization regimen (Fig. 3*F*). Unfortunately, although other studies reported IgG isotype analysis of macaque sera (48, 49), we were unable to identify any anti-human IgG2, IgG3, or IgG4 secondary Ab reagents that cross-reacted with recombinant reference rhesus macaque IgG isotypes, despite testing a range of commercially available clones (Supplemental Fig. 3). Only the anti-human IgG1 clone HP6091 cross-reacted with the rhesus IgG1 counterpart. Although the functional role of IgG isotypes cannot be directly compared between species, the development of such reagents is necessary before such studies of macaque IgG isotype profiles can be undertaken.

In vitro GIA following viral vector/protein immunization

The functional activity of IgG purified from serum was measured using the standardized assay of in vitro GIA against the 3D7 and FCR3 strains of *P. falciparum* (41). The AMA1 sequence of FCR3 differs from that of the FVO strain by only a single amino acid (41). Negligible levels of GIA (<20%) were detectable in the serum before immunization and 10 wk after a single AdCh63 AMA1 administration (Fig. 4*A*, 4*B*). Conversely, the nine monkeys that received the MVA AMA1 boost immunization at week 8 showed moderate GIA at week 10 against both strains (median 44.7%, range: 27.1–72.4% against 3D7 and median 36.5%, range: 24.5–44.1% against FCR3), indicating the induction of functional IgG against these two diverse allelic families of AMA1 by the biallelic viral-vectored vaccines. However, this in vitro efficacy waned to preimmunization levels by week 18 in the three animals that only received AdCh63-MVA immunization. Conversely, the highest levels of in vitro GIA were achieved at week 18 against both strains, following the protein-boost immunization in CoVaccine HT at week 16 (either the AdCh63-MVA-protein or AdCh63-protein regimen), and against 3D7, following the protein boost in Alhydrogel. This increase in GIA was not apparent against FCR3 following the protein boost in Alhydrogel, in agreement with the ELISA titers for the FVO allele of AMA1, which showed comparable titers after the AdCh63-MVA and AdCh63-MVA-protein regimens (Fig. 3*B*). IgG end point titers against both alleles of AMA1 correlated with GIA measured at 5 mg/ml (data not shown) and 10 mg/ml, showing a sigmoidal relationship as seen before in such studies (Fig. 4*C*, 4*D*) (50). Overall, in vitro GIA was lower in all samples when purified IgG was assayed at 5 mg/ml, in agreement with other studies (41, 51), and confirming that the growth inhibitory effect can be titrated out. In agreement with the trend for better maintenance of IgG titers following protein immunization in CoVaccine HT (Fig. 3*A*, 3*B*), higher levels of GIA were maintained in these monkeys at week 24 in comparison with those that received viral-vectored vaccines alone or the protein boost in Alhydrogel (Fig. 4*E*). There was also a highly significant correlation across all samples between GIA against the 3D7 and FCR3 strains, although the overall magnitude of the GIA was higher against 3D7 than against FCR3 (Fig. 4*F*).

mBC responses correlate with Ab longevity

mBC levels were assessed in PBMCs at various time points, including week 16 (prior to protein-in-adjuvant immunization), week 18 (2 wk after protein immunization), and at a late time point (week 31). No ASCs were detectable in PBMCs using the ex vivo B cell ELISPOT assay prior to culture (data not shown); however, following a standard 6-d culture period, ASCs developed from mBCs and specific for the 3D7 allele of AMA1 were detectable (FVO was not assayed). Although low numbers of ASCs were measured at the early and late time points, a significant increase in ASCs was observed following protein immunization across all of the groups ($p = 0.014$) (Fig. 5*A*). There were no significant differences observed between the groups, with some relatively strong responders and some low responders present in each group. mBC levels specific for 3D7 AMA1, as measured using this assay at week 18, correlated significantly with 3D7 AMA1 IgG titers 6 and 13 wk later (weeks 24 and 31, respectively) (Fig. 5*B*).

Effects of pre-existing adenoviral immunity on Ab and T cell immunogenicity

In this study, 10 of the 11 macaques had been immunized with AdCh3 or AdHu6 viral-vectored vaccines against hepatitis C or HIV-1 prior to immunization with AdCh63 AMA1 (Table I) (S. Capone, A. Folgori, S. Colloca, and A. Nicosia, unpublished data). Pre-existing NAb titers against AdHu6, AdCh3, and AdCh63 were assayed in the serum preimmunization (Fig. 6*A*), and T cell responses against the AdHu5 hexon protein were assayed by ex vivo IFN-γ ELISPOT in PBMCs (Fig. 6*B*). AdHu5 hexon peptides were used to detect AdHu6-, AdCh63-, or AdCh3-induced T cell responses (in the absence of serotype-specific peptides), because it is known that T cell epitopes are present within regions of the immunodominant hexon protein that are conserved across serotypes (52). Group A (who had received an AdHu6-AdHu6-AdCh3 regimen 1 y previously) and group B (who had received an AdCh3-AdCh3-AdHu6 regimen 1 y previously) showed detectable vector-specific NAb titers that corresponded to the previous immunization schedules. Group C (who had received an AdCh3-AdCh3 regimen 5 y previously) only showed low-level NAbs against AdCh3. The single macaque in group D, which was adenovirus naive, showed no detectable NAb against any vector. Only minimal cross-reactive NAbs were detected in two macaques against AdCh63, and the rest were negative. T cell responses were also detectable against the AdHu5 hexon protein, but these were comparable across all of the groups. Overall, there was no discernable influence of pre-existing adenoviral immunity on the potency of AdCh63 AMA1 immunization, with peak AMA1-specific IFN-γ T cell responses at week 10 and AMA1 (3D7)-specific IgG titers postprime showing no significant correlation with pre-existing NAb titers against any serotype (Fig. 6*C*, 6*D*). Overall, these data indicate that prior immunization with adenoviral vectors of heterologous serotype does not detrimentally affect the immunogenicity of other subsequent adenoviral vector-based immunization regimens.

Discussion

Despite extensive efforts, the blood-stage malaria vaccine field has failed to demonstrate significant high-level vaccine efficacy in human clinical trials using stand-alone subunit vaccine technologies (4). This study in rhesus macaques aimed to assess the merits of combining the two leading and most clinically relevant subunit vaccine platforms: recombinant protein-in-adjuvant and viral vectors. We previously showed in mice, using such vaccines encoding the MSP1 blood-stage malaria Ag, that this approach can achieve Ab and T cell responses that equal, or in some cases surpass, the best immune responses achieved by either vaccine technology alone (31).

The ability of the AdCh63-MVA heterologous prime-boost regimen to induce potent Ag-specific T cell responses was docu-

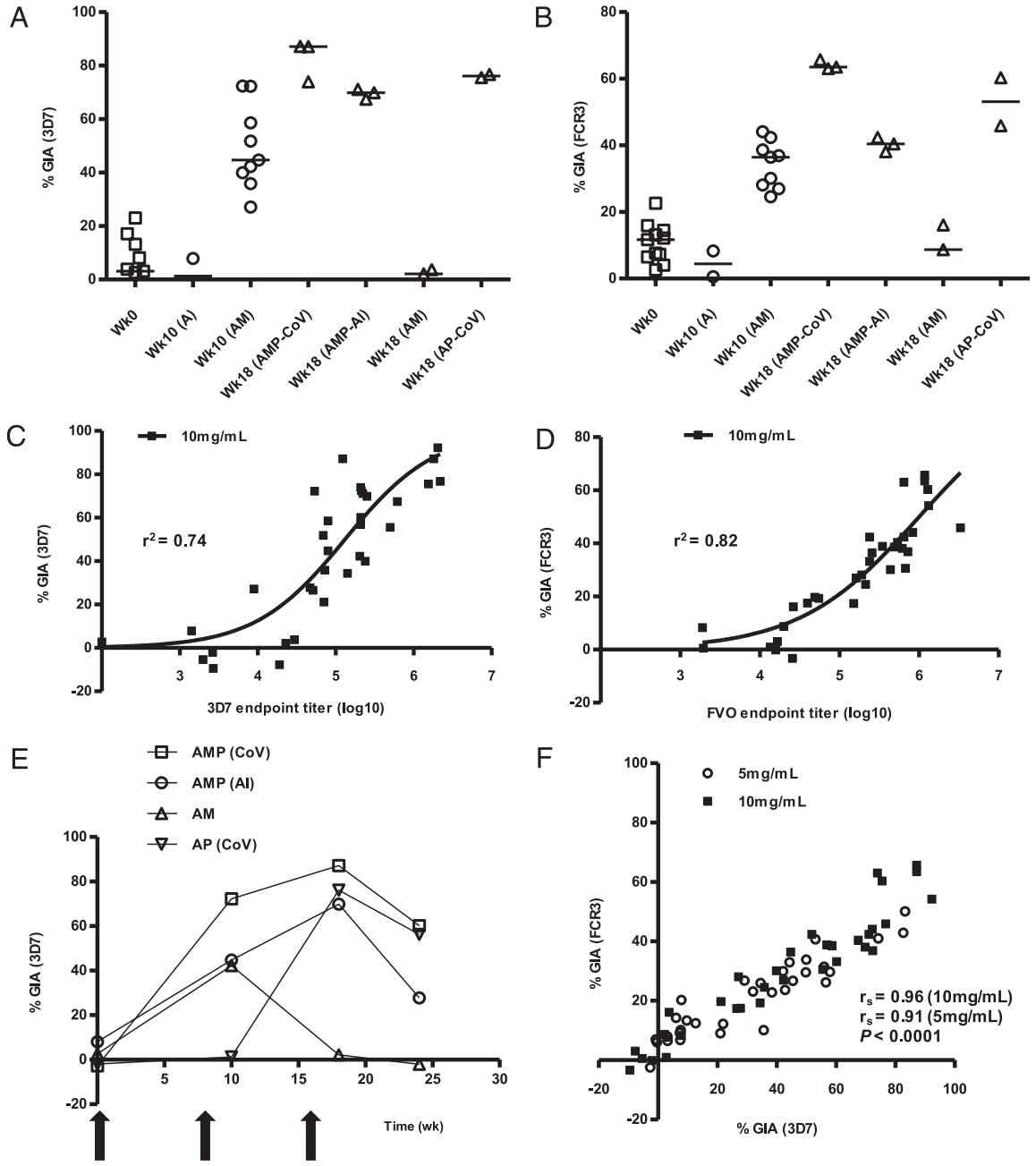

FIGURE 4. Functional activity of IgG induced by viral vector/protein immunization as measured using the in vitro assay of GIA. The functional activity of IgG purified from serum was measured at 10 mg/ml using the standardized assay of GIA against *P. falciparum* strain 3D7 (*A*) and strain FCR3 (*B*). Median and individual data points are shown for the indicated regimens and time points. Relationship between 3D7 GIA at 10 mg/ml and serum 3D7 AMA1-specific IgG ELISA titer (*C*) and FCR3 GIA at 10 mg/ml and serum FVO AMA1-specific IgG ELISA titer (*D*). Individual data points and nonlinear regression curve are shown. *E*, Median 3D7 GIA over time is shown for each group. *F*, Spearman correlation of 3D7 and FCR3% GIA over the course of the entire study ($n = 33$).

mented in mice for the liver-stage malaria Ag ME-TRAP (53) and blood-stage Ag MSP1 (11, 31). Vaccine developers targeting malaria and other immunologically challenging diseases, including HIV-1, have struggled for many years to translate promising rodent immunogenicity into high-magnitude primate and human responses. In this study, this regimen was shown to perform similarly in rhesus macaques to our experience in mice, inducing median T cell responses >2600 SFU/million PBMCs at the peak of the response, as measured by IFN-γ ELISPOT. Recently described data for a candidate HIV-1 vaccine also showed T cell responses of similar magnitude when using two heterologous adenoviruses of human serotype in rhesus macaques (46). Given the recent concerns surrounding the use of AdHu5 in humans (8), it remains encouraging that comparable immunogenicity can be achieved in primates by using simian adenovirus and MVA vectors.

Encouragingly, T cell responses against the AMA1 Ag were detected in multiple pools of peptides that spanned the vaccine Ag insert, and they were not substantially affected by amino acid substitutions used to remove potential sites of N-linked glycosylation. These data indicate that the substituted amino acids are not critical for peptide binding to MHC molecules or for TCR:peptide:MHC recognition in these animals. Alternatively, the substituted amino acids may not form part of the T cell epitopes at all

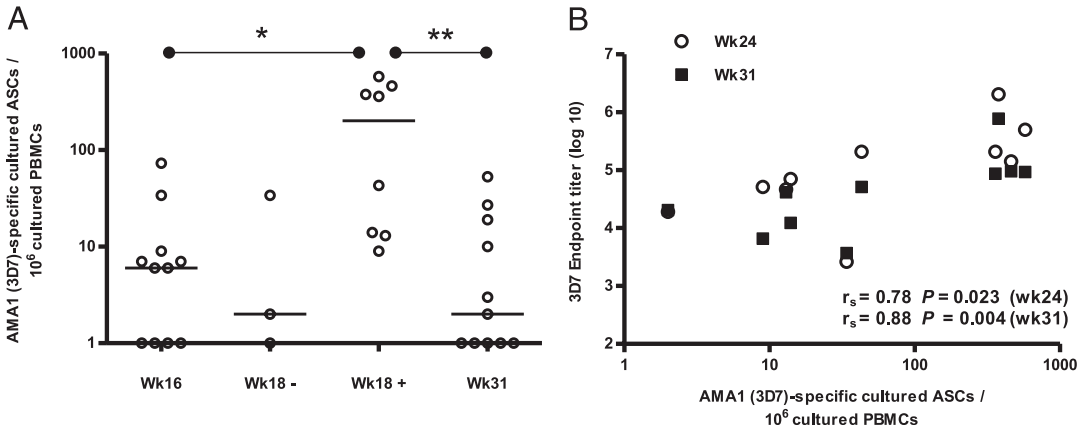

FIGURE 5. mBC responses correlate with Ab longevity. mBC responses were assayed by ELISPOT at weeks 16, 18, and 31. *A*, Individual and median responses are shown following AdCh63-MVA immunization at week 16 ($n = 11$); at week 18 and following the protein-in-adjuvant boost (Wk18+) (data combined for AdCh63-MVA-protein and AdCh63-protein groups; $n = 8$); at week 18 following AdCh63-MVA immunization and no protein boost (Wk18−) ($n = 3$); and at week 31 for all groups ($n = 11$). Results are shown as the number of 3D7 AMA1-specific ASCs per million original cultured PBMCs. *B*, Spearman correlation of 3D7 AMA1 mBC responses at week 18 against 3D7 AMA1 serum IgG ELISA titers at weeks 24 and 31 ($n = 11$). $^*p < 0.05$; $^{**}p < 0.01$, Wilcoxon signed-rank test.

following processing and presentation of the 15mer peptides, although the small reduction in responses at week 10 indicates that at least some of these amino acids are within recognized T cell epitopes. In any case, vaccine-induced T cells against such epitopes should still be capable of recognizing the corresponding native epitopes within the parasite AMA1 Ag upon *P. falciparum* malaria challenge. Recognized epitopes were also spread between peptide sequences that were common and unique to the 3D7 and FVO alleles. One study showed an important role for $CD4^+$ T cells against AMA1 in mediating efficacy against blood-stage *P. chabaudi* infection in mice (13). The induction of such responses against epitopes within AMA1 that are common to multiple alleles bodes well for human immunization if such strain-transcending $CD4^+$ T cells contribute significantly to protection in humans, especially given the issues surrounding the allele-specific protective effects of IgG against this Ag (6).

Advances in multiparameter flow cytometry now allow for the dissection of different T cell populations, phenotypically and in terms of cytokine production and effector function (54). Studies in mice showed correlations between multifunctional (IFN-γ^+TNF-α^+IL-2^+) $CD4^+$ T cells and protection against *Leishmania major* (55), whereas in comparison, $CD8^+$ T cells that secrete IFN-γ but not TNF-α and IL-2 are reported to correlate with protection against *P. berghei* liver-stage malaria (53). Associating the protective outcomes of T cell phenotypes and quality versus quantity in human studies remains a significant challenge for vaccine development (56). Although protective outcome against *P. falciparum* cannot be assessed in rhesus macaques, it remains encouraging that multifunctional $CD8^+$ and $CD4^+$ T cell subsets with a long-term memory phenotype were induced by AdCh63-MVA immunization. These observations are also in agreement with those obtained using recently reported human adenovirus vaccines against HIV-1 (46).

Ab induction by human and simian adenoviral-vectored vaccines has been documented in mice, rabbits, and macaques using vectors recombinant for Ags from pathogens, such as malaria, HIV-1, and rabies virus (9–11, 46, 57). High-Ab titers have been reported following heterologous prime-boost regimens using adenovirus-MVA or two adenoviral vectors of heterologous serotype (9, 11). We previously reported in mice using vaccines against MSP1 that even higher Ab titers can be achieved by combining vectored vaccines with protein-in-adjuvant vaccines (31), similar to that seen in studies of HIV-1 vaccine candidates (32–36). The data in this study of two viral-vectored vaccines and protein-in-adjuvant vaccines in rhesus macaques agreed with the previous murine data, demonstrating that moderately high IgG was induced against both alleles of AMA1 by AdCh63-MVA immunization and that even higher titers could be induced by protein-in-adjuvant boosting. This represents an important test of the adenovirus-protein and adenovirus-MVA-protein regimens in macaques, prior to evaluation of these promising regimens in clinical trials. Further studies will be needed to compare the titers induced by vector-protein regimens with protein-only regimens in macaques, although other murine and macaque vaccine studies with different constructs have suggested that they may perform comparably (34, 37). Interestingly, boosting with protein formulated in CoVaccine HT only led to moderately higher-peak and long-term IgG titers in comparison with formulation in Alhydrogel. Comparative studies of these two adjuvants using protein vaccines alone showed CoVaccine HT to be far superior to alum for Ab induction in mice (S.C. de Cassan, S.C. Gilbert, A.V.S. Hill, and S.J. Draper, unpublished data). It may be that less-potent chemical adjuvants are required to boost IgG responses following viral-vector priming. Given the small group sizes and use of only two adjuvants in this study, it is impossible to conclude whether new experimental adjuvants may outperform alum in the context of vector prime-protein boost regimens, but this observation warrants further investigation. In the meantime, numerous new experimental adjuvants remain in development for use in malaria vaccines (58), and their comparative assessment and accessibility remain essential for the success of this subunit vaccine platform in humans.

The AMA1 vaccine field has suffered from the polymorphic nature of this Ag, as well as the fact that vaccine-induced Abs inhibit growth in a vaccine-allele–specific manner (6). The IgG induced in this study by the 3D7 and FVO biallelic vaccines showed correspondingly high-level GIA against the 3D7 and FCR3 vaccine strains, especially after the AdCh63-MVA-protein and AdCh63-protein immunization regimens. Further studies are required to assess whether these Abs mediate growth inhibition against alternative strains. Encouraging and recent data suggested a benefit of biallelic vaccines for targeting Ab epitopes that are common to both alleles (41), as well as better strain-transcending cross-reactive responses in higher primates compared with IgG induced in mice and rabbits (59). Specific IgG isotypes have also

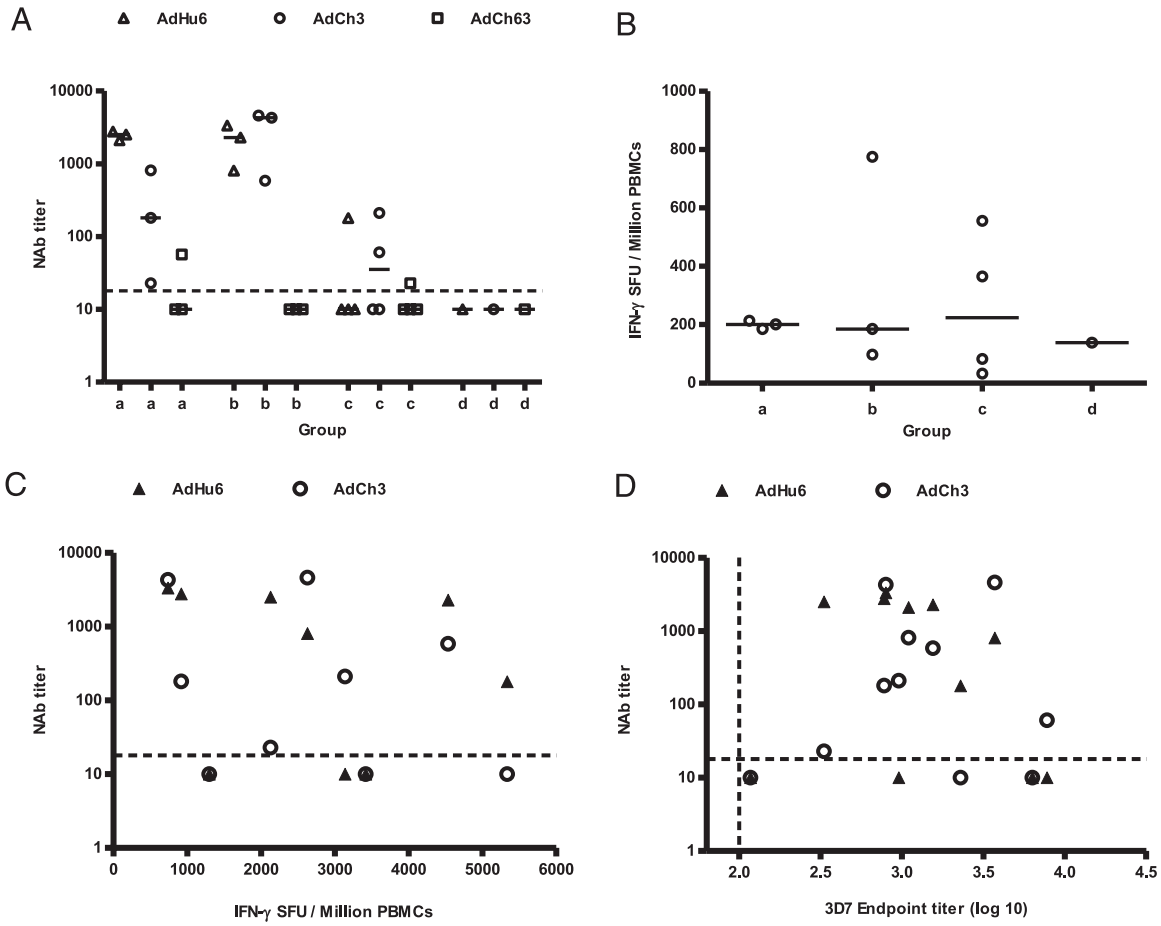

FIGURE 6. Pre-existing immunity to adenoviral vectors at week 0. Ten of the 11 macaques had been immunized with AdCh3 and/or AdHu6 viral-vectored vaccines prior to immunization with AdCh63 AMA1. Macaques were grouped (a–d) according to prior immunization status at week 0 of this study (Table I). *A*, Pre-existing NAb titers against AdHu6, AdCh3, and AdCh63 were assayed in the serum of each macaque at week 0. *B*, T cell responses against AdHu5 hexon peptides were assayed by ex vivo IFN-γ ELISPOT in PBMCs at week 0. *C*, Ex vivo IFN-γ ELISPOT data for AMA1-specific T cells in PBMCs at week 10 (following AdCh63-MVA AMA1 immunization) against pre-existing NAb titers for AdHu6 and AdCh3 at week 0 ($n = 9$). Spearman correlation for AdHu6: $r_s = -0.53$, $p = 0.15$; AdCh3: $r_s = -0.27$, $p = 0.48$; and AdCh63: $r_s = -0.02$, $p = 0.95$. *D*, 3D7 AMA1-specific serum IgG ELISA titers at week 4 (following AdCh63 AMA1 immunization) against pre-existing NAb titers for AdHu6 and AdCh3 at week 0 ($n = 11$). Spearman correlation for AdHu6: $r_s = -0.41$, $p = 0.21$; AdCh3: $r_s = +0.06$, $p = 0.85$; and AdCh63: $r_s = -0.39$, $p = 0.23$.

been associated with protection against blood-stage infection in humans (16), possibly through the induction of Ab-dependent cellular inhibition (23, 60) or Ab-dependent respiratory burst (22) mechanisms via interaction with FcγRs on innate effector immune cells (61). Although little is known about how rhesus IgG1-4 isotype function corresponds to their human counterparts, this study has highlighted the need for the development of rhesus-specific reagents to assess these.

mBC responses were shown to correlate with IgG Ab levels in some studies (62, 63) but not others (64–66). We showed in mice immunized with viral vector and protein MSP1 vaccines that serum IgG levels correlate with uncultured Ab-secreting plasma cell levels in the spleen (31). In this study, we showed that mBC levels increased significantly postimmunization at the time points assessed and that these responses correlated with longer-term serum IgG levels, similar to that seen with the AMA1 protein vaccine in a phase Ia clinical trial (63). Although the direct role for these cells in immediate and long-term immunity to malaria remains unclear (27, 28, 67), their evaluation in blood-stage malaria vaccine clinical trials is becoming more routine (63, 68, 69). Such studies should shed more light on the role that these cells play in the maintenance and boostability of vaccine-induced IgG responses and how these cells are modulated by chronic malaria infection.

A number of adenoviral serotypes of human and simian origin are now in advanced stages of preclinical and clinical development as vaccine vectors for a wide range of infectious diseases, including malaria, HIV-1, tuberculosis, influenza, and hepatitis C (8). Concerns are frequently raised regarding the induction of vector-specific or cross-reactive antivector immune responses that may affect vaccine efficacy if multiple vectors were licensed and deployed for use against different pathogens. In this study, we showed that prior immunization with AdHu6 or AdCh3 vaccines against HIV-1 or hepatitis C in macaques had no major impact on subsequent AdCh63 AMA1 vaccine efficacy. These data are encouraging for the viral vector vaccine field in general and agree with other clinical data for MVA showing that this vector could be reused in Gambian adults just 1 y after a previous MVA vaccination (70).

In summary, these data have strengthened the evidence that combinations of clinically relevant subunit vaccine platforms can achieve broad, functional, and potent immune responses in macaques and that these responses can also be tailored by modulating

immunization regimens. These data have important implications for vaccine development against numerous immunologically challenging infectious diseases and cancer and further encourage the coadministration of Ab-inducing recombinant protein vaccines with T cell- and Ab-inducing recombinant viral vectors as a next-generation strategy that may achieve significant protective blood-stage malaria immunity in humans.

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Disclosures

S.J.D., S.C.G., and A.V.S.H. are named inventors on patent applications covering malaria vectored vaccines and immunization regimens. S.C., M.N., A.F., and A.N. are employees of and/or shareholders in Okairòs, which is developing vectored vaccines for malaria and other diseases.

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