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Priming with an Adenovirus 35-Circumsporozoite Protein (CS) Vaccine followed by RTS,S/AS01B Boosting Significantly Improves Immunogenicity to *Plasmodium falciparum* CS Compared to That with Either Malaria Vaccine Alone▽

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The RTS,S/AS02A protein-based vaccine consistently demonstrates significant protection against infection with *Plasmodium falciparum* malaria and also against clinical malaria and severe disease in children in areas of endemicity. Here we demonstrate with rhesus macaques that priming with a replication-defective human adenovirus serotype 35 (Ad35) vector encoding circumsporozoite protein (CS) (Ad35.CS), followed by boosting with RTS,S in an improved MPL- and QS21-based adjuvant formulation, AS01B, maintains antibody responses and dramatically increases levels of T cells producing gamma interferon and other Th1 cytokines in response to CS peptides. The increased T-cell responses induced by the combination of Ad35.CS and RTS,S/AS01B are sustained for at least 6 months postvaccination and may translate to improved and more durable protection against *P. falciparum* infection in humans.

Plasmodium falciparum malaria affects millions of people throughout the world annually, and very young children are particularly vulnerable to anemia, cerebral malaria, and death. An effective *P. falciparum* malaria vaccine could have a profound impact on the lives of the estimated 2 billion at risk (34). The feasibility of development of an effective subunit vaccine against *P. falciparum* malaria has been convincingly demonstrated by a protein-based antigen (RTS,S), comprising part of the preerythrocytic circumsporozoite (CS) protein, in the AS02A adjuvant system (RTS,S/AS02A; GlaxoSmithKline Biologicals). The RTS,S antigen incorporates part of the CS central tetrapeptide repeat region and C-terminal flanking region, known to contain both B- and T-cell epitopes, into a chimeric gene expressed in *Saccharomyces cerevisiae*. This construct was named RTS,S to indicate the presence of the CS repeat region (R), T-cell epitopes (T), and hepatitis B virus surface antigen (S) in a mixture of the RTS fusion protein and the S protein that assembles into virus like particles (14, 16).

RTS,S formulated in AS02A, a proprietary adjuvant system containing an oil-in-water emulsion and the immune stimulants MPL and QS21, protects approximately 41% of malaria-naïve humans against challenge with *Plasmodium falciparum* sporozoites (18). RTS,S/AS02A efficacy in a field trial was 35% (95% confidence interval [95% CI], 22 to 47%; $P < 0.0001$) for protection against first clinical episodes and 49% (95% CI, 12 to 71%; $P = 0.02$) for protection against severe malaria during an 18-month period for 1- to 4-year-old African children (1, 2). While the unprecedented protection conferred by RTS,S/AS02A remains partial, several approaches to increasing the efficacy of the vaccine are being studied (16), including new adjuvant formulations and new vaccination strategies.

The immune correlates of RTS,S-induced protection are not well defined. However, protection induced by the RTS,S/AS02A vaccine has been associated with high anti-CS antibody titers, perhaps via inhibition of binding (7) or paralysis of sporozoites (13), or by their opsonization and destruction by phagocytes (32). RTS,S/AS02A also induces $CD4^+$ T-cell responses in clinical and field trials (20, 28, 40). The results of flow cytometric analyses by Sun and coworkers suggested that the protective immunity induced by RTS,S/AS02A is associated with CS-specific $CD4^+$ and $CD8^+$ T-cells producing gamma interferon (IFN-γ) (36).

To improve the induction of T-cell immunity, RTS,S was evaluated in a more potent liposomal MPL-QS21 adjuvant system designated AS01B. In rhesus macaques, RTS,S formulated in AS01B induced equivalent antibody titers and four-fold-higher numbers of T cells expressing type 1 cytokines than the RTS,S/AS02A formulation (35); a similar increase was seen in another recent rhesus macaque trial (P. Mettens, P. M. Dubois, et al., submitted for publication). Preliminary data obtained in clinical challenge studies conducted at the WRAIR from 2003 to 2005 indicate that RTS,S/AS01B increases CS-specific antibody and $CD4^+$ T-cell responses and protects a higher proportion of volunteers against infection following

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challenge with *P. falciparum* sporozoites than does RTS,S/AS02A (vaccine efficacy, 50% with RTS,S/AS01B versus 32% with RTS,S/AS02A [two-sided $P = 0.11$]) (K. Kester, unpublished data). A statistically significant association between efficacy and the level of both humoral and cellular immune responses is also observed (K. Kester and U. Krzych, unpublished data). It is therefore conceivable that further improvements in immune responses to CS protein could translate into further increases in efficacy against infection.

We have shown recently that immunization with a recombinant human nonreplicative adenovirus serotype 35 (Ad35)-based malaria vaccine expressing the *Plasmodium yoelii* CS protein (Ad35PyCS) induced dose-dependent and potent, CS-specific $CD8^+$ cellular and humoral immune responses and conferred significant inhibition (92 to 94%) of liver infection upon high-dose sporozoite challenge in a *Plasmodium yoelii* mouse malaria model (26). In our studies, Ad35PyCS protected mice better than did the Ad5-based vector Ad5PyCS, even in the absence of preexisting Ad5 antibodies. Preexisting immunity to Ad5 dampens the immune responses to transgene products delivered by Ad5, although such preexisting immunity can partially be overcome by using Ad5 in combination with other vectors. However, Ad35 is still immunogenic despite preexisting immunity to Ad5 (21, 26). The prevalence of Ad5 antibodies ranges from 30 to 90% worldwide, while the prevalence of Ad35 antibodies is much less, ranging from zero to 6% in the developed world up to 8 to 25% in sub-Saharan Africa (19, 25). In addition, the geometric mean titers (GMTs) against Ad35 proved to be approximately 20-fold lower than the GMTs against Ad5 when sera from Ad5- and Ad35-seropositive individuals were compared (19, 38). Thus, we chose the nonreplicative Ad35 vector for the CS protein with the idea that this vector might prove useful in a variety of different geographical regions. We created a codon-optimized vector construct using a consensus amino acid sequence for the N-terminal half of the *P. falciparum* CS molecule and a peptide sequence identical to that found in RTS (but shorter by 4 amino acids) for the C terminus (Ad35.CS). We used this vector to evaluate the immunogenicity of heterologous prime-boost combinations with RTS,S/AS01B compared with repeated doses of either vaccine alone.

MATERIALS AND METHODS

Ad35 vectors. The replication-defective Ad35 vectors have deletions early in regions 1 and 3 and were generated in PER.C6/55K cells as described elsewhere (15, 38). Expression of the synthetic, codon-optimized *CS* gene is under the control of an immediate-early cytomegalovirus promoter and a simian virus 40 polyadenylation signal. The synthetic insert encodes a CS protein (based on EMBL DNA sequence CQ830509 and EMBL protein sequence CAH04007) in which the C-terminal 14 amino acids are truncated. The N-terminal sequence of this CS protein is a consensus assembled by alignment of various sequences present in GenBank, while the C terminus is based on the 3D7 *P. falciparum* clone sequence. To optimize T-cell responses, the amino acid sequence of the C terminus encoded in Ad35.CS is precisely the same as that present in the RTS,S antigen.

Immunization protocol. Animal work was performed under a protocol approved by the WRAIR institutional animal care and use committee. All animal experimentation described was in compliance with the Animal Welfare Act, adhering to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals*, and in accordance with all applicable USDA, OLAW, and DoD guidelines.

Rhesus macaques of both sexes were determined to be healthy by physical examination and by normal values on cell blood counts and a serum chemistry panel. They were segregated into groups of seven animals, with one group of four animals for a negative control, and all groups were as closely matched as possible for average age, sex, and weight. Each group was randomly assigned to a specific treatment regimen. All animals were naïve to *P. falciparum* CS exposure by history and an enzyme-linked immunosorbent assay (ELISA) for antibodies to the CS repeat region.

TABLE 1. Study design

Group	*n*	Vaccine administered at the following mo of the study:		
		Time zero	1	3
1	7	Ad35.CS		Ad35.CS
2	7	RTS,S/AS01B	RTS,S/AS01B	RTS,S/AS01B
3	7	RTS,S/AS01B	RTS,S/AS01B	Ad35.CS
4	7	Ad35.CS	RTS,S/AS01B	RTS,S/AS01B
5	4	Ad35 empty		Ad35 empty

The vaccines were administered intramuscularly in the right rectus femoris muscle. They were given at time zero and at 1 and 3 months, except for the groups receiving two doses of Ad35.CS or empty Ad35 alone, which were given at time zero and 3 months (Table 1).

All adenovirus vectors were administered at 10^{11} virus particles per dose suspended in 0.5 ml saline for injection. Doses of RTS,S/AS01B were the standard full adult dose of 50 μg RTS,S in 0.5 ml AS01B, as supplied by the manufacturing partner in premeasured single-dose vials and single-dose adjuvant syringes for mixing immediately prior to immunization. A standard operating procedure was followed to ensure uniformity of preparation.

ELISA. Sera were collected and frozen to determine the nature and magnitude of the antibody response to CS R32 (part of the tetrapeptide repeat region of the CS protein [11]) by ELISA (41), with an anti-rhesus macaque secondary antibody (Sigma A-2054) at 1:2,000. ELISAs were performed in triplicate in serial twofold dilutions, by fitting the mean optical densities to a 4-parameter equation (minimum R^2, 0.990). Titers were defined as the dilution factor required to yield an optical density of 1.0 in our assay.

IFN-γ enzyme-linked immunospot (ELISPOT) assay. Peripheral blood mononuclear cells (PBMCs) were harvested prior to the first immunization, 2 weeks after each immunization, and 3 and 6 months after the final immunization. Whole blood, mixed with the anticoagulant EDTA, was layered over Lymphoprep density centrifugation reagent (Axis-Shield, PoC, Oslo, Norway) within 3 h of collection. Next, the harvested PBMCs were enumerated and cryopreserved in 45% (vol/vol) autologous serum–45% phosphate-buffered saline with 10% dimethyl sulfoxide.

Short-term ELISPOT assays were performed to quantify antigen-specific IFN-γ-producing cells. Briefly, cells were thawed, washed once in complete RPMI (BioWhittaker Cambrex) supplemented with 1% penicillin-streptomycin (Invitrogen), L-glutamine (Sigma-Aldrich), nonessential amino acids (Invitrogen), sodium pyruvate (Invitrogen), 2-mercaptoethanol (Invitrogen), and sodium bicarbonate (Sigma-Aldrich) with 20% fetal bovine serum (FBS; HyClone), and incubated with various stimuli in 10% FBS. The negative control was media+ (M+), consisting of complete RPMI supplemented with 10% FBS and anti-CD28 and anti-CD49d antibodies (BD-Pharmingen), at 1 μg/ml. Stimulants, diluted in M+, were CS-C, a pool of 30 15-mer peptides overlapping by 11 amino acids that covers the C terminus and part of the NANP repeat region of the CS molecule (concentration of each peptide, 1.25 μg/ml), and concanavalin A (Sigma) at 1.25 μg/ml. Cells were preincubated with the stimulants for 5 h (37°C, 95% humidity, 5% CO_2), and washed with fresh supplemented medium with the same stimulants. Subsequently, the cells were plated at a density of 200,000/well in 96-well MultiScreen-IP plates (Millipore) that had previously been coated with a monoclonal antibody specific for simian IFN-γ (U-CyTech). After a 16-h incubation, plates were developed at room temperature using a biotinylated polyclonal antibody against monkey IFN-γ (U-CyTech), alkaline phosphatase-conjugated streptavidin (Southern Biotech), nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (Pierce), and phosphate-buffered saline–0.05% Tween 20 washes. Cells were enumerated using the high-resolution AID ELISPOT reader system (Cell Technology, Inc), and each well was visually corrected for lint or other debris. Cells from each thawed vial were also immediately tested for viability on the flow cytometer (FACSCaliber; BD Immunocytometry Systems) using the viability stain 7-aminoactinomycin D (Sigma) and anti-CD4 and anti-CD8 antibodies (BD Immunocytometry Systems). Average viability was over 95%, and vials with an overall (wide lymphocyte gate) or subset viability less than 90% were rejected and the procedure repeated up to three times. Plates with

average conconavalin A responses of fewer than 500 spots per 10^6 PBMCs were similarly rejected and the procedure repeated. The background was extremely low: the average response by CS-C-stimulated cells at time zero for all groups was less than 10 spots/10^6 PBMCs. Baseline responses to peptide pools at time zero were not predictive of later responses by immunized animals, and in unstimulated medium controls at later time points, the average spot count was also less than 10 spots/10^6 PBMCs. Thus, no adjustment to the raw spot count needed to be made.

Intracellular cytokine staining assay. Ex vivo antigen-driven amplification of cytokine expression was used to detect T cells expressing interleukin 2 (IL-2) and/or IFN-γ and/or tumor necrosis factor alpha (TNF-α). Briefly, the PBMCs were stimulated with the same 15-mer CS-C peptide pool as in the ELISPOT assay, at 1 μg/ml each peptide and 1 μg/ml each anti-CD28 and anti-CD49d (Becton Dickinson). Controls were unstimulated cells (in M+ alone) and staphylococcus enterotoxin B at 1.0 μg/ml. Brefeldin-A (GolgiPlug; Becton Dickinson) was added to cell cultures 2 h later. Cultured cells were harvested after 20 h of incubation at 37°C under 5% CO_2, washed, and stained with phycoerythrin-coupled anti-CD4 (Becton Dickinson). Cells were then fixed and permeabilized for 20 min at 4°C using Cytofix-Cytoperm (Becton Dickinson) and stained with anti-IFN-γ coupled to fluorescein isothiocyanate, anti-IL-2 coupled to allophycocyanin, and phycoerythrin-Cy7 coupled to anti-TNF-α (Becton Dickinson). Cells were then washed and analyzed on an LSR1 flow cytometer (Becton Dickinson). A minimum number of 30,000 cells were acquired in the CD4 subset so as to detect a difference between medium background values of 0.05% and peptide-induced responses of 0.11% by using a one-sided chi-square test with continuity correction and a significance level of 0.05.

Statistical method. Both ELISA and ELISPOT data were log transformed, confirmed to be normally distributed, and then subjected to parametric statistical analysis. ELISA and ELISPOT data of the four test groups were compared using single-factor analysis of variance for each time point (the negative control was omitted from analyses to increase stringency). When the analysis of variance results were statistically significant, a posthoc analysis followed using Dunnett's comparison. Finally, ratios of geometric mean T-cell responses with 95% CIs were calculated where both the Ad35.CS-alone group and the RTS,S/AS01B-alone group served as comparators.

RESULTS

We compared the immunogenicities of heterologous prime-boost regimens consisting of Ad35.CS followed by RTS,S/AS01B or RTS,S/AS01B followed by Ad35.CS in rhesus macaques. Homologous comparator groups were two doses of Ad35.CS or three doses of RTS,S/AS01B. Healthy, malaria antigen-naïve rhesus macaques were stratified by age, sex, and weight, distributed evenly into groups of seven, and randomly assigned to regimens (Table 1). The first group received two doses of Ad35.CS at time zero and 3 months. The second group received three sequential administrations of RTS,S/AS01B at time zero and 1 and 3 months. The third group received two sequential administrations of RTS,S/AS01B at time zero and 1 month, followed by a single heterologous injection of Ad35.CS at month 3. The fourth group received a single administration of Ad35.CS followed by two heterologous administrations of RTS,S/AS01B on the same schedule (time zero, 1 month, and 3 months). Finally, a smaller negative-control group ($n = 4$) receiving two doses (at time zero and 3 months) of the parental Ad35 vector without the CS insert (empty Ad35) was included but was excluded from the statistical analysis. Two additional study groups involving only adenovirus vaccinations will not be presented here, because they did not modify the conclusion upon statistical analysis.

To evaluate IFN-γ production by ELISPOT, PBMC were stimulated with a pool of 15-mer peptides, overlapping by 11, against the C-terminal region of CS.

Two weeks after the final immunization (week 14), the group primed with Ad35.CS and boosted twice with RTS,S/AS01B had 553 geometric mean spots per million (GMSM) approximately 16-fold higher than the GMSM of 34 for the comparator group receiving three doses of RTS,S/AS01B ($P < 0.0001$) (Fig. 1a; Table 2). The heterologous prime-boost regimen in which two doses of RTS,S/AS01B preceded the single Ad35.CS dose resulted in a GMSM of 167, which was also significantly higher than that for the RTS,S/AS01B protein-only group ($P = 0.02$). Two doses of the Ad35.CS construct resulted in a GMSM of 83, 2.5 times higher than that for the protein-only group but no longer significantly different ($P = 0.28$).

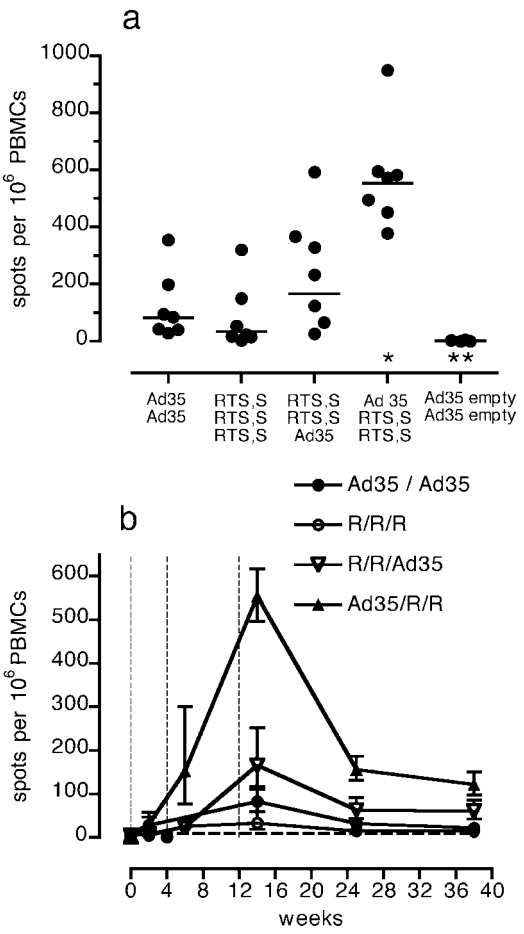

FIG. 1. ELISPOT data. Shown are antigen-specific IFN-γ ELISPOT results for CS C-terminal 15-mer peptides, expressed as the number of spots per 10^6 PBMCs. (a) Two weeks after the last immunization. Geometric means (horizontal lines) were compared to those for the RTS,S-alone group. Statistical significance is indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$. RTS,S, RTS,S/AS01B; Ad35, Ad35.CS. (b) Time course of geometric mean IFN-γ ELISPOT responses. Error bars, standard errors of the means, calculated on log-transformed data; vertical dashed lines, vaccination time points; horizontal dashed line, average background. Week 14 is 2 weeks after the final immunization; weeks 25 and 38 are 3 and 6 months later, respectively. R, RTS,S/AS01B.

In order to further characterize the T-cell-mediated immune response, we performed an ex vivo antigen-driven amplification of cytokine expression that was measured by intracellular staining with flow cytometry analysis (ICS). Figure 2a shows the numbers of $CD4^+$ T cells expressing at least two cytokines among IL-2, IFN-γ, and TNF-α. These results demonstrated

TABLE 2. Ratios of geometric mean IFN-γ ELISPOT responses against CS C-terminal peptides of test groups versus the RTS,S/AS01B comparator group[a]

Wk	Result for the following immunization protocol:					
	RTS,S/AS01B, RTS,S/AS01B, Ad35.CS		Ad35.CS, RTS,S/AS01B,RTS,S/AS01B		Ad35.CS,Ad35.CS	
	Ratio (95% CI)	*P*	Ratio (95% CI)	*P*	Ratio (95% CI)	*P*
6	Pooled[b]		6.0 (1.5–25.4)	0.017		
14	4.9 (1.2–20.4)	0.02	16.4 (4.0–67.8)	0.0001	2.5 (0.6–10.2)	0.28
25	4.1 (1.3–12.7)	0.01	10.0 (3.2–31.2)	<0.0001	2.0 (0.7–6.3)	0.029
38	3.6 (0.95–14.1)	0.06	7.3 (1.9–28.3)	0.003	1.3 (0.3–5.1)	0.091

[a] The comparator group received three immunizations, each with RTS,S/AS01B.
[b] The RTS,S/RTS,S/RTS,S and RTS,S/RTS,S/Ad35 results were pooled for week 6 analysis, since the prime and first boost treatments were identical. Statistical analysis is based on Dunnett's comparison using log-transformed data. Week 25 represents the 3-month time point after final immunization.

the same relationship between groups as the ELISPOT data in Fig. 1a. The observed $CD4^+$ T-cell response was a mix of effector cells expressing IFN-γ and/or TNF-α and memory T cells expressing IL-2 (Fig. 2b to d) (31). Although the magnitude differs, the relative responses measured by ELISPOT and by ICS are strikingly similar.

We also evaluated the IFN-γ T-cell response by ELISPOT assays at 25 weeks (13 weeks, or approximately 3 months, after the last immunization) and 38 weeks (26 weeks, or approximately 6 months, after the last immunization) (Fig. 1b). The T-cell responses for the RTS,S/AS01B-only group dropped to 16 and 17 GMSM at 3 and 6 months following the last injection, respectively. The responses for the recipients of two homologous doses of Ad35.CS dropped to 32 and 22 GMSM, respectively. For group 3 (the RTS,S/AS01B, RTS,S/AS01B, Ad35.CS group), the T-cell response fell to 63 GMSM by 3

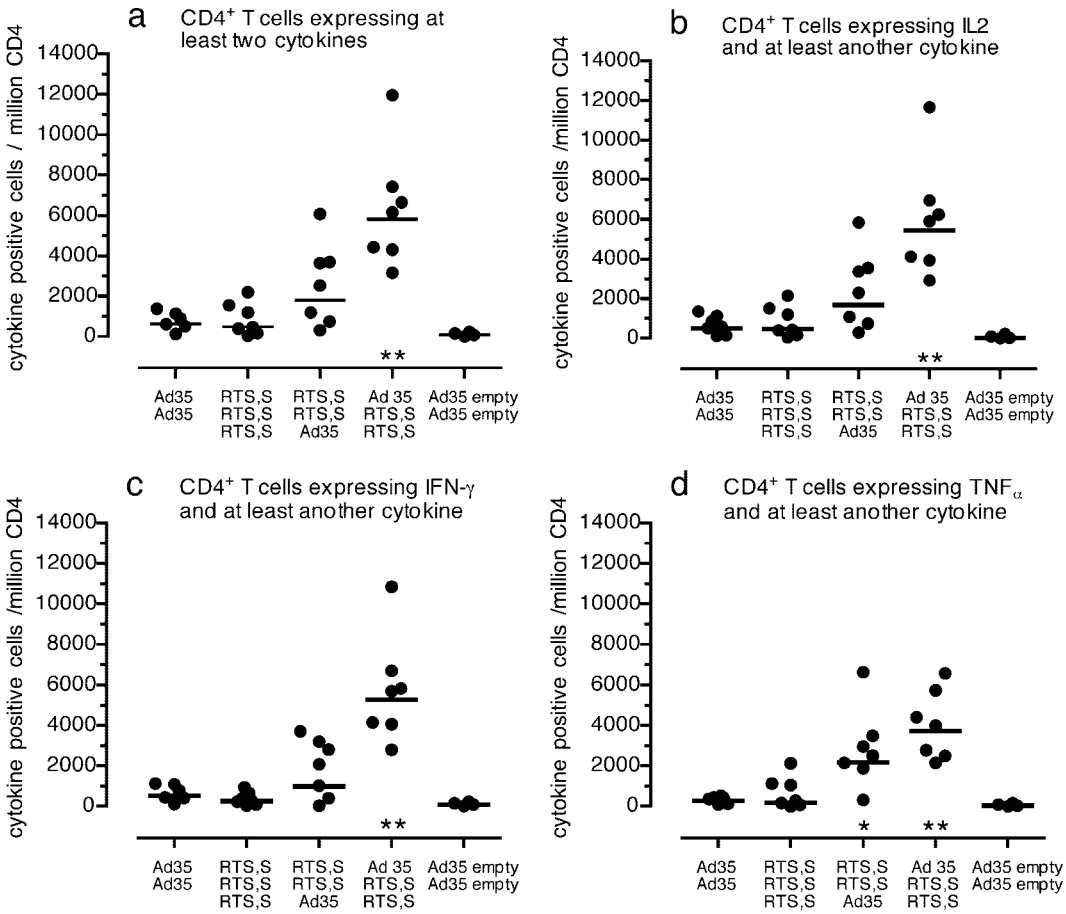

FIG. 2. Intracellular cytokine staining data. Results are numbers of cytokine-expressing $CD4^+$ T cells per million $CD4^+$ cells. (a) All cells expressing any two or all three cytokines evaluated. (b, c, and d) Cells expressing two or three cytokines including at least IL-2, IFN-γ, or TNF-α, respectively. Cells were harvested 2 weeks after the last vaccination. Geometric means were compared to those for the RTS,S/AS01B comparator group. *, $P < 0.05$; **, $P < 0.01$.

months later but was then maintained well above background at 61 GMSM at 6 months after vaccination. Lastly, the long-term T-cell response was by far the best for the heterologous group receiving Ad35.CS followed by two RTS,S/AS01B administrations, with 156 GMSM at 3 months, and 122 GMSM at 6 months, after the last vaccination. At 6 months after the last vaccination, the T-cell response for the heterologous Ad35.CS–RTS,S/AS01B group was 7.3-fold higher than that for the protein-alone group at the same time point and 6-fold higher than that for the Ad35.CS-alone group ($P = 0.003$) (Table 2).

Some differences between groups in the IFN-γ ELISPOT results appeared early, after only one or two doses. A single RTS,S/AS01B priming injection resulted in a T-cell response indistinguishable from background, while a single Ad35.CS immunization resulted in a response of 28 to 31 spots per million at 2 weeks (Fig. 1b, week 2). Boosting the RTS,S/AS01B prime with RTS,S/AS01B 4 weeks later resulted in a response of 26 GMSM, while boosting the Ad35.CS prime with one dose of RTS,S/AS01B resulted in a significantly better boost, to 152 GMSM ($P = 0.017$) (Fig. 1b, week 6).

We also measured the antibody responses to the immunodominant repeat region of CS protein for the different vaccination groups (Fig. 3). Comparison of the three RTS,S/AS01B-containing regimens showed that the B-cell response to CS protein did not differ statistically significantly among them either at 2 weeks or at 3 months following the last vaccination ($P = 0.14$ and 0.35, respectively). Thus, all groups receiving at least two doses of protein with adjuvant had similarly good antibody responses. By comparison, both at 2 weeks and at 3 months following the last vaccination, the antibody titers for the Ad35.CS-only group were 10- and 7-fold lower than the titers for the RTS,S/AS01B comparator group ($P \leq 0.0001$ and $P = 0.0001$, respectively, by Dunnett's comparison).

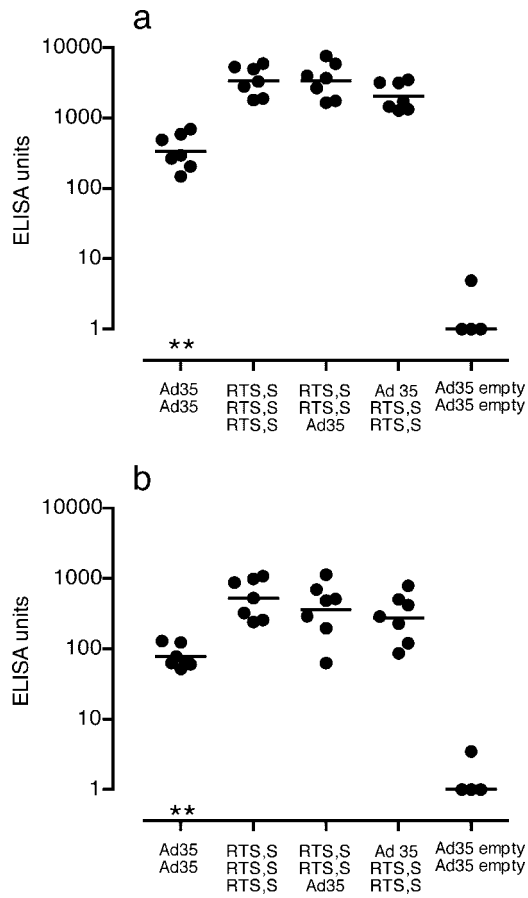

FIG. 3. Antibody responses 2 weeks (a) and 3 months (b) after final immunization. RTS,S, RTS,S/AS01B; Ad35, Ad35.CS. **, $P < 0.01$ by Dunnett's comparison with RTS,S alone as the comparator and with the empty Ad35 group omitted. Horizontal lines represent geometric means (or the median in the case of the empty Ad35 group).

DISCUSSION

The early stages of development of the malaria parasite occur in the few minutes that it takes for the mosquito-introduced, motile sporozoite to find its way to a suitable hepatocyte and in the week-long schizont development phase within the hepatocyte. These two phases together, lumped into the term "preerythrocytic," are characterized by a total lack of host symptoms. Thus, preerythrocytic antigens are considered ideal for development of traveler's vaccines, because prevention of the completion of all of these stages should entirely prevent symptoms. In contrast, the blood-stage antigens have been thought to be ideal for disease-limiting strategies, which are of particular interest in areas of endemicity. The CS protein, the most abundant surface antigen on the sporozoite, continues to be detectable for at least the first 3 days of schizogony and thus has been considered an attractive vaccine target for both antibody-mediated (against the sporozoite) and cell-mediated (against the schizont) immunity since the mid-1980s (24). The checkered career of CS protein through numerous failed clinical vaccine trials until its more recent successes as the RTS,S/AS02A formulation could serve as an object lesson in making malaria vaccines work through persistence. The observation that RTS,S/AS02A given to children in an area of endemicity not only reduced reinfection rates but also reduced the incidence of severe disease (1, 2) leads to the hypothesis that increasing the overall immunogenicity of RTS,S/AS02A may increase efficacy both for populations in areas where *P. falciparum* malaria is endemic and for those in areas where it is not.

The use of different vaccine constructs in heterologous combination is increasingly being evaluated for diseases in which good T-cell responses are thought to be important, such as tuberculosis, human immunodeficiency virus disease, and malaria (12, 29, 30). Nonreplicative adenovirus vectors are particularly attractive (37) due to their expected safety profile (10) and their efficiency at boosting a number of other kinds of vaccine constructs (3). To date, they have been primarily paired in heterologous combinations with DNA plasmid vaccines and with other viral constructs in which the heterologous combination has shown an excellent capacity to increase cellular immune responses (22).

Malaria CS protein-based adenovirus vaccines consistently induced complete and long-lasting protection when administered in heterologous combinations in the murine model (5) and were subsequently further refined (6, 26). Although the murine model of malaria has been only par-

tially useful at predicting protective immunity in humans, the existence of the RTS,S/AS02A protein vaccine against *P. falciparum* malaria, already on the road to licensure, made the further evaluation of a heterologous adenovirus-protein combination attractive as a potential strategy to enhance the partial sterile protection against malaria that the protein provides by itself. In studies of naïve adults rendered immune to live-parasite challenge with RTS,S/AS02A, both anti-CS antibody levels and cellular IFN-γ responses have been partially, albeit imperfectly, correlated with protection (18, 36), and both are considered important. Since the cellular responses to RTS,S/AS02A are detectable but rather weak, the addition of a heterologous construct to the protein vaccine is a rational approach (23, 29). DNA priming has been tested as an improvement on the RTS,S/AS02A protein alone. The timing of potential DNA priming was explored with the rhesus macaque, and a regimen was optimized for enhancing serologic and delayed-type hypersensitivity responses after a single, suboptimal boost of RTS,S/AS02A (39). In humans, priming with DNA can result in the subsequent recall of cellular responses by a RTS,S/AS02A boost given approximately a year later, but no challenge data were collected (9, 40). The combination of an MVA poxvirus-based CS construct with RTS,S/AS02A has already been demonstrated to provide very modest improvements in in vitro T-cell analyses and no increase in protection for humans (8). The demonstration of the superiority of adenovirus constructs in the generation of IFN-γ responses compared with DNA and other types of viral vectors in a different disease system (22) supports our adoption of this approach.

Having chosen the nonreplicative Ad35 vector for reasons of utility and preexisting immunity (19, 25, 38), we created a construct containing a codon-optimized full-length insert. Although the CS N terminus was based on a consensus sequence among known alleles, the amino acid sequence for the portion of the repeat and C terminus that is also in the RTS construct was intentionally an exact match, to optimize T-cell boosting. Having determined that the glycosylphosphatidylinositol anchor sequence could be problematic during expression (6), and in order to avoid a putative glycosylation site, the final 14 residues of CS protein were not included, making the C terminus 4 residues shorter than the C terminus of the RTS protein (14).

Macaques can be used as challenge models for malaria, but only with other species of macaque malaria that are congeners of *Plasmodium vivax* rather than *P. falciparum*. However, the rhesus macaque, by virtue of the relatively close homology of its class II and, to a lesser extent, class I major histocompatibility complex molecules to those of humans (4, 17, 33), has been a very useful predictor of immune responses in humans. Most recently, the improvement in IFN-γ responses seen in two macaque studies on RTS,S in a newer adjuvant formulation (35; P. Mettens et al., submitted) did translate to a partial improvement both in the same kinds of in vitro responses and in improved protection rates in a human challenge trial (K. Kester, unpublished), thus adding credence to the macaque model. Finally, macaques (unlike mice) do express CD46, the cellular surface receptor for Ad35, on their dendritic cells and thus are a good model for human immunogenicity with this vector.

Although heterologous adenovirus-protein prime-boost combinations have been used for primates in other diseases (27, 42), in this head-to-head comparison of giving adenovirus first and protein second or the reverse, the adenovirus as the prime and the two injections of protein as the boost were clearly the winning immunization strategy.

Thus, priming with Ad35.CS followed by boosting with RTS,S/AS01B results in IFN-γ T-cell responses that are highly significantly increased in both magnitude and duration over those observed with three doses of RTS,S/AS01B or with two doses of Ad35.CS. The improvement in IFN-γ responses when RTS,S is formulated with AS01B instead of AS02A has already been demonstrated to be a fourfold increase (35). Although the techniques used to assess the T-cell responses have improved in precision since that earlier study took place, the additional 16-fold increase seen here when the RTS,S/AS01B formulation is preceded by the Ad35.CS construct implies almost a 2-log-unit total increase over the response to the existing RTS,S/AS02A formulation. In addition, antigen-specific effector cells clearly circulate for a much longer period when the adenovirus vector is involved. There was no significant effect on the magnitude of the serologic response when one of the protein immunizations was replaced with the adenovirus vector, regardless of the order of administration. All immunizations were uneventful and safe; these data will be reported in detail elsewhere. We are continuing to evaluate the specificity and kinetics of the immune responses in these macaques, as well as generating more-detailed data on the cell types involved in the IFN-γ response by using additional intracellular cytokine staining assays.

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