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The Requirement for Potent Adjuvants To Enhance the Immunogenicity and Protective Efficacy of Protein Vaccines Can Be Overcome by Prior Immunization with a Recombinant Adenovirus

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A central goal in vaccinology is the induction of high and sustained Ab responses. Protein-in-adjuvant formulations are commonly used to achieve such responses. However, their clinical development can be limited by the reactogenicity of some of the most potent preclinical adjuvants and the cost and complexity of licensing new adjuvants for human use. Also, few adjuvants induce strong cellular immunity, which is important for protection against many diseases, such as malaria. We compared classical adjuvants such as aluminum hydroxide to new preclinical adjuvants and adjuvants in clinical development, such as Abisco 100, CoVaccine HT, Montanide ISA720, and stable emulsion-glucopyranosyl lipid A, for their ability to induce high and sustained Ab responses and T cell responses. These adjuvants induced a broad range of Ab responses when used in a three-shot protein-in-adjuvant regimen using the model Ag OVA and leading blood-stage malaria vaccine candidate Ags. Surprisingly, this range of Ab immunogenicity was greatly reduced when a protein-in-adjuvant vaccine was used to boost Ab responses primed by a human adenovirus serotype 5 vaccine recombinant for the same Ag. This human adenovirus serotype 5–protein regimen also induced a more cytophilic Ab response and demonstrated improved efficacy of merozoite surface protein-1 protein vaccines against a Plasmodium yoelii blood-stage challenge. This indicates that the differential immunogenicity of protein vaccine adjuvants may be largely overcome by prior immunization with recombinant adenovirus, especially for adjuvants that are traditionally considered poorly immunogenic in the context of subunit vaccination and may circumvent the need for more potent chemical adjuvants. The Journal of Immunology, 2011, 187: 2602–2616.
are thus being developed that are aimed at inducing strong Ab and T cell responses including TLR agonists, liposomes, and novel emulsions (9). However, it is unclear whether these adjuvants will demonstrate reactogenicity profiles that are acceptable for vaccine licensure.

Viral vectored vaccines, although not without their own developmental and regulatory challenges, have been explored as another avenue to generate strong immune responses through subunit vaccination (10). For example, sequential immunizations of recombinant human adenovirus type 5 (AdHu5) and modified vaccinia virus Ankara (MVA) vectors encoding the blood-stage malaria Ag merozoite surface protein-1 42-kDa region (MSP-123) have been shown to generate strong T cell responses as well as high-titer Abs that are protective against both a lethal Plasmodium yoelii sporozoite and blood-stage challenge (11, 12). The ability of viral vectors to induce strongly both the humoral and cellular arms of the immune system has led to their use in various heterologous prime-boost strategies (13–18).

Adenoviral prime–protein boost (AP) regimes, whereby the two leading subunit vaccine platforms are combined, have more recently been shown to induce improved Ab responses compared with the use of either strategy on its own. We have demonstrated in mice that this AP immunization strategy can lead to improved Ab responses, with a moderate T cell response induced by the adenoviral vector, when using P. falciparum MSP-1 vaccines (14). These Ab responses were found to be more consistently primed by an adenoviral vector and also induced a more cytolytic Ab response dominated by IgG2a. In agreement with these murine data, nonhuman primate studies of similar regimes, for candidate malaria and HIV-1 vaccines, have also shown particular promise (15, 19, 20).

In this study, we initially compared the potency of several promising adjuvants (both preclinical and clinically tested/approved for clinical trial) in a head-to-head manner to provide comparative immunogenicity data on leading adjuvant formulations when administered in a three-shot protein-in-adjuvant regime (PPP). We then extended this work to carry out a detailed characterization of the immunogenicity of AP regimes in comparison with PPP regimes, using the model Ag OVA as well as the blood-stage malaria vaccine candidate Ags MSP-1 and erythrocyte binding Ag (EBA)-175 (2, 21, 22). We showed that the marked differential immunogenicity of adjuvants seen in PPP regimes can be largely overcome by priming Ab responses with a recombinant adenoviral vector encoding the same Ag, so that weaker adjuvants now perform more comparably to strong adjuvants. Irrespective of the protein adjuvant used, the AP regimen induced more cytolytic Abs and, in the case of using a saponin-based adjuvant, was capable of inducing strong humoral and cellular immunity simultaneously. This consistently improved immunogenicity, particularly when using less potent adjuvants, and also translated into improved protective efficacy of MSP-1 vaccines in a P. yoelii blood-stage challenge model in mice.

**Materials and Methods**

**Animals and immunizations**

All procedures were performed in accordance with the terms of the United Kingdom Animals (Scientific Procedures) Act Project License and were approved by the University of Oxford Animal Care and Ethical Review Committee. Six- to 8-wk-old female BALB/c (H-2<sup>d</sup>) and C57BL/6 (H-2<sup>b</sup>) mice (Harlan Laboratories, Oxfordshire, U.K.) were anesthetized before immunization with Isoflu (Abbot Animal Health). All immunizations were administered i.m. with vaccine divided equally into each musculus tibialis, unless otherwise specified. Immunization doses and intervals varied between experiments and are explained in the text and figure legends. Immune responses were assayed 2 wk after each immunization and before the protein vaccine boost in AP regimes.

**Viral vectors and protein vaccines**

The generation of AdHu5 and MVA viral vectors expressing P. yoelii MSP-142 and MSP-1 33-kDa region (MSP-113), as well as P. falciparum MSP-1, has been previously described (11, 16). AdHu5 and MVA viral vectors expressing P. falciparum EBA-175 (F2 region) (A. Douglas, A. Williams, J. Illingworth, G. Kamuyu, S. Biswas, A. Goodman, S. Yorke, C. Crosnier, G. Wright, F. Osier, K. Marsh, A. Turner, A.V. Hill, and S.J. Draper, submitted for publication) and OVA were made as described elsewhere (A.L. Goodman, A.M. Blagborough, S. Biswas, Y. Wu, A. V.S. Hill, R.E. Sinden, and S.J. Draper, submitted for publication). The OVA vectors express the full 1188- bp coding sequence of hen OVA (GenBank accession number MN205152, http://www.ncbi.nlm.nih.gov/genbank/). An N311Damino acid substitution was carried out to prevent N-linked glycosylation as described elsewhere (23). The final construct was codon optimized for human expression and synthesized by GeneArt (Regensburg, Germany). For protein vaccinations, grade VII OVA was obtained from Sigma-Aldrich. P. yoelii MSP-1 19-kDa region (MSP-119)-GST fusion protein was made as previously described using an Escherichia coli expression system (12). P. falciparum EBA-175 (F2 region) and MSP-119 protein were produced as previously described (16, 25). Endotoxin levels for P. falciparum proteins were measured using the Limulus amebocyte lysate gel clot assay according to the manufacturer’s instructions (Salesworth). The endotoxin content of purified P. falciparum EBA-175 protein was <21 endotoxin units/25 μg protein and <6.7 endotoxin units/25 μg P. falciparum MSP-119 protein.

**Adjuvants**

Adjuvants used in this study were dosed and prepared in low-phosphate PBS (pH 7) (Life Technologies-Invitrogen) as described in Table I. In brief: Abisco 100 (26) (Isconova) (12 μg/dose) was gently mixed with Ag in PBS. Adju-Phos (Brentag) (75 μg Al<sub>3+</sub>/dose) and Alhydrogel (Brentag) (75 μg Al<sub>3+</sub>/dose) were combined with Ag in PBS and spun at 4°C for 30 min before administration. CoVaccine HT (27) (a novel proprietary vaccine adjuvant of Protherics Medicines Development, a BTG International Group company, London, U.K.) was mixed gently 1:1 with Ag in PBS (2 mg sucrose fatty acid sulfate esters/dose). Complete and IFA (Sigma-Aldrich) were mixed vigorously through vortexing 1:1 with Ag in PBS. CFA was used only once, and mice were subsequently boosted with IFA. Immunizations were administered s.c. for the complete and IFA adjuvants. Montanide ISA720 (Seppic) and Ag in PBS was emulsified using a T10 ULTRA-TURRAX (IKA) homogenizer under sterile conditions at 25,000 rpm for 6 min, keeping the sample on ice in a recirculating bath at 3°C. Adjuvants based on a stable emulsion (SE) with different TLR agonists incorporated into the emulsion (28) (Infectious Disease Research Institute; 20 μg/dose) were mixed with Ag in PBS and vortexed for 30 s. All vaccines were kept on ice until administration. For all vaccines, the protein dose was incorporated into the PBS fraction of the vaccine. Adsorption of Ag to aluminum-based adjuvants was assessed as previously described (15). Using this method, OVA was found to adsorb to Alhydrogel by 89%. OVA only adsorbed to Adju-Phos by 9%, and P. yoelii MSP-1<sub>1-19</sub>-GST adsorbed to Adju-Phos by 40% (data not shown).

**ELISA**

Total IgG ELISAs were carried out as described previously (12). OD was read at 405 nm using a Model 550 Microplate Reader (Bio-Rad). Serum Ab endpoint titers were taken as the -axis intercept of the dilution curve at an absorbance value 3 SDs greater than the OD 405 for serum from a naive mouse. A standard positive serum sample and naive serum sample were added as controls for each assay. Naive mouse serum was negative for Ag-specific responses to all Ags (data not shown). P. yoelii MSP-1<sub>1-19</sub>-specific Abs, following immunization of mice with GST–PyMSP-1<sub>1-19</sub>, were measured using P. yoelii MSP-1<sub>1-19</sub>-IMX108 protein (29), which does not contain the GST-tag present in the protein used for immunization. P. fal- ciparum MSP-1<sub>1-19</sub>-specific Ab responses were measured using P. falcipa- rum MSP-1<sub>1-19</sub>-GST (QKNG) made as previously described in an E. coli expression system (16).

**Isotype ELISA**

To detect Ag-specific IgG1 and IgG2a responses, plates were coated at a concentration of 2 μg/ml with protein overnight at room temperature. A standard curve comprised of isotype-purified mouse IgG1 or IgG2a mAb (eBioscience) was added in duplicate to separate plates at a concentration of 20 μg/ml and diluted 1:3 fold. A positive control of mAb at a dilution of 1:6075 for IgG1 and 1:2025 for IgG2a was also added to each separate plate. After blocking, serum diluted in PBS/Tween was added in duplicate to the plate for 2 h at room temperature. Plates were then washed, and...
either biotin anti-mouse IgG1 or IgG2a (BD Biosciences) was added to the test plates. Following a 30-min incubation with Extravidin Alkaline Phosphatase (Sigma-Aldrich), plates were developed using the same reagents as for total IgG ELISA. Plates were developed until the monoclonal positive control reached an OD405 of 1.0. This point was defined as 1 unit, and units were read off the standard curve similar to published methodology (30). Samples were diluted to fall on the linear part of the curve. Low-titer samples from the experiments using OVA were diluted 1:100 and developed according to the same positive control as before. Isotype responses for these samples are reported as OD 405 nm.

### Avidity ELISA

Ab avidity was assessed using a sodium thiocyanate (NaSCN)-displacement ELISA as described previously (14). Sera were individually diluted to a level calculated to give a titer of 1:100, based on known total IgG titers, and exposed to an ascending concentration (0–7 M) of the chaotropic agent NaSCN (Sigma-Aldrich). Plates were developed as for total IgG. The intercept of the OD405 curve for each sample with the line of 50% reduction of the OD405 in the NaSCN-free well for each sample (i.e., the concentration of NaSCN required to reduce the OD405 to 50% of that without NaSCN) was used as a measure of avidity.

### Ex vivo IFN-γ ELISPOT

IFN-γ ELISPOTs were carried out using PBMC isolated from the blood and spleen as previously described (31). In brief, MAIP ELISPOT plates (Milipore) were coated with anti-mouse IFN-γ mAb (Mabtech) at 5 µg/ml in carbonate-bicarbonate buffer. Plates were blocked with complete DMEM (Sigma-Aldrich; 10% FBS from Biosera; 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate; all from Invitrogen) for 1 h at 37°C. PBMC and splenocytes were resuspended in complete medium and counted using a Casy counter (Schräf Systems). A total of 50 µl PBMC harvested from the blood was plated into duplicate wells, and 50 µl peptide diluted in medium plus 0.25 × 10⁶ naive splenocytes was added to test wells. Medium and naive splenocytes only were plated into negative control wells. Spleen cells were resuspended in complete medium plus 1 × 10⁶ cells/ml, and 50 µl cells were plated in duplicate. A total of 50 µl peptide diluted in complete medium was added to test wells, and complete medium alone was added to control wells. OVA-specific CD4⁺ T cell peptides (ISQAVHAAHAEINEQGTEIVDWTSSVMNKEEKIKVY) (32, 33) were pooled, and OVA-specific CD8⁺ T cell peptide (SIINFEKL) (32) was added at a final concentration of 5 µg/ml. Plates were incubated at 37°C, 5% CO₂ in a humidified incubator for ~18 h. Plates were then washed and incubated with biotinylated anti-mouse IFN-γ mAb (Mabtech), followed by an incubation with a streptavidin alkaline phosphatase polymer (Mabtech). Spots were developed by addition of color development buffer and counted using ELISPOT reader system (InCell 1000 and development diagram program). Results were expressed as spot-forming cells (SFC) per million cells. Background responses in media-only wells were subtracted from those measured in peptide-stimulated wells.

### Intracellular cytokine staining

Analysis of the percentage of cytokine-producing peripheral blood (PB) CD4⁺ and CD8⁺ T cells by intracellular cytokine staining has previously been described (11). Briefly, cells were stimulated for 5 h with pools of 15-mer peptides overlapping by 10 aa corresponding to PyMSP-1g15 at a final concentration of 5 µg/ml for each peptide (11). Cells were surface stained with anti-CD8α PerCP-Cy5.5 and anti-CD4 PB (eBioscience). After permeabilization using Cytofix/Cytoperm (BD Biosciences), cells were stained intracellularly with anti–IFN-γallophycocyanin, anti–TNF-α PerCP-Cy5.5, and anti–IL-2 PE (eBioscience). Samples were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star). Background responses in unstimulated cells were subtracted from the stimulated responses prior to analysis.

### CD4⁺ T cell depletion

CD4⁺ T cells were depleted using an anti-CD4 antibodies mixture purified using protein G affinity chromatography from hybridoma culture supernatants as previously described (11). The degree of CD4⁺ T cell depletion was assessed by flow cytometry using anti-CD4 PB (clone RM5.4), anti-CD3e APC, and anti-CD8 PerCP-Cy5.5 (eBioscience) in the PBMC of vaccinated depleted mice and unvaccinated control mice on day +1 with respect to challenge on day 0. Depletion was assessed by gating on CD3⁺CD4⁺ cells and shown to be >98%.

### Ab-secreting cell assay

Cells isolated from the spleen and bone marrow were tested for Ab-secreting cells (ASCs) by ELISPOT assay as previously described (14, 34), except that bone marrow was flushed from dissected femurs with complete DMEM using a 26-gauge needle and passed through a sterile 70-µm cell strainer. ELISPOT plates were counted using the AID plate reader software (AID; Cadama Medical), and counts were visually confirmed. Ab-forming spots were relatively large and spherical in size with fuzzy granular edges.

### Parasites

P. yoelii YM parasitized RBC (pRBC) challenges were carried out as previously described (12). Mice were infected by i.v. injection with 10⁷ pRBC. Blood-stage parasitemia was monitored from day 3 postchallenge by Giemsa-stained thin-blood smear and calculated as a percentage of infected RBC. Mice were considered uninfected if no parasites were observed in 50 fields of view and were sacrificed by a humane method at ≥80% parasitemia.

### Statistical analysis

All statistical analysis was carried out using Prism version 5 (GraphPad). All ELISA titers were log10 transformed prior to analysis. For nonparametric data, a Kruskal–Wallis test with Dunn’s multiple comparison posttest was used to compare more than two groups. A one-way ANOVA was used for multiple comparisons of parametric data with Bonferroni’s multiple comparison posttest for comparison of groups as stated. An unpaired t test was used to compare the means of two groups for parametric data, and a Mann-Whitney U test was used for nonparametric data. A two-way ANOVA with Bonferroni’s multiple comparison posttest was used to explore the effect of two variables. Correlations were tested using Spearman’s rank correlation. A p value <0.05 was considered significant (*p < 0.05, **p < 0.01, and ***p < 0.001).

### Results

#### Novel adjuvants can improve the immunogenicity of protein vaccines

Though numerous adjuvants have been selectively tested with a diverse set of Ags (24, 35–38), a comparative assessment of the immunogenicity of a leading panel of adjuvants is lacking. To address this shortcoming, 11 adjuvants (both preclinical and clinically tested/approved for clinical trial) were assessed in a three-shot protein-in-adjuvant regimen (PPP regimen) using the model Ag OVA. C57BL/6 mice were immunized with three shots of 20 µg of OVA, 2 wk apart, formulated with adjuvant as described in Table I. Serum total IgG titers were assayed 2 wk after each immunization in response to OVA protein by ELISA. After one shot of protein-in-adjuvant only some mice in select groups seroconverted (Fig. 1A). A second shot of protein-in-adjuvant, mice in all groups had detectable Ab responses. These IgG titers were significantly boosted by a third shot of protein-in-adjuvant for Abisco 100 and CoVaccine HT (p < 0.01 by two-way ANOVA with Bonferroni’s multiple comparison posttest) (Fig. 1A). Two weeks after the final immunization, a broad range of Ab responses was seen, with a 156-fold difference in median titers observed between the strongest and weakest responding groups. All adjuvants induced significantly higher total IgG titers than OVA in PBS (p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison posttest). CoVaccine HT, Montanide ISA720, SE plus TLR4, -7, and -8, and SE plus TLR 4, -7, -8, and -9 induced the highest IgG titers. CoVaccine HT induced titers surpassing those induced with Freund’s adjuvant and induced significantly higher IgG titers than the classical aluminum-based adjuvant Adju-Phos (p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison posttest) (Fig. 1B). The remaining SE-based adjuvants induced IgG titers comparable to Freund’s adjuvant, with Adju-Phos (based on aluminum phosphate) inducing by far the lowest titers. Only two mice receiving OVA in PBS seroconverted after three immunizations, though these responses appeared to be transient, as they returned to baseline when Ab titers were assayed 6 wk after the final immunization (data not shown).
T cell responses were also assessed in the blood against the known H-2\textsuperscript{b} CD8\textsuperscript{T} cell epitope and pooled CD4\textsuperscript{T} cell epitopes present in OVA 2 wk after the final immunization. Though the hierarchy of T cell responses induced by the adjuvants differed to that of IgG, it was comparable for CD4\textsuperscript{T} cell and CD8\textsuperscript{T} cell responses (Fig. 1C, 1D). As previously shown, Alhydrogel and Adju-Phos were poor T cell inducers (35). Emulsion-based adjuvants such as Freund’s adjuvant and Montanide ISA720 induced a median of 431 and 692 CD4\textsuperscript{T} spot-forming units (SFU)/10\textsuperscript{6} PBMC, indicating that AP regimes can impact both the humoral and cellular immune responses induced by more classical protein vaccine adjuvants can be improved upon by using new experimental adjuvants.

An alternative immunization with a recombinant adenoviral vector reduces the differential Ab immunogenicity of protein-in-adjuvant vaccines

Recently, the addition of a protein-in-adjuvant boost following a recombinant adenoviral prime has been shown to enhance Ab titers when compared with protein-in-adjuvant regimes on their own as well as recombinant adenoviral prime–MVA boost regimens (14, 15). C57BL/6 mice were thus primed with 1 \times 10\textsuperscript{10} viral particles (vp) of recombinant AdHu5 vector expressing OVA (AdHu5-OVA) and boosted 8 wk later with 20 µg of OVA protein-in-adjuvant as prepared previously. The aim was to assess whether a similar effect in terms of improved immunogenicity is seen over a range of different adjuvants. Three control groups primed with AdHu5-OVA and either not boosted, boosted with OVA in PBS, or boosted with 1 \times 10\textsuperscript{7} PFU MVA-OVA were also included. In the AdHu5-OVA only group, Ab responses peaked at week 8 and then plateaued out to week 10, corresponding to 2 wk post–protein-in-adjuvant boost (Fig. 2A). Adenoviral-primed Ab responses were significantly boosted by all protein-in-adjuvant vaccines, apart from in the SE plus TLR4, -7, -8, and -9 group, as well as by MVA-OVA (p < 0.01 by two-way ANOVA with Bonferroni’s multiple comparison posttest) (Fig. 2B). Analysis of the mean fold change from preboost to postboost IgG titers in individual mice showed that some adjuvants boosted adenoviral-primed responses more efficiently than others. SE plus TLR4/9 induced a significantly higher mean fold change in IgG titer than Adju-Phos, Alhydrogel, and CFA. Abisco 100 also induced a higher mean fold change in titer than Alhydrogel (both: p < 0.01 by Kruskal-Wallis test with Dunn’s multiple comparison posttest on fold change of significantly boosted groups). This indicates that some adjuvants can perform particularly well in the context of an AP immunization regimen. However, overall, the differential immunogenicity observed following PPP immunization was greatly reduced, with most responses across the different groups now being of a comparable and relatively high magnitude (Fig. 2C). Only an 8-fold difference in median titers was observed within the AP groups that had showed significant boosting. Interestingly, using a recombinant adenoviral prime improved the boosting ability of the aluminum-based adjuvant Adju-Phos, which now induced similar Ab responses to CoVaccine HT, a potent inducer of Ab responses in the PPP experiments (8-fold increase in the mean, 95% confidence interval [CI]: 2–33 for CoVaccine HT; 6-fold increase in the mean, 95% CI: 3–12 for Adju-Phos; p > 0.05, one-way ANOVA with Bonferroni’s multiple comparison posttest). Protein-in-adjuvant boosting also surpassed boosting with MVA-OVA, in agreement with our previous data (14, 15). Surprisingly, OVA in PBS also slightly boosted adenoviral-primed IgG responses (2-fold increase in the mean, 95% CI: 0.5–10.9).

T cell responses were also assessed 2 wk after the protein-in-adjuvant boost (Fig. 2D, 2E). Interestingly, Abisco 100, in addition to inducing strong IgG titers, induced a median of 6799 CD4\textsuperscript{T} SFU/10\textsuperscript{6} PBMC, indicating that AP regimes can impact both the humoral and cellular arm of the immune system. This AP regimen induced T cell responses equivalent to the adenoviral prime–MVA boost regimen, which has been optimized for T cell induction (p > 0.05 for CD4\textsuperscript{T} and CD8\textsuperscript{T} T cell responses by Kruskal-Wallis test with Dunn’s multiple comparison posttest Abisco 100 versus MVA). Overall, these data indicate that the broad range of Ab induction seen with adjuvants following the administration of protein vaccines is greatly reduced when responses are first primed with an adenoviral vector.
Effect of adenoviral priming is consistent in other mouse strains and other Ags

To further investigate the immunogenicity of selected adjuvants and to assess whether the effects seen with OVA are Ag or mouse strain specific, the *P. falciparum* blood-stage malaria Ags EBA-175 (F2 region) and MSP-1 were also assessed in BALB/c mice immunized with PPP and AP regimes (Fig. 3). The MSP-1 C terminus undergoes proteolytic cleavage during RBC invasion and is cleaved into 33-kDa (MSP-133) and 19-kDa (MSP-119) fragments (39). Ab responses against MSP-119, but not MSP-133, are protective against blood-stage malaria (11, 40). However, a role has also been reported for MSP-1–specific CD8+ T cells against liver-stage parasites, as MSP-1 is also expressed during late liver-stage infection (11, 41). MSP-133–specific CD4+ T cells have also been shown to provide help for B cells and aid the development of de novo Ab responses (11). EBA-175 binds to sialic acid residues on glycophorin A on the surface of erythrocytes and can mediate invasion by malaria parasites (42). Abs induced against this Ag have been shown to inhibit *P. falciparum* invasion of erythrocytes in vitro (24). In this study, these two Ags were given as a mixture (10 μg each) formulated with adjuvant as previously described and were administered 3 wk apart in the PPP regimen. A total of 1 × 10^9 vp AdHu5–MSP-1 and 1 × 10^9 vp AdHu5–EBA-175 were also administered as a mixture in the AP regime and boosted with protein vaccines 8 wk later as previously. All mice in the PPP groups seroconverted in response to EBA-175 after one immunization (Fig. 3A) and followed the same hierarchy as seen after two shots of OVA (with Montanide ISA720 and CoVaccine HT inducing the strongest IgG titers; Fig. 1B). IgG titers were significantly boosted by a second immunization of protein-in-adjuvant (*p*, 0.001, two-way ANOVA with Bonferroni’s multiple comparison posttest) but not by a third, after which all adjuvants induced comparable Ab responses to EBA-175, indicating that the Ab responses had reached a plateau (*p*, 0.05, two-way ANOVA with Bonferroni’s multiple comparison posttest) (Fig. 3B). Though mice immunized with EBA-175 in PBS had detectable Ab responses, IgG titers in the adjuvant groups were significantly higher (*p*, 0.05, one-way ANOVA with Bonferroni’s multiple comparison posttest) but not by a third, after which all adjuvants induced comparable Ab responses to EBA-175, indicating that the Ab responses had reached a plateau (*p*, 0.05, two-way ANOVA with Bonferroni’s multiple comparison posttest) (Fig. 3B).

In accordance with previous data (14, 15), and in contrast to the PPP regimes, mice in the AP groups seroconverted 2 wk after the...
adenoviral prime in response to both Ags (Fig. 3D, 3E). Following a protein immunization, total IgG titers were significantly boosted in response to both Ags by > 1 log in all adjuvant groups (16-fold increase in the mean across all adjuvant groups, 95% CI: 13–20 for EBA-175; 31-fold increase in the mean across all adjuvant groups, 95% CI: 23–41 for MSP-1 19; p, 0.001, two-way ANOVA with Bonferroni’s multiple comparison posttest). Interestingly, though mice only responded to MSP-1 19 in Montanide ISA720 following three shots of protein vaccine (Fig. 3C), Ab responses to MSP-1 19 in mice primed with the adenoviral vector were successfully boosted to high levels by each protein-in-adjuvant vaccine when administered in the AP regimen (Fig. 3E). There was no significant difference in the mean fold change from preboost to postboost IgG titers in relation to the use of a particular adjuvant with the two different Ags (data not shown). Also, as seen with OVA, adenoviral-primed responses were also boosted by protein in PBS (4-fold increase in the mean, 95% CI: 3–7 for EBA-175; 13-fold increase in the mean, 95% CI: 5–35 for
despite some minor differences, the improved boostability of IgG responses observed following a recombinant adenoviral prime is consistent and can be observed in different mouse strains and when using different Ags.

Longevity of responses

The induction of not only high titer but also sustained Ab responses is desirable for efficacious vaccines. The longevity of the Ab responses induced by the different adjuvants deployed in PPP and AP regimes were thus measured 10 wk after the final immunization (Fig. 4A,4B). Ab responses were generally higher 10 wk post-vaccination in the AP groups, which is most likely a reflection of initially higher titers 8 wk earlier. The reduction in log10 titers from the peak of the response to the last time point in the OV A system was compared for the different adjuvants administered either in AP or PPP regimes (Fig. 4C). There was a mean reduction of 0.3 and 0.5 log10 titers in AP and PPP regimes, respectively. A significant difference in the reduction of log10 IgG titers over time between the two regimes was found for 3 out of the 11 adjuvants tested (CoVaccine HT, Freund’s adjuvant, and SE and TLR4/9; p < 0.01 two-way ANOVA with Bonferroni’s multiple comparison posttest). However, in most cases, there was no difference in the decline of Ab responses between AP and PPP regimes as has been previously found (14). Thus, almost all vaccine-induced IgG, irrespective of method of induction, seems to be subject to the same rate of decay/t1/2 over time.

The levels of plasma cells (ASCs) in the spleen and bone marrow were also investigated to determine if there is a correlation between Ab titers and plasma cells as has been suggested previously (14, 43). In C57BL/6 mice immunized with OVA, ASCs were only detected above baseline in a few mice across different groups (data not shown). In contrast, strong MSP-119–specific ASC responses were detected in the bone marrow (Fig. 4D) and spleens (Fig. 4E) of BALB/c mice immunized with the AP regimen 10 wk after the protein vaccine boost (postboost, dark gray bars). Median responses are shown with range. *p < 0.05 by one-way ANOVA with Bonferroni’s multiple comparison posttest, **p < 0.001 by two-way ANOVA with Bonferroni’s multiple comparison posttest.

FIGURE 3. The effect of an adenoviral prime is consistent in other mouse strains and with other Ags. BALB/c mice (n = 5/group) were immunized twice i.m. 3 wk apart with a mixture of 10 µg P. falciparum EBA-175 protein and 10 µg P. falciparum MSP-119 protein. IgG titers were measured in the serum to EBA-175 protein 2 wk after each immunization (Imx) (A). Median responses are shown with range. IgG titers were measured in the serum in response to EBA-175 protein (B) and MSP-119 (QKNG allele) protein (C) 2 wk after the final immunization. Median responses are shown. BALB/c mice were primed i.m. with a mixture of 1 × 10⁹ vp AdHu5–MSP-1 and AdHu5–EBA-175 and boosted i.m. 8 wk later with a mixture of 10 µg P. falciparum EBA-175 protein and 10 µg P. falciparum MSP-119 protein. IgG titers were measured in the serum in response to EBA-175 protein (D) and MSP-119 (QKNG allele) protein (E) 8 wk after the prime (pre-boost, light gray bars) and 2 wk after the protein vaccine boost (postboost, dark gray bars). Median responses are shown with range. *p < 0.05 by one-way ANOVA with Bonferroni’s multiple comparison posttest.
ASC levels were not explored in PPP-vaccinated mice because Ab responses were only detectable in the Montanide ISA720 group for this Ag.

An adenoviral prime skews adjuvants toward the induction of cytophilic Ab isotypes
As adjuvants are known to skew the immune response toward either a Th1-type or Th2-type Ab response (dominated in mice by IgG2a or IgG1, respectively), and as it has previously been shown that viral vector-containing regimes induce a more cytophilic Ab response that is maintained better over time (14, 45), the induction of IgG Ab isotypes by AP regimes was compared with PPP regimes.

IgG isotype ELISAs were carried out using the serum of mice immunized with PPP or AP regimes with OVA, EBA-175, and MSP-1 vaccines 2 wk after the final vaccination. Moderate IgG1 and IgG2a Abs were induced across the different adjuvants and regimes (Fig. 5A). The effect of regimen on the log isotype ratio was significant as well as the effect of adjuvant (p < 0.001, two-way ANOVA with Bonferroni’s multiple comparison posttest), driven by TLR agonist-containing adjuvants, which induced a greater ratio of IgG2a/IgG1 in both regimes (Fig. 5D). The induction of isotypes was also investigated for MSP-1 and EBA-175 vaccines, in which the same trend toward a greater induction of IgG2a Abs was found with AP regimes (Fig. 5B, 5C).
Overall, the adjuvants induced comparable isotype responses to both Ags, and SE plus TLR4 again induced a greater ratio of IgG2a/IgG1 (as seen in the OVA system) in response to EBA-175 PPP vaccination (Fig. 5E). The effect of regimen on the log isotype ratio was again significant for EBA-175 ($p < 0.001$, two-way ANOVA with Bonferroni’s multiple comparison posttest), indicating a skew toward cytophilic Abs after AP immunization. This was not investigated for MSP-1 vaccines because only mice immunized with Montanide ISA720 in a PPP regimen had detectable Ab responses.

The effect of dose and immunization interval on Ab responses

After showing that an adenoviral prime mediates improved boosting of IgG titers by protein-in-adjuvant vaccines, we next sought to address whether a difference in the dose of Ag exposed to the immune system (between protein vaccines and adenoviral vectors) and/or extended immunization intervals might be mediating this effect. In order to address this, C57BL/6 mice were immunized with either 5 or 20 µg OVA in selected adjuvants at an interval of 8 wk or immunized with 100 µg OVA 2 wk apart. Serum Ab responses were assessed 2 wk after the second immunization and compared with responses seen after two shots of 20 µg of OVA (Fig. 1A). Ab responses to OVA in PBS were negative at all doses, and Ab responses preboost in mice receiving two vaccinations 8 wk apart were also negative (data not shown). For the adjuvants investigated, there was no enhancement of Ab responses with an increased dose of 100 µg OVA given 2 wk apart (Fig. 6). There was a trend for an improvement of Ab responses using the standard 20 µg dose with an extended immunization interval of 8 wk, although this was only significant for Abisco 100 and SE plus TLR4/9 ($p = 0.0091$ for Abisco 100 and $p = 0.0027$ for SE plus TLR4/9, $t$ test) (Fig. 6B, 6D). This indicates that a prolonged time interval between immunizations, rather than dose of Ag, may improve Ab induction by a subset of adjuvants when used in protein-only PPP regimes. However, these data are insufficient to explain why adjuvants such as Adju-Phos (Fig. 6C) were better able to boost IgG responses in the context of an AP immunization regimen. Interestingly, however, priming mice with 20 µg OVA in CoVaccine HT (a good primer of Ab responses in PPP regimes) followed by a boost 8 wk later of 20 µg OVA formulated in Adju-Phos did not result in improved Ab responses (Fig. 6C). Overall, these data suggest that the improved boosting of

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**FIGURE 5.** An adenoviral prime skews adjuvants toward the induction of cytophilic Ab isotypes. BALB/c and C57BL/6 mice ($n = 5$ to 6/group) were immunized as described previously with AP and PPP regimes. IgG1 and IgG2a Ab responses were measured in the serum 2 wk after the last immunization in response to OVA (A), EBA-175 (B), and MSP-1 (QKNG allele) (C) protein. Median and individual responses are shown (A–C). IgG2a/IgG1 ratios were calculated for OVA (D) and EBA-175 (E) and log transformed. Mean responses are shown (D, E). The dotted line indicates the threshold for responses above background in A. ND, no data.
IgG responses, seen with most adjuvants in the AP immunization regimen, appears to be inherent to the adenoviral prime rather than due to differences in immunization schedules and/or Ag dosing.

**AP immunization improves the efficacy of MSP-1 protein vaccines following *P. yoelii* blood-stage challenge**

To investigate whether AP regimes could lead to enhanced vaccine efficacy, BALB/c mice were immunized with *P. yoelii* MSP-1 protein vaccines as outlined in Fig. 7 and subsequently challenged with 10^5 *P. yoelii* pRBCs 2 wk after the final immunization. The protein vaccines, used in this study at 1.5 μg/dose, were formulated in CoVaccine HT and Adju-Phos, as these adjuvants induced very different Ab titers when screened in PPP regimes (Fig. 1B). In agreement with the studies of other Ags at higher doses at the time of challenge, IgG titers were significantly higher in mice immunized with AP regimes and the PPP CoVaccine HT regimen compared with mice immunized with the PPP Adju-Phos regimen and control vaccines (p < 0.05, one-way ANOVA with Bonferroni’s multiple comparison posttest) (Fig. 7A). Following challenge, all mice immunized with control vaccines or the PPP Adju-Phos regimen succumbed to infection (Fig. 7B). Survival was seen in four out of six mice (67%) immunized with AP regimes and in two out of six mice (33%) immunized with the PPP CoVaccine HT regimen (Fig. 7C–E). There was a significant reduction in parasitemia, as measured by an area under the curve (AUC) analysis of parasitemia between days 3 and 5, in both AP regimes and the PPP CoVaccine HT regimen as compared with the PPP Adju-Phos regimen and mice immunized with control vaccines/ regimes (Fig. 7F). Peak parasitemia was shown to significantly correlate with Ag-specific total IgG titers (Fig. 7G). As seen with OVA, EBA-175, and *P. falciparum* MSP-1, more cytotrophic IgG2a Abs were again induced by AP regimes against *P. yoelii* MSP-1, which were also shown to correlate with protection, but not for IgG1 (Supplemental Fig. 1A–C). Ab avidity was also assessed at the time of challenge. There was a trend toward higher avidity in groups receiving an AP regimen compared with a PPP regimen, though this was only significant against PPP CoVaccine HT (p < 0.05) by Kruskal-Wallis test with Dunn’s multiple comparison posttest (Supplemental Fig. 1D). There was no correlation between Ab avidity and total IgG titers (r = −0.3; p = 0.1) or peak parasitemia (r = 0.1; p = 0.5). Overall, these data indicate that the improved immunogenicity in terms of Ab responses due to the adenoviral prime can also lead to improved efficacy against a blood-stage challenge.

**AP regimen efficacy following *P. yoelii* blood-stage challenge is not CD4+ T cell dependent**

In agreement with previous data, we have shown that Ab responses against PyMSP1.19 associate with protection against blood-stage malaria (12, 36). However, a role has also been shown for CD4+ T cells directed against MSP-1,33, which can aid the development of de novo anti-parasite Ab responses (11), as well as mediate some control of blood-stage parasitemia (46). To rule out that the improved efficacy of the AP regimen was not due to CD4+ T cells directed against MSP-1,33, which can aid the development of de novo anti-parasite Ab responses (11), as well as mediate some control of blood-stage parasitemia (46). To rule out that the improved efficacy of the AP regimen was not due to CD4+ T cells directed against the PyMSP-1,33 fragment included in the AdHu5–PyMSP-1,42 vaccine (but absent from the MSP-1,9 protein vaccine), mice were immunized with the regimes outlined in Fig. 8. CD4+ T cells were depleted prior to pRBC challenge in mice receiving an AP Adju-Phos regimen to explore the effect of PyMSP-1,33–specific T cells in mediation of protection. In a separate group, mice were primed with an AdHu5 expressing PyMSP-1,33 (11) and boosted with the previously used PPP Adju-Phos regimen to determine whether the supplementation of T cell help (in the absence of priming protective PyMSP-1,19–specific Abs) through the adenoviral prime could improve this regimen. To confirm that the AdHu5 vectors expressing PyMSP-1,42 and PyMSP-1,33 induced comparable T cell responses, PBMC from the blood of immunized mice were phenotyped 2 wk postprime in response to an overlapping peptide pool of PyMSP-1,33. CD8+ IFN-γ+ T cell responses measured 2 wk after the prime in response to both vectors were not significantly different (p > 0.05, Mann–Whitney U test) (Fig. 8A), suggesting comparable T cell
immunogenicity of the two vectors (as has been observed before following AdHu5-MVA immunization) (11). However, Ag-specific CD4+ T cell responses could not be detected after the single AdHu5 immunization, which was not surprising given these have been shown to be low even after the AdHu5-MVA prime-boost PyMSP-133 regimen (11). The depletion of CD4+ T cells was assessed 24 h postchallenge in the PBMC of depleted mice (Fig. 8B) and was 98% successful. An AUC analysis of parasitemia on days 3–5 revealed that the depletion of CD4+ T cells in mice receiving an AP Adju-Phos regimen resulted in no significant difference in comparison with mice receiving the same regimen and control rat IgG (p = 0.05, Mann–Whitney U test; Fig. 8C). Crosses indicate when mice were sacrificed. F, AUC analysis of parasitemia. Median responses are shown. G, IgG titers measured 2 wk after the final immunization in each regimen were correlated with percentage peak parasitemia. Spearman’s rank correlation is shown. The dotted line indicates the threshold for responses above background in A. *p < 0.05 by one-way ANOVA with Bonferroni’s multiple comparison posttest.

Discussion
The comparative assessment of new vaccine adjuvants remains essential for subunit vaccine development. A panel of 11 leading and accessible vaccine adjuvants (both preclinical and clinically tested/approved for clinical trial) has been assessed in this study in a PPP regimen and compared with an AP regimen for the induction of high and sustained Ab responses.

We have shown that novel adjuvants can induce potent Ab responses surpassing aluminum-based adjuvants and the classical reference adjuvant, Freund’s adjuvant, formulated with OVA in a PPP regimen. Though most licensed adjuvants to date predominantly induced the humoral arm of the immune system, we have also shown that the SE plus TLR4/9 emulsion induces very strong CD4+ and CD8+ T cell responses with the soluble Ag OVA, as has been shown for TLR agonists coupled to other Ags (47, 48). Interestingly, the differential immunogenicity of IgG titers induced by adjuvants in PPP regimes was greatly reduced when an AdHu5 vector was used to prime Ab responses, although some adjuvants still boosted adenovirus-primed responses more efficiently than others. Importantly, this effect was observed for aluminum-based adjuvants such as Adju-Phos, which performed poorly in a PPP regimen but which boosted Ab responses primed by an AdHu5 vector as efficiently as the potent adjuvant CoVaccine HT. Recently, a similar trend was reported when rhesus macaques primed with the simian adenovirus ChAd63 vector expressing the blood-stage malaria apical membrane Ag 1 (AMA1) were boosted either with AMA1 protein formulated in Alhydrogel or CoVaccine HT. The use of ChAd63 in this study also suggests that the results in this study with AdHu5 could potentially be extended to other adenovirus vectors, given the
concerns surrounding pre-existing immunity to AdHu5 in humans and its use as a clinical vaccine vector (10). AdHu5 was chosen as a model adenovirus in this study as it has no intellectual property restrictions and induces immunogenicity comparable to simian adenoviruses in preclinical models (16, 17).

The improved immunogenicity of adjuvants following an adenoviral prime was also extended to P. falciparum MSP-1 and EBA-175 vaccines in a different strain of mouse, indicating that this effect can be translated to other Ags and is unlikely to be strain specific. It is possible that the absence of Ab responses to P. falciparum MSP-1 with adjuvants other than with Montanide ISA720 in a PPP regimen could be due to the fact that MSP-1 has been shown to be refractory to Ag processing (49). The observation that this can be overcome by Montanide ISA720 warrants further investigation.

The two vaccine delivery platforms used in this study are inherently different, and it is possible that the increased immunogenicity of protein vaccine adjuvants seen following an adenoviral prime is due to an adenoviral vector producing more Ag in vivo at the time of priming than a given dose of protein vaccine. This dose effect could prime a quantitatively greater memory B cell response than a given dose of protein vaccine. This dose effect could prime a quantitatively greater memory B cell response that can subsequently be boosted more effectively. Alternatively, adenoviral vaccines may inherently prime a better quality of memory B cell response, likely related to the profile of innate adjuvants (12, 53). However, priming mice with the potent adjuvant CoVaccine HT and boosting mice 8 wk later with Adju-Phos did not lead to increased IgG responses, which suggests that the priming effect is inherent to the adenovirus vector and that the prime-boost interval alone in the AP regimen is unlikely to account for the improved boosting effect seen with most adjuvants. In agreement with the data in this study, no significant differences were also observed in a phase Ia clinical trial of an AMA1 vaccine formulated in Alhydrogel plus CpG7909 using a 4-wk versus 8-wk immunization interval (54). It would thus appear that adenoviral-primed Ab responses are more easily boosted, which has been suggested previously (14, 15).

The induction of Ab isotypes by vaccine adjuvants was also explored in this study. Th2-type responses (dominated by IgG1) are thought to function through neutralizing Abs, whereas Th1-type responses (dominated by IgG2a) are thought to activate complement and function through Fc receptors leading to Ab-dependent cellular inhibition as well as phagocytosis. We have found that a more Th1-type Ab response is induced following an AP regimen in comparison with a PPP regimen, in agreement with previous data (14). Adjuvants containing TLR4 agonists were also able to

FIGURE 8. Improved efficacy of AP regimes following P. yoelii blood-stage challenge is not CD4+ T cell dependent. BALB/c mice (n = 6/group) were immunized i.m. with either 1.5 μg P. yoelii MSP-119-GST protein in Adju-Phos 3 wk apart (PPP) or primed with 1 × 10^10 vp AdHu5–PyMSP-1A2 (Ad42) and boosted 8 wk later with 1.5 μg P. yoelii MSP-119-GST protein in Adju-Phos. One group of mice immunized with the AP Adju-Phos regimen was depleted of CD4+ T cells, the other received normal rat IgG as a control. Separately, BALB/c mice (n = 6/group) were primed i.m. with 1 × 10^10 vp AdHu5–PyMSP-1A3 (Ad33) and boosted with three shots of 1.5 μg P. yoelii MSP-119-GST protein in Adju-Phos 3 wk apart. All mice were challenged with 10^5 pRBCs i.v. 2 wk after the final immunization, and parasitemia was measured as the percentage of infected RBCs over time. A. The percentage of CD8+ IFN-γ+ T cells was measured by intracellular cytokine staining in the PBMC of mice 2 wk after the AdHu5 vaccines. Median responses are shown. B. Representative flow plots from log transformed one group depleted and control mouse showing the percentage of single and double CD3+CD4+ and CD3+CD8+ positive cells. C. AUC analysis of parasitemia. Median and individual responses are shown. *p < 0.05 by one-way ANOVA with Bonferroni’s multiple comparison posttest.
induce a more Th1-type Ab response in PPP regimes as has been previously shown (47, 55, 56). Surprisingly, CoVaccine HT did not induce a skewed Th1 response, despite evidence showing that some of its action is dependent on TLR4 (57). TLR agonist containing adjuvants also induced relatively strong T cell responses in the PPP OVA regimen, which may account for the isotype switch as seen in T-dependent Ab responses. The improved induction of CD4+ T cell help [essential for T-dependent Ab responses (58, 59)] by the adenovirus in the AP regimes could also be another explanation for improved B cell priming. A study by Galli et al. (60) has recently demonstrated that the induction of CD4+ T cells following an adjuvanted influenza vaccine predicted the persistence of Ab responses, highlighting a potential link between these two cell types. However, data using OVA-specific transgenic CD4+ T cells has indicated that transferred transgenic T cells can only help Ag-experienced (and not naive) B cells and that the size of the secondary Ab response is restricted by the amount of T cells present at the time of Ag re-exposure (61). Further studies will thus be necessary to confirm whether the induction of better cellular immunity at the time of B cell priming is an important contributing factor.

Following immunization with either the AP or PPP regimes, there appeared to be no difference in the rate of decline of IgG titers as has previously been suggested (14). However, a difference in the rate of decline has been suggested for some IgG isotypes, with IgG2a being shown to be better maintained over time compared with IgG1 (14, 62). It would be interesting to investigate whether this phenomenon is also evident when comparing the different vaccination regimes and adjuvants used in this study. However, total IgG titers were not evidently better maintained over time in the case of AP regimes, despite the enhanced cytotoxic IgG2a Ab response. For Plasmodium falciparum MSP-119, we also found a correlation of IgG titers with ASC levels in the spleen and bone marrow for the peak and late time points assayed, as has previously been shown for this Ag (14). This implies that serum Ab titers may be maintained by long-lived plasma cells, as has been suggested for other Ags (63–65). However, though not explored in this study, serum Ab titers have also been shown to correlate with memory B cell levels, as measured by a cultured ELISPOT assay, for some acute infections and vaccines (63, 66, 67). This suggests that the maintenance of serological Ab titers may be under differential control by these two cell populations. As virally vectored vaccination regimes, as well as AP regimes, have been shown to induce memory B cells (15), the contribution of this cell type to Ab responses and their boostability in AP regimes warrants further investigation.

The AP regimen was also shown to lead to improved Ab responses and protection in the P. yoelii model utilizing vaccines formulated with CoVaccine HT and Adju-Phos, and this was not associated with a protective contribution from adenoviral-induced PyMSP-1–specific CD4+ T cells. Total IgG and IgG2a titers, but not IgG1 or avidity, were shown to correlate with protection, as has been reported previously for P. yoelii blood-stage infection (36, 68). It remains of interest to explore whether the induction of a more cytotoxic Ab response may account for the increase in protection seen with the AP regimen over a PPP regimen, although the contribution of different isotypes to protection in this model system is disputed (68, 69).

In summary, we have shown that novel emulsion-based adjuvants as well as adjuvants containing TLR agonists can induce both strong humoral and cellular immune responses in a classical subunit vaccination approach. More importantly, we have found that the differential immunogenicity of these protein vaccine adjuvants can be largely overcome through an adenoviral priming immunization. This approach could therefore enhance the clinical immunogenicity and utility of adjuvants that are traditionally considered poorly immunogenic and circumvent the need for more potent and experimental chemical adjuvants that are currently required to deliver candidate protein vaccines for difficult diseases such as blood-stage malaria.

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