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Adenovirus 5 and 35 vectors expressing *Plasmodium falciparum* circumsporozoite surface protein elicit potent antigen-specific cellular IFN-γ and antibody responses in mice☆

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ABSTRACT

Falciparum malaria vaccine candidates have been developed using recombinant, replication-deficient serotype 5 and 35 adenoviruses (Ad5, Ad35) encoding the *Plasmodium falciparum* circumsporozoite surface protein (CSP) (Ad5.CS, Ad35.CS) (Crucell Holland BV, Leiden, The Netherlands). To evaluate the immunogenicity of these constructs, BALB/cJ mice were immunized twice with either Ad5.CS, Ad35.CS, empty Ad5-vector (eAd5), empty Ad35 vector (eAd35), or saline. Another group received the CSP-based RTS,S malaria vaccine formulated in the proprietary Adjuvant System AS01B (GlaxoSmithKline Biologicals, Rixensart, Belgium). Here we report that Ad5.CS, Ad35.CS, and RTS,S/AS01B, elicited both cellular and serologic CSP antigen-specific responses in mice. These adenoviral vectors induce strong malaria-specific immunity and warrant further evaluation.

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1. Introduction

The *Plasmodium falciparum* circumsporozoite protein (CSP) is the immunodominant antigen on the surface of sporozoite-stage parasites and is expressed during the sporozoite and early liver stages of infection [1]. The full-length *P. falciparum* CSP contains a tetrapeptide central repeat region containing B-cell epitopes, and C- and N-terminal flanking regions containing B- and T-cell epitopes [2]. The RTS,S/AS02A vaccine, which represents part of central repeat and the C-terminal portions of the CSP formulated with the AS02A Adjuvant System, has shown efficacy in clinical [2–6] and field trials [7–10] against falciparum malaria. The RTS,S/AS02A vaccine elicits strong CSP-specific cellular immune responses that have been associated with protection in clinical trials [11] and strong CSP-specific antibody responses associated with protection in clinical trials [3,4,6] (and Kester unpublished) and in field trials in adults and infants [9,10] but not in young children [7,8].

Recently, a new formulation of the RTS,S antigen with the AS01B Adjuvant System was compared to RTS,S/AS02A and elicited equivalent antibody responses, a 4-fold increase in CSP-specific IFN-γ ELISPOT responses, and significant increases in CSP-specific delayed type hypersensitivity responses in the rhesus macaque vaccine model [12–14]. In a subsequent clinical challenge study, RTS,S/AS01B, in comparison to RTS,S/AS02A, increased CSP-specific antibody and CD4+ T-cell responses and protected a higher proportion of volunteers against infection

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following challenge with *P. falciparum* sporozoites than did RTS,S/AS02A (vaccine efficacy 50% with RTS,S/AS01B versus 32% with RTS,S/AS02A [Fisher's exact, 2-tailed $p = 0.11$]). There was a statistically significant association between vaccine efficacy and the magnitude of the humoral and cellular immune responses (Kester KE, personal communication). These results suggest that further improvements in immune responses to the CSP may lead to an increase in protection against infection.

Recombinant, non-replicating viral vectors are a promising means to improve antigen presentation/processing, immunogenicity, and protection. Among myriad potential platforms, adenoviral vectors offer the potential to safely induce antigen-specific antibody, CD4+ and CD8+ T-cells and immunologic memory in humans [15,16] In the initial proof of concept for an adenovirus based CSP vaccine in mice, immunization with a single-dose of a Ad5-vectored *P. yoelii* CSP induced high titer CSP-antibody, CSP-specific IFN-γ-secreting splenic CD4+ and CD8+ T-cells, and 40% protection against lethal *P. yoelii* challenge [15]. This Ad5-vector induced protection was largely dependent on CD8+ T-cells [17] Later, an Ad35-vectored *P. yoelii* CSP vaccine showed a 92–94% CSP-specific inhibition of hepatic schizogony after challenge with a high-dose of *P. yoelii* sporozoites [18].

Encouraged by these results, we produced replication-deficient, Ad5 and Ad35 vectors encoding the full-length *P. falciparum* CSP 3D7 clone minus the GPI anchor as described elsewhere [16,19]. As a prequel to a larger heterologous prime-boost study in rhesus macaques, we first evaluated the cellular and humoral immunogenicity elicited by two homologous immunizations with Ad5.CS or Ad35.CS in BALB/cJ mice, and compared the results with those elicited by two injections of the adjuvanted protein malaria vaccine RTS,S/AS01B, an improved formulation related to RTS,S/AS02A, as positive controls. Empty vectors and saline were used as negative controls.

2. Materials and methods

2.1. Vaccines

Generation of replication-deficient Ad vector on PER.C6/55K cells was performed as described previously [16,20,21]. Briefly, expression of the synthetic, codon-optimized CSP gene is under the control of an immediate-early cytomegalovirus promoter and a simian virus 40 polyadenylation signal. The synthetic insert encodes CSP (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 CSP 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. The amino acid sequence of the CSP C-terminus encoded in Ad35.CS and Ad5.CS was engineered to yield precisely the same amino acid sequence as that of the RTS,S vaccine antigen, but was 4 amino acids shorter at the C-terminus. Adenovirus vector aliquots were supplied frozen in PBS, and were diluted in Dulbecco's phosphate buffered saline (DPBS, BioWhittaker, Walkersville, MD) to achieve a final dose of 10^{10} viral particles in 50 μL for injection.

The RTS,S antigen is a recombinant fusion protein of part of the central repeat region of *P. falciparum* CSP (3D7 strain) (R), most of the C-terminal non-repeat region of CSP containing multiple T-cell epitopes (T), and the hepatitis B surface antigen (S), which is co-expressed with unfused hepatitis B surface antigen (S). These molecules self-assemble into virus-like particles [22]. RTS,S is supplied as a lyophilized cake in a single human dose vial. The AS01B Adjuvant System was supplied as a single-dose vial. After mixing, the final dose was one-tenth of the adult dose, and contained 5 μg of RTS,S, in a total volume of 50 μL AS01B. All vaccines were formulated just before administration and were delivered by intramuscular injection.

2.2. Animal care and use

Animal work was performed under a protocol approved by the WRAIR Institutional Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition.

2.3. Experimental design

Thirty-six male BALB/cJ mice (Jackson Laboratories, Bar Harbor, ME), age 3–4 weeks, were allocated into six equal groups, and vaccinated with two doses of vaccine, 4 weeks apart. The six groups received two doses each of one of the following vaccine preparations: Ad5.CS, Ad35.CS, empty vector Ad5 (eAd5), empty vector Ad35 (eAd35), saline, or RTS,S/AS01B. All injections were intramuscular in the right, anterior thigh. Serum was collected on day 0 (T0), 2 weeks after the first immunization on day 14 (Week 2), and 2 weeks after second immunization on day 42 (Week 6). At Week 6, mice were euthanized and trunk blood and spleens removed aseptically. Splenocytes were collected and cryopreserved, and serum separated and frozen, for subsequent analysis.

2.4. Protocol deviations

One mouse from the RTS,S/AS01B immunized group was discovered to have a greatly enlarged (misshapen and about five times normal volume) spleen at the time of euthanasia. On subsequent assays, the cells harvested from this spleen were non-responsive to all stimulants, including phytohemagglutinin. This individual mouse's data was excluded from subsequent evaluation of cellular responses due to presumptive neoplasia. ELISpot wells from one other mouse in the RTS,S/AS01B group were entirely black and unreadable even upon repetition, and this mouse's data was also excluded due to being unquantifiable. Serology was not performed on three serum samples from Week 6 of the Ad35.CS immunized group due to accidental loss of sample.

2.5. ELISPOT procedures

ELISPOTS were performed to quantify antigen-specific IFN-γ producing splenocytes. All assays were conducted blindly with no knowledge of the animal groups. Thawed, washed splenocytes were stimulated overnight in 10% FBS in complete RPMI 1640 with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (all Sigma), and 0.055 mM 2-mercaptoethanol, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (all Gibco, Grand Is., NY) (cRPMI). Stimulants included peptide pools of 15-mers overlapping by 11 amino acids spanning the CSP N-terminus (CS-N) (Crucell Holland BV) at 1. 25 μg/mL, and spanning the CSP C-terminus into the repeat region (CS-C) (GlaxoSmithKline Biologicals) at 1.0 μg/mL. Controls included PHA (Sigma) at 5 μg/mL and unstimulated complete medium.

Membranes of 96-well, ELISPOT plates (MultiScreen™ ELIIP10SSP, Millipore Corp., Bedford, MA) were pre-wet with 70% ethanol, then washed with DPBS and coated overnight at 4 °C

with 100 μL of 15 μg/mL capture AN18 anti-mouse IFN-γ monoclonal antibody (Mabtech, Mariemont, OH) in DPBS. The following day, plates were washed and blocked with cRPMI containing 5% FBS for 1 h at 37 °C, 95% humidity, 5% CO_2. Stimulated cells were transferred to the ELISPOT plate at 200,000 viable cells per well, and cells were allowed to incubate overnight (37 °C, 95% humidity, 5% CO_2).

The following day, plates were washed with DPBS and labeled with 100 microliters/well of 1 μg/mL secondary monoclonal biotinylated anti-mouse IFN-γ R4-6A2 (Mabtech) in DPBS containing 0.5% FBS for 2 h at room temperature while rocking. After washing with DPBS, 100 μL/well of streptavidin-alkaline phosphatase conjugate (Mabtech) in DPBS with 0.5% FBS were added and plates rocked at room temperature for 1 h. Plates were then washed with DPBS and then twice with distilled water before 100 μL/well BCIP/NBT plus developer (Mabtech) were added. Spot development usually occurred within 5–10 min. Plate reactions were stopped with tap water and air dried. Data was collected using an ELISPOT plate reader (AID Autoimmun Diagnostika GmbH2003, Strassberg, Germany) and analyzed by ELISpot Software Version 3.1 (AID). Blinded laboratory personnel corrected each plate scan for lint, debris, or membrane damage. A maximum number of spots counted per well was set at 350, as accuracy and reproducibility decreased significantly above that number.

ELISPOT assays were repeated if a particular sample failed with a high response in plain media (>20 spots per million (SPM)), low cell viability (<90% viable under trypan blue), or a PHA (positive control) response less than 2 S.D. (S.D. = 337 SPM) under the mean PHA response (840 GMSPM). The geometric mean IFN-γ response to plain media was 12 spots per million cells.

2.6. ELISA procedures

Samples were assayed on day 0, 2 weeks after the first vaccination, and 2 weeks after the second vaccination. All assays were conducted blindly with no knowledge of the animal group assignments. All reagents except coating buffer and substrate contained boiled casein (BC) as a blocking agent (5 g/L skim milk casein (Sigma) in PBS), and were performed substantially as reported elsewhere [23] Briefly, 96-well, polystyrene ELISA plates (Immunlon 2 HB®, microtiter flat-bottom, Thermo Labsystems, Franklin, MA) were coated with 100 μL of 2 μg/mL of the central repeat region of CSP, R32-LR (GlaxoSmithKline Biologicals) [24] in antigen diluent (1:1000 BC in 1× PBS) overnight. After casein blocking, samples were serially diluted down the plate in triplicate, from 1:50 to 1:64,000. Secondary antibody was a 1:3000 dilution of HRP-conjugated goat anti-mouse heavy and light chain IgG (Kierkegaard & Perry Labs, Gaithersburg, MD). Plates were developed with 100 μL of ABTS (2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonate, KPL) for 1 h, and stopped by adding 10 μL 20% sodium dodecyl sulfate (Sigma) in water to each well. Plates were immediately read at 414 nm in a Vmax Plate Washer (Molecular Devices) and data analyzed with SoftMaxPro 4.6 program (Molecular Devices). Antibody titers were defined as the serum dilution required to yield an optical density of 1.0 in our assay.

2.7. Statistical methods

GraphPad Prism ver. 4.0 was used to do the analysis (GraphPad Software, San Diego, CA). All data were log-transformed prior to analysis, to normalize distribution and variance. Data were analyzed by performing ANOVA on log-transformed data. Where indicated, negative control groups were omitted from the ANOVA to increase stringency of comparison. When the ANOVA results were statistically significant, a pair-wise post-test analysis was performed using Tukey's Multiple Comparison test. One comparison using student's *t*-test was performed on the N-term ELISpot data.

3. Results

3.1. ELISPOT results

ELISPOT analysis of splenocytes from mice immunized with Ad5.CS, Ad35.CS, or RTS,S/AS01B revealed high levels of CSP-specific, IFN-γ secreting splenocytes in response to stimulation with the CS-C peptide pool (Fig. 1a). Geometric mean spots per million splenocytes for RTS,S/AS01B, Ad5.CS, and Ad35.CS were 302, 400 and 179 SPM, respectively. There was a significant difference among just these three responding groups by ANOVA ($p = 0.025$), with Ad35.CS responses being significantly ($p < 0.05$) lower than Ad5.CS using Tukey's test. No differences among the negative groups were observed.

Both Ad35.CS (547 GMSPM) and Ad5.CS (1563 GMSPM) recipients developed excellent ELISPOT responses to the CS-N peptide

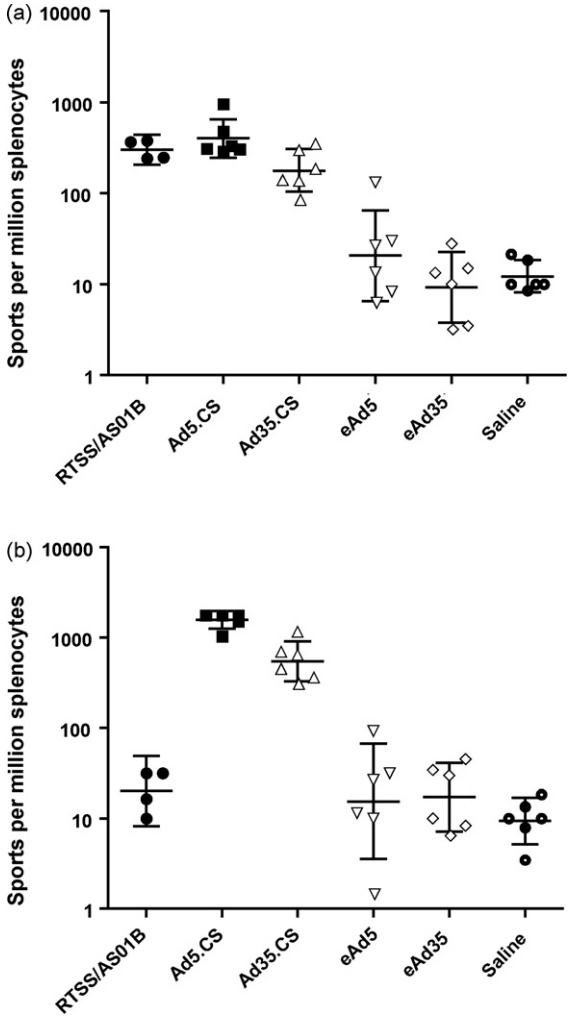

Fig. 1. IFN-γ ELISPOT responses to CS-C peptide and CS-N peptide pools 2 weeks after second vaccination. Groups of 6 BALB/cJ mice received two vaccinations, 4 weeks apart. eAd5 and eAd35 are the empty Ad5 and Ad35 vectors. Points represent individual mouse responses, bars represent geometric mean with 95% confidence interval: (a) IFN-γ ELISPOT responses to CS-C peptide pool and (b) IFN-γ ELISPOT responses to CS-N peptide pool.

pool, with a high degree of significance ($p < 0.0001$ by ANOVA) when compared with the negative groups. Four of the six mice in the Ad5.CS group reached the upper limit of 350 spots per well, thus substantially and artificially decreasing the variance in this group. Although a post hoc Student's *t*-test of just these two groups makes them appear different ($p < 0.001$), the Tukey's test comparison was not able to distinguish between them when all possible combinations of the six groups were included in the analysis (Fig. 1b). RTS,S/AS01B (which does not contain the N-terminus of CSP) and saline group responses were low and indistinguishable from parental empty vectors eAd5 and eAd35 responses to CS-N.

3.2. ELISA results

Mice immunized with either Ad-CS vector constructs or with RTS,S/AS01B developed high levels of antibody to R32-LR. 2 weeks after the initial vaccination, responses were seen in groups receiving RTS,S/AS01B, Ad5.CS and Ad35.CS with geometric mean titers (GMT) of 2579, 933, and 1105, respectively ($p < 0.0001$ by ANOVA across all six groups) (Fig. 2a). After just the initial vaccination, the three responding groups were statistically similar to each other ($p = 0.084$ by ANOVA). 2 weeks after the second vaccination, GMTs in the responding groups had all risen, to 10,344 in the RTS,S/AS01B group, 2283 in the Ad5.CS group, and 4195 in the Ad35.CS group ($p < 0.0001$ by ANOVA across all six groups) (Fig. 2b). After boosting, a significant difference was observed among just the three responding groups by ANOVA ($p = 0.0031$); Tukey's post-test comparisons indicated that the response from the RTS,S/AS01B group was significantly higher than the response from the Ad5.CS group only ($p < 0.01$; all other $p > 0.05$). GMT in eAd5, eAd35, and saline groups were indistinguishable from each other at all timepoints.

4. Discussion

Recent studies have described in detail the ability of Ad5 and Ad35 vectors to efficiently deliver *Plasmodium* antigens and elicit immunogenicity as well as protect laboratory animals from sporozoite challenge with *P. yoelii* [15,17,18]. We have demonstrated the ability of Ad5.CS and Ad35.CS constructs to deliver both T-cell and B-cell epitopes of *P. falciparum* CSP in BALB/cJ mice, resulting in strong antigen-specific cellular responses as measured by IFN-γ ELISPOT and high antibody titers as measured by ELISA. These data are similar to those reported for an Ad35-vectored *P. yoelii* CSP construct that conferred 92–94% inhibition of liver infection upon high-dose sporozoite challenge with *P. yoelii* [15], and for another Ad35 based construct for a different falciparum malaria antigen, LSA-1 [25].

Ad35 interacts with CD46 on dendritic cells (DC) for endocytic transport and proper antigen presentation [26]. The immunokinetics of virus vector uptake by DC in mice is not fully known, however, it is known that the high-affinity Ad35 binding receptor, CD46, is not expressed in the murine immune system [27]. In the mouse, Ad35 vectors invade DC less efficiently than Ad5 will, and less efficiently than it will invade primate and human DC. This biological difference could easily account for the various differences in immune responses seen between the Ad5.CS and Ad35.CS groups in our study. For this reason, a CD46-transgenic mouse was developed for the evaluation of Ad35 vectors, subsequent to the initiation of this study [27]. Our study thus is underestimating the potential immunogenicity of the Ad35 vaccine vector in humans and other primates.

In our mouse immunogenicity model, both Ad5.CS and Ad35.CS immunizations yielded significant antibody titers, although these were lower than those elicited by the comparator malaria vaccine RTS,S/AS01B. Adjuvanted RTS,S is so far the only malaria vaccine

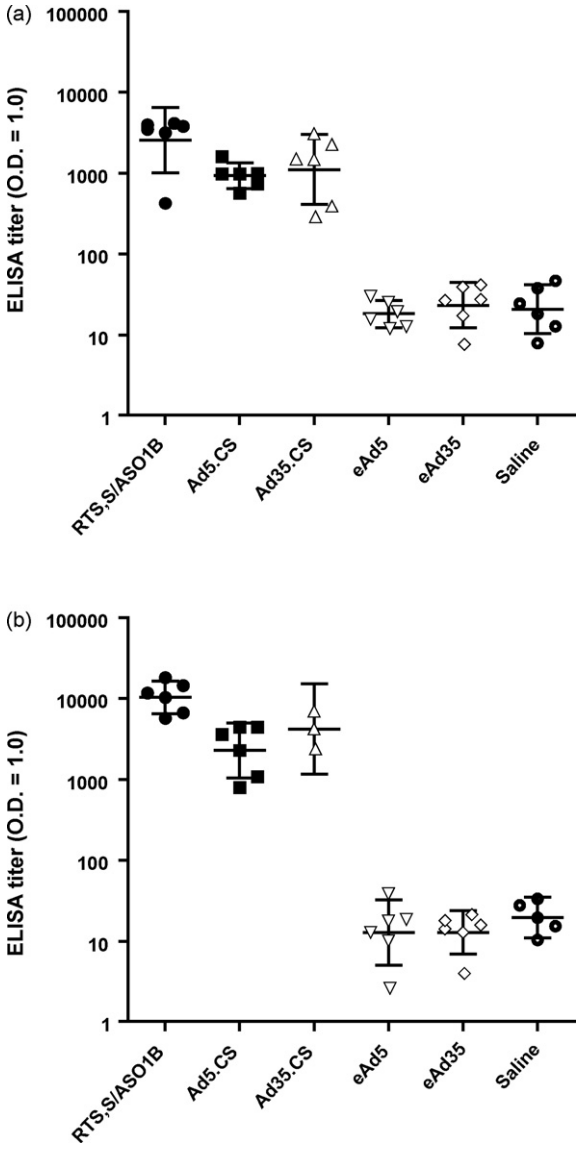

Fig. 2. Geometric mean ELISA titers to R32 2 weeks after first and second vaccination. Groups of 6 BALB/cJ mice received two vaccinations, 4 weeks apart. eAd5 and eAd35 are the empty Ad5 and Ad35 vectors. Points represent individual mouse responses, bars represent geometric mean with 95% confidence interval. (a) Geometric mean ELISA titers to R32 after first vaccination and (b) geometric mean ELISA titers to R32 after second vaccination.

to have reduced rates of infection in malaria-naïve and malaria-experienced adults [3–6,10] and to have protected young children and infants in endemic areas [7–10]. The protection mediated by RTS,S/AS02A has been linked to antibody to the CSP-repeat region in malaria-naïve [3,4,6] and malaria-experienced adults [10] and infants [9], suggesting that the production of anti-CSP antibody is an important criterion for CSP-based vaccines.

Both Ad5.CS and Ad35.CS induced IFN-γ secreting cells specific for both N-terminal and C-terminal epitopes in mice. These regions contain well-described T-helper and T-cytolytic epitopes which are thought to be involved in protective immunity acquired by humans after irradiated-sporozoite immunization [1,2]. In the case of adjuvanted RTS,S-based protection, T-cell responses to the C-terminus also have been associated with protection against infection [11] (and Kester, KE, unpublished). Infants and young children, who bear the burden of nearly 80% of falciparum malaria mortality in Africa [28], have little or no neutralizing antibodies to Ad35,

further supporting its use as a vector for *P. falciparum* antigens in these populations [18,29–31]. In contrast, the higher prevalence of neutralizing antibody to Ad5 in both malaria free and malaria endemic populations could result in a less predictable response to a Ad5-based malaria vaccine [31–33].

Since this study on Ad5.CS, A35.CS and RTS,S/AS01B was performed in BALB/c mice, we have also described the immunogenicity of these vaccines either singly or in various combinations in *Macaca mulatta* [16]. In that study, the combination of a single priming dose of Ad35.CS followed by two boosting doses of RTS,S/AS01B yielded equivalent anti-CSP antibodies and a 16-fold greater T-cell response to peptides representing the CSP C-terminal region than did 3 doses of RTS,S/AS01B. By comparison, two homologous doses of Ad35.CS yielded only 1/10th the anti-CSP antibodies and a 2.5-fold greater T-cell response to peptides representing the CSP C-terminal region in comparison to 3 doses of RTS,S/AS01B.

In conclusion, we have demonstrated potent immune responses to *P. falciparum* CSP induced by immunization of mice with Ad5.CS, Ad35.CS and RTS,S/AS01B vaccine candidates. The protective efficacy of these candidates in combination has not yet been determined in humans.

Disclosures

Jaap Goudsmit, Maria Grazia Pau, Olga Ophorst, and Jerome H.V. Custers are employees of Crucell Holland BV, manufacturer of Ad5.CS and Ad35.CS. Joe Cohen, Marie-Claude Dubois, and Marie-Ange Demoitié are employees of GSK Biologicals, manufacturer of RTS,S and AS01B. Patrice Dubois was an employee of GSK Biologicals at the time of this research and is currently a consultant to GSK Biologicals. V. Ann Stewart, Patrice Dubois, Joe Cohen, D. Gray Heppner, Maria Grazia Pau and Jaap Goudsmit have patents pending for the use of Ad35.CS and RTS,S/AS01B.

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