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Evaluation of a prime-boost vaccine schedule with distinct adenovirus vectors against malaria in rhesus monkeys

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ABSTRACT

A vaccine that elicits both specific antibodies and IFN-γ-producing T cells is required to protect against pre-erythrocytic malaria. Among the most promising approaches to induce such complex immunity are heterologous prime-boost vaccination regimens, in particular ones containing live viral vector. We have demonstrated previously that adenovectors serotype 35 (Ads35) encoding the circumsporozoite (CS) antigen or liver-stage antigen-1 (LSA-1) are highly effective in improving the T-cell responses induced by immunizations with protein-based vaccines in a heterologous prime-boost schedule. Here we evaluated the potential of a heterologous prime-boost vaccination that combines the Ad35.CS vector with the serologically distinct adenovector Ad5.CS, in rhesus macaques, after establishing the potency in mice. We show that the heterologous Ad35.CS/Ad5.CS prime-boost regimen elicits both antibody responses and robust IFN-γ-producing $CD8^+$ T-cell responses against the CS antigen. Analysis of the quality of the antibody responses in rhesus macaques, using indirect immunofluorescence assay (IFA) with *Plasmodium falciparum*-coated slides, demonstrated that this heterologous prime-boost regimen elicits a high titer of antibodies that are able to bind to *P. falciparum* sporozoites. Level of the IFA response was superior to the response measured with sera of an adult human population living in endemic malaria region. In conclusion, the combination of Ad35.CS, a vaccine based on a rare serotype adenovirus, with Ad5.CS or possibly another adenovector of a distinct serotype, induces a complex immune response that is required for protection against malaria, and is thus a highly promising approach for pediatric vaccination.

1. Introduction

Every year malaria kills one million people, from which a large majority are children under 5 years of age [1]. Pediatric vaccination against malaria is thus desperately needed. Although the feasibility of a malaria vaccine was demonstrated using irradiated sporozoites more than 30 years ago [2], an effective malaria vaccine has not been generated as yet. The most advanced of the malaria vaccine candidates currently being tested in human clinical trials is the pre-erythrocytic circumsporozoite (CS)-protein-based vaccine RTS,S, formulated with AS adjuvants. Albeit this vaccine demonstrated only a limited protection in field trials [3–7], a large phase III trial has been initiated and, provided that positive results are achieved, the licensure of the vaccine is expected in a few years. The limited protection induced with RTS,S is probably due to the vaccine failure to induce $CD8^+$ T-cell responses while eliciting strong humoral immunity, a feature attributable to protein-based vaccines in general [8,9]. The results obtained with RTS,S support the feasibility of a pre-erythrocytic vaccine based on the CS antigen, but also reinforce the vision that an efficacious vaccination against the liver-stage malaria will unquestionably require induction of both antibody responses and cellular immunity.

Heterologous prime-boost vaccinations involving viral vectors, specifically designed to elicit both antibody and T-cell responses, are being evaluated for malaria [10–13]. Rationale for this approach is based on the notion that different types of vaccines engage different antigen-processing pathways, with heterologous combinations efficiently stimulating multiple components of the immune system. Among the heterologous prime-boost combinations, schedules involving adenoviral vectors are particularly interesting. The appealing attributes of adenovectors for vaccination lay in their safety and their ability to infect a broad range of both actively

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dividing as well as non-dividing mammalian cells, to express high levels of the genes incorporated and to induce potent antibody and cellular responses when administered by different immunization routes [14–17]. An additional important advantage is the availability of a mature industrial manufacturing platform based on the PER.C6® cell line, which enables cost-effective production of sufficient vaccine dosages for mass vaccination [18,19]. Adenovectors have proven successful when combined in heterologous prime-boost regimens with other type of vaccines, such as DNA [20–22], poxvirus vectors [23,24] or BCG [25,26]. In recent animal studies we have demonstrated the advantage of combining adenovectors with protein-based vaccines. In a non-human primate study, immunizations combining the adenovector serotype 35 expressing the CS antigen (Ad35.CS) and RTS,S were highly effective in eliciting strong and sustainable T-cell as well as antibody responses [10]. The superiority of the heterologous combination with Ad35 and protein versus homologous counterparts has been corroborated in a mouse study with another malarial antigen, the liver-stage antigen-1 (LSA-1) [11].

Recently, promising heterologous prime-boost regimens that combine SIV antigen-expressing adenovectors of different serotypes have been described, demonstrating an advantage of the heterologous combination in eliciting a potent T-cell immunity with a broad polyfunctional phenotype and a protection in a stringent rhesus macaques challenge model [27].

In the current study, we have evaluated the ability of a heterologous prime-boost schedule using two serologically distinct adenovectors, Ad35 (B type) and Ad5 (C type), expressing *Plasmodium falciparum* CS antigen to elicit the humoral and cellular immune responses that are required for protection against malaria.

2. Materials and methods

2.1. Adenoviral vectors and immunizations

The E1/E3-deleted rAd35 and rAd5 adenovectors encoding for the *P. falciparum* CS antigen were generated using a procedure described elsewhere [19,28,29]. The CS gene is a synthetic codon-optimized insert encoding a CS protein based on the EMBL protein sequence CAH04007, and truncated for the last 14 amino acids at the C-terminus [30]. The N-terminus of this CS protein is a consensus assembled by alignment of various sequences present in the GenBank, while the repeat region and the C-terminus is based on the sequence of the 3D7 *P. falciparum* clone.

The potency of the heterologous Ad35.CS/Ad5.CS prime-boost regimen was first determined in mice, followed by evaluation of immunogenicity in rhesus macaques. For the mouse studies, BALB/c female mice, 6–8 weeks old, were purchased from Harlan (Zeist, The Netherlands) and kept at the Animal Facility of Crucell Holland B.V. under specified pathogen-free conditions. Institutional Committees for Animal Care and Use reviewed and approved the mouse experiments. The heterologous prime-boost regimen in mice consisted of a prime immunization with Ad35.CS followed by a boost with Ad5.CS (prime-boost group, eight mice). As control for boosting, a group of mice received a prime with Ad35.CS followed by an immunization with an empty (without the transgene) adenovector (AdE) (prime-only group, eight mice). An additional group of mice received two immunizations with the corresponding empty adenovectors (negative control group, 4 mice). A homologous prime-boost control group, receiving two subsequent immunizations with the Ad35.CS vector, would have been ideal as comparator group for the heterologous regimen. However, as published in earlier studies such type of homologous prime-boost immunization regimen is not feasible in mice due to the high levels of adenovector neutralizing antibodies that are induced upon the prime immunization, which hamper the effect of the boost immunization [29,31,32]. Mice were intramuscularly (IM) immunized, at 0 and 4 weeks, with 10^9 vp of the adenovectors prepared in a total volume of 100 μl of PBS containing 5% sucrose (50 μl/quadriceps of hind legs). Two weeks after the last immunization sera and spleen cells were collected for determination of the CS-specific antibody and T-cell responses.

For evaluation of immunogenicity of heterologous Ad35.CS/Ad5.CS prime-boost regimen in the non-human primates, rhesus macaques (6 animals) were selected from an available animal pool based on following criteria: (i) physical exam demonstrating a good health, (ii) no prior exposure to malaria and malaria antigens (no background in CS-specific immunological assay), and (iii) no detectable neutralizing antibodies against Ad35 or Ad5 in serum. All animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* [33], and with the approval of the Institutional Animal Care and Use Committee of Harvard Medical School. Animals were housed in quarantine for 5 weeks, to allow complete acclimatization of animals prior to vaccination. Rhesus macaques were immunized twice, at 0 and 12 weeks, with 10^{11} vp of Ad35.CS and at 25 weeks with 10^{11} vp of Ad5.CS. The adenovectors were prepared in 0.5 ml of PBS containing 5% sucrose. Upon immunizations, animals were monitored by relevantly trained personnel for vaccination-related adverse effects, including monitoring of behaviour and body weight (daily for 1 week after each vaccination), hematology and clinical chemistry analysis (at 1, 3, 7 and 14 days after each vaccination). Adverse effects were neither observed after Ad35.CS nor after Ad5.CS vaccination (*data not shown*). At defined time points upon immunizations, serum and peripheral blood mononuclear cells (PBMC) were collected for evaluation of the CS-specific antibody and T-cell responses and the neutralizing antibodies against the adenovectors.

2.2. Human serum samples

Liberian human serum samples from healthy adults (>15 years) living in a *P. falciparum* malaria holoendemic and perennial area were kindly provided by Professor Marita Troye-Blomberg (Stockholm University, Sweden).

2.3. Neutralization assays

The neutralizing antibodies induced against the adenovectors were determine using neutralizations assays for Ad35 and Ad5 adenovectors as previously described [34]. Briefly, 1/32 diluted sera were added to 96-wells flat-bottom microtiter plates (Greiner Bio-one, Alpen a/d Rijn, The Netherlands) and serially two-fold-diluted. Recombinant adenovirus containing the luciferase reporter gene (rAd35.Luc, rAd5.Luc) and A549 cells were added to the plates. The luciferase reporter gene expression in the A549 cells was measured using luciferase substrate (PerkinElmer, Waltham MA) and a Trilux luminescence detector (according to the manufacturer's instructions). Data was analyzed using non-linear regression to calculate the antibody inhibitory concentration of 90% (IC_{90}) in the sera samples.

2.4. CS-specific ELISA

The CS-specific antibody responses were measured using ELISA. The ELISA procedure used for the mouse samples was performed as described elsewhere [35]. Ninety-six-well microtiter plates (Maxisorp, Nunc) were coated overnight at 4 °C with 2 μg/ml of CS specific peptide $(NANP)_6C$ in 0.05 M carbonate buffer. After washing the plates, 1:100 diluted individual serum samples were added to the wells and serially two-fold-diluted. Plates were incubated for

2 h at room temperature. Plates were washed again and incubated with biotin-labelled anti-mouse IgG (Dako, Denmark) and afterwards with HRP-conjugated streptavidin (Pharmingen San Diego, CA), 30 min at 37 °C each. Finally, the plates were washed and 100 μl of o-phenylenediamine dihydrochloride (OPD) substrate (Pierce, Rockford, IL) was added to each well. After 10 min, the reaction was stopped by adding 100 μl/well of 1 M H_2SO_4. The optical density (OD) was measured at 492 nm using a Bio-Tek reader (Bio-Tek Instruments, Winooski, VT). In the case of the rhesus macaques and human samples a similar procedure was performed, with a few modifications. Dilution of serum samples was performed using blocking buffer containing 1% normal human sera (Harlan, Horst, The Netherlands). As detection antibody we used an alkaline phosphatase-conjugated goat anti-human IgG antibody (Pierce, Rockford, IL) that was cross-reactive with non-human primate antibodies. ELISA plates were developed using *p*-Nitrophenyl Phosphate, Disodium Salt (PNPP) substrate (Pierce, Rockford, IL). The optical density (OD) was measured at 405 nm using a Bio-Tek reader (Bio-Tek Instruments, Winooski, VT). In all ELISA assays the sera obtained from animals before vaccinations were used as negative control for the assay specificity. As expected, these control sera were found to be negative.

2.5. Indirect immunofluorescence assay (IFA)

The binding of vaccination-induced antibodies to the native CS protein on the surface of *P. faciparum* sporozoites was evaluated using IFA as described elsewhere [35]. Briefly, 20 μl of twofold serially diluted serum samples were added to air-dried NF-54 *P. falciparum* sporozoite-coated multi-spot slides and incubated for 30 min at room temperature. Excess serum was removed by washing three times for 5 min with PBS pH 7.2. Detection of bound antibodies was performed using a FITC-conjugated goat anti-human IgG antibody (Pierce, Rockford, IL). After incubation for 30 min the excess of antibody was removed by washing as described above and slides were air-dried and cover-slipped using mounting fluid (Dako Cytomation). The slides were directly analyzed using an Axioplan 2 Imaging microscope (Zeiss). IFA titter was defined as the highest dilution of the serum with which the sporozoites were visible. Sera obtained from animals before vaccinations were used as negative control for IFA specificity. As expected, these control sera were found negative in the IFA.

2.6. T-cell assays

The CS-specific T-cell responses were evaluated using IFN-γ ELISPOT and intracellular cytokine staining (ICS) as described previously [11,36]. For the stimulation of cells a CS total pool, consisting of 59 overlapping 15-mer peptides covering the whole sequence of the CS protein, was used. As negative control of the stimulation, cells from individual animals were incubated with medium alone.

For the ELISPOT assay, 96-well multiscreen plates (Millipore, Bedford, MA) were coated overnight with a rat anti-mouse IFNγ antibody (Pharmingen, San Diego, CA) or with a rabbit anti-human IFNγ antibody (B27; Pharmingen, San Diego, CA). After washing plates were blocked with D-PBS containing 5% FBS for 2 h at 37 °C. Cells from individual animals were then stimulated for 18 h at 37 °C in a 5% CO_2 environment. Following incubation, the plates were washed and incubated for 1.5 h with a biotinylated rat anti-mouse IFNγ antibody (Pharmingen, San Diego, CA) or with a biotinylated rabbit anti-human IFNγ antibody (Biosource). Plates were washed and incubated for 1.5 h with streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The specific staining was developed with nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce, Rockford, IL), stopped by washing with tap water, air-dried, and analyzed using an AELVIS ELISPOT reader (AELVIS GmbH). Background levels of cytokine production shown by the negative controls were around the 55 spots/10^6 PBMC.

In the case of ICS assay, cells from individual animals were stimulated with the CS-specific peptide pool or cultured with medium alone, in the presence of anti-CD49d and anti-CD28 (BD Biosciences). After 1 h of incubation, monensin (GolgiStop; BD Biosciences) was added to all cultures and further incubation was performed for 5 h. After washing, cultured cells were stained with monoclonal antibodies specific for cell surface molecules (CD4 and CD8). After fixing with Cytofix/Cytoperm solution (BD Biosciences), cells were permeabilized and stained with antibodies specific for IFNγ. Approximately 200,000–1,000,000 events were collected per sample. The background level of cytokine staining varied from sample to sample, but was typically less than 0.01% of the $CD4^+$ T cells and less than 0.05% of the $CD8^+$ T cells.

2.7. Statistical analysis

All variables have been log transformed prior to the analyses, to account for the log normal distribution of the observations. Statistical analysis for Figs. 1 and 5 was performed using SPSS version 15.0. Experimental groups were compared using student *t*-test, upon log transformation of the data. Differences were considered significant when $p < 0.05$. In the case of Figs. 2–4, all results have been analyzed using a mixed model, with successive measurements within each subject while accounting for the correlation between successive observations. In this case, all computations were conducted in SAS version 9.1 (procedures PROC MIXED, PROC NLIN and PROC ROBUSTREG).

3. Results

3.1. Potency of the Ad35.CS/Ad5.CS heterologous prime-boost regimen in mice

We first determined the immunogenicity of the heterologous Ad35.CS/Ad5.CS prime-boost regimen in mice. For this purpose, a group of mice was primed with Ad35.CS and boosted with Ad5.CS (prime-boost), another group of mice was primed with Ad35.CS and boosted with an empty adenovector (prime only) and a third group received two consecutive immunizations with empty vectors (negative control). In the case of the mouse study the heterologous regimen involved only one immunization with Ad35.CS, instead of two immunizations. Our choice for this regimen in mice was based upon previous studies showing that a single immunization of mice with Ad35 induces considerably high anti-vector responses, which hamper the effect of a second vaccination with this same vector [29,31,32]. The Ad35.CS/Ad5.CS prime-boost regimen elicited CS-specific antibody responses that were significantly higher ($p = 0.005$) than responses induced by the prime-only immunization. In line with the antibody responses, analysis of the CS-specific IFN-γ-producing T-cell responses by ELISPOT (Fig. 1B) showed superior ($p < 0.001$) responses in the Ad35.CS/Ad5.CS prime-boost group compare to the prime-only group. Further evaluation of the T-cell responses using ICS (Fig. 1C and D) revealed that the CS-specific IFN-γ producing T cells induced upon vaccination with either with prime-only or prime-boost schedule were primarily $CD8^+$ cells. CS-specific IFN-γ producing $CD4^+$ cells were also detected, but at very low levels. Overall, the data indicate that the potency of the prime-boost combination of Ad35.CS with Ad5.CS was higher than the single immunization with Ad35.CS, thus improving the CS-specific immune responses.

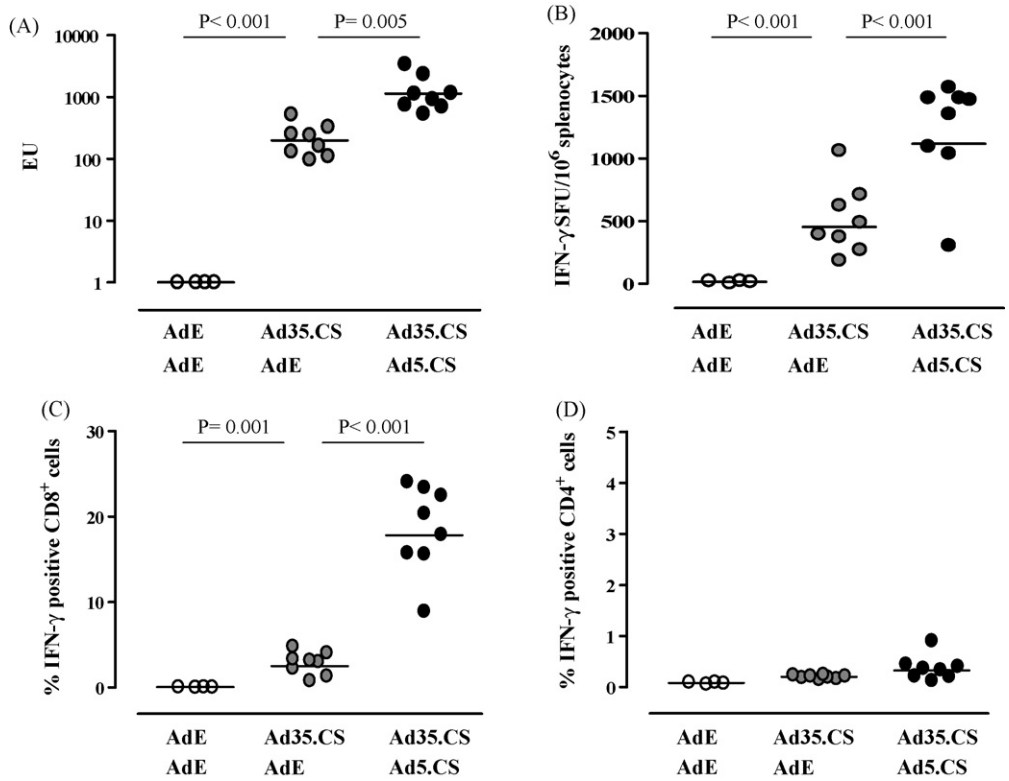

Fig. 1. Evaluation of the heterologous Ad35.CS/Ad5.CS prime-boost regimen in mice. For the prime-boost regimen, a group of mice (prime-boost group, eight mice) was primed with Ad35.CS followed by a boost with Ad5.CS. As control for boosting, a group of mice (prime-only group, eight mice) was primed with Ad35.CS and later injected with an empty adenovector (AdE). The negative control group (negative control group, 4 mice) received two immunizations with empty adenovectors. Mice were immunized I.M. at 0 and 4 weeks with 10^9 vp of the corresponding adenovectors. Two weeks after the last immunization the CS-specific antibody and T-cell responses were assessed using ELISA (A), IFN-γ ELISPOT (B) and ICS (C and D). The bars represent the geometric means of the EU (A), SFU (B) and percentage of $CD8^+$ cells (C) or $CD4^+$ cells (D). EU = ELISA Unit; SFU = spot forming units; *P* = statistical significance between vaccination schedules.

3.2. Immunogenicity of the Ad35.CS/Ad5.CS heterologous prime-boost regimen in rhesus macaques

3.2.1. Study design and screening

We next evaluated the ability of the heterologous Ad35.CS/Ad5.CS prime-boost regimen to induce CS-specific T-cell and antibody responses in non-human primates. A group of 6 rhesus macaques received two consecutive immunizations with Ad35.CS 12 weeks apart, followed by an Ad5.CS immunization at week 25. Prior to immunizations, all available animals were pre-screened for neutralizing antibodies against Ad35 or Ad5, for ELISA antibodies against CS, and for a background in the ELISPOT assay upon stimulation with the CS peptide pool (*data not shown*). Only seronegative monkeys, with no background in ELISPOT (less than 55 spots/10^6 PBMC), were selected for the study. At different time points upon immunizations serum and peripheral blood mononuclear cells (PBMC) were collected for evaluation of the induced immune responses against the CS antigen and the adenovectors. The chosen immunization schedule was based on a previous non-human primate study [10] showing induction of immune responses when following this set-up. We have performed an analysis of immune response at numerous time points, in order to obtain an insight into the kinetics of the immune response

3.2.2. Ad35 and Ad5 neutralizing antibodies induced by the prime-boost regimen

The neutralizing antibody titers against the adenoviral vectors were measured using Ad35 and Ad5 neutralization assays, at several time points throughout the study, both to confirm the vaccine uptake and to evaluate feasibility of second immunization with Ad35. As depicted in Fig. 2, the first vaccination with Ad35.CS (Ad35 prime) evoked low titers of Ad35 neutralizing antibodies (mean titer 143 at 2 weeks), indicating the feasibility of a second vaccination with the same vector. Upon the second Ad35.CS vaccination (Ad35 boost) the levels of neutralizing antibodies increased significantly showing maximal values at 14 weeks time point (mean titer 8460). This increase was transient, as the Ad35 neutralizing antibodies decreased towards the 37 weeks time point (mean titer 562). However, the raise of Ad35 neutralizing antibodies observed upon the second Ad35.CS vaccination indicated that a third vaccination with the same vector at week 25 might not be successful due to possible neutralization of Ad35 vaccine. In contrast to the Ad35 vector, a single immunization with Ad5.CS (Ad5 boost) induced a

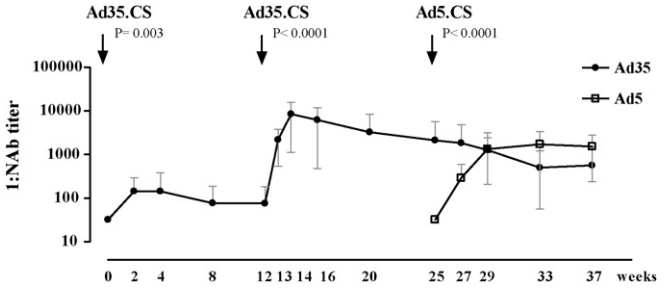

Fig. 2. Neutralizing antibody responses against Ad35 and Ad5 adenovectors induced in rhesus macaques by the heterologous prime-boost regimen. Rhesus macaques (6 animals) were immunized twice, at 0 and 12 weeks, with 10^{11} vp of Ad35.CS and at 25 weeks with 10^{11} vp of Ad5.CS. At defined time points upon immunizations serum was collected for evaluation of the anti-vectors responses. Results are expressed as the mean of the NAb titters from 6 animals and bars represent the standard deviation. NAb = neutralizing antibodies; *P* = statistical significance versus week 0 or the previous vaccination.

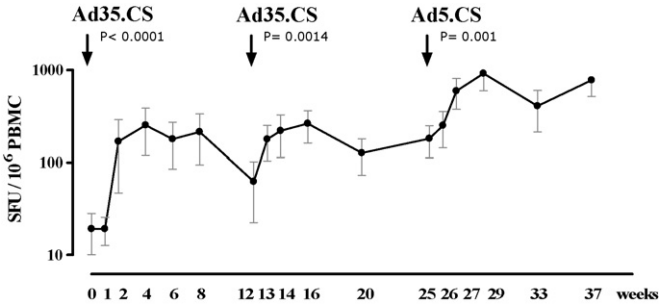

Fig. 3. T-cell responses induced by the heterologous Ad35.CS/Ad5.CS prime-boost in rhesus macaques. Rhesus macaques (6 animals) were immunized twice, at 0 and 12 weeks, with 10^{11} vp of Ad35.CS and at 25 weeks with 10^{11} vp of Ad5.CS. At defined time points upon immunizations PBMCs were collected for evaluation of the T-cell responses using IFN-γ ELISPOT. Results are expressed as the mean SFU from 6 animals and bars represent the standard deviation. SFU = spot forming units; *P* = statistical significance versus week 0 or the previous vaccination.

high titer of Ad5 neutralizing antibodies that reached maximal values at 29 weeks time point (mean titer 1329) and remained stable towards the later time points

3.2.3. Ad35.CS/Ad5.CS prime-boost regimen induces CS-specific IFN-γ-producing T cells

At several time points throughout the study we have determined CS-specific IFN-γ-producing T cells, an important component of the protective response against malaria [37] using the ELISPOT assay. As illustrated in Fig. 3 the Ad35.CS prime immunization induced a significant number of the IFN-γ-producing T cells (mean = 253 spots per 10^6 PBMC at week 4, *p* versus week 0 <0.0001). The induced T-cell response remained high up to week 8, after which a decrease of the response was detected. The boost vaccination with Ad35.CS restored the T-cell response which remained relatively stable for approximately 12 weeks after the Ad5.CS boost. The heterologous boost with Ad5.CS elicited a tremendous increase of the IFN-γ producing T cells (mean = 922 spots per 10^6 PBMC at week 29, *p* versus prior immunization = 0.0009), which corroborated the capacity of this vector to boost the CS-specific responses elicited with two vaccinations with Ad35.CS. The T-cell response only slightly decreased towards week 37 (mean = 781 spots per 10^6 PBMC) indicating long duration of the response.

3.2.4. The heterologous prime-boost regimen with Ad35.CS and Ad5.CS induces high CS-specific antibody responses.

In Fig. 4, the titer of CS-specific antibodies, as induced during the course of immunizations with Ad35.CS and Ad5.CS, is depicted.

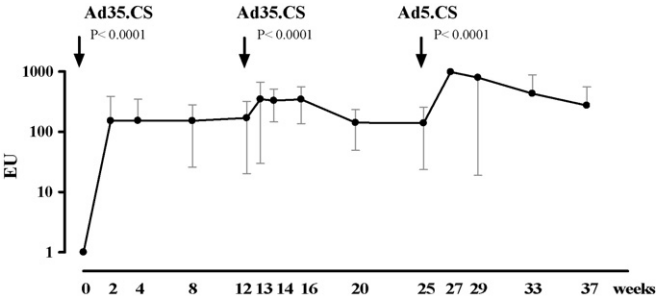

Fig. 4. CS-specific antibody responses induced by the heterologous Ad35.CS/Ad5.CS prime-boost regimen in rhesus macaques. Rhesus macaques (6 animals) were immunized twice, at 0 and 12 weeks, with 10^{11} vp of Ad35.CS and at 25 weeks with 10^{11} vp of Ad5.CS. At defined time points upon immunizations serum was collected for evaluation of the CS-specific antibody response using ELISA. Results are expressed as the mean EU response from 6 animals and bars represent the standard deviation. EU = ELISA Unit; *P* = statistical significance versus week 0 or the previous vaccination.

It is clear that already a single immunization with Ad35.CS evoked significant CS antibody responses, which reached the peak at two weeks after immunization (mean EU = 153 at week 4, *p* versus prior immunization <0.0001). The levels of the CS antibody response were maintained until the 12 weeks time point, when the Ad35 boost immunization lead to a further increase of the response (mean EU = 347 at week 16, *p* versus prior immunization <0.0001). The Ad5.CS boost vaccination elicited a substantial increase of the CS antibody responses, reaching the maximum level 2 weeks after the immunization (mean titer = 988 EU, *p* versus prior immunization <0.0001). The CS-specific antibody response decreased only about half log towards week 37 (mean titer = 274 EU). This result demonstrates the ability of the Ad5.CS vector to further augment CS antibody responses achieved with two vaccinations with Ad35.CS.

3.2.5. The CS antibody titers induced by the heterologous regimen are higher than the titers found in sera of individuals from malaria endemic area

People living in malaria endemic areas gradually acquire immunity to malaria parasite that controls high parasitaemia and protects from severe malaria. Although correlates of protection against malaria are not known, some epidemiological studies have shown an association between CS-specific antibodies and protection against the disease [38,39]. In order to estimate the significance of the CS antibody titers in sera of immunized rhesus macaques, we compared them with the CS-specific titers in sera of adults from a malaria endemic region (Liberia) using ELISA and IFA assays (Fig. 5). As shown in Fig. 5A, the ELISA assay that was used is suitable both for human and non-human primate sera. Analysis of the CS responses in human and non-human primate sera by ELISA (Fig. 5B) shows that CS titers induced upon two immunizations with Ad35.CS in rhesus macaques were comparable to the CS titers in the human samples from Liberia. Importantly, the heterologous Ad5.CS immunization boosted CS titers to a significantly higher level ($p = 0.005$) than the titers found in human sera. Using the IFA method, we determined the ability of the CS-antibodies to bind the native CS protein on the surface of P. falciparum sporozoites, in selected human and non-human primate sera (Fig. 5B). The IFA titer induced after one Ad35.CS immunization was somewhat lower (1/100) than the titer measured in the human serum (1/200). After the Ad35.CS boost immunization, the IFA titer was significantly increased and clearly higher (1/1600) than the titer of the human sample. The Ad5.CS boost further increased the IFA titer (1/3200) in non-human primate serum. Overall these results indicated that the heterologous Ad35.CS/Ad5.CS prime-boost regimen induces CS-antibodies able to bind *P. falciparum* sporozoites.

4. Discussion

High-level protection against malaria requires induction of a strong and complex cellular and humoral immunity, which most probably can only be achieved by heterologous prime-boost vaccinations. In this study we demonstrate the ability of a heterologous prime-boost regimen that combines the Ad35 (type B vector) and Ad5 (type C vector) adenovectors, expressing the malaria pre-erythrocytic CS antigen, to induce a potent antigen-specific IFN-γ-producing T-cell response. In addition, we show that this heterologous prime-boost regimen evokes significant levels of CS-specific antibodies, which are able to bind *P. falciparum* sporozoites.

One of the major obstacles for the development of a vaccine against malaria is the lack of immune correlates of protection. Thus far, studies in mice have indicated that IFN-γ producing $CD8^+$ T cells and antibodies play a role in protection against liver-stage parasites [37,40]. In humans, immunity induced by the most protective malaria vaccine so far, the irradiated sporozoites, have been

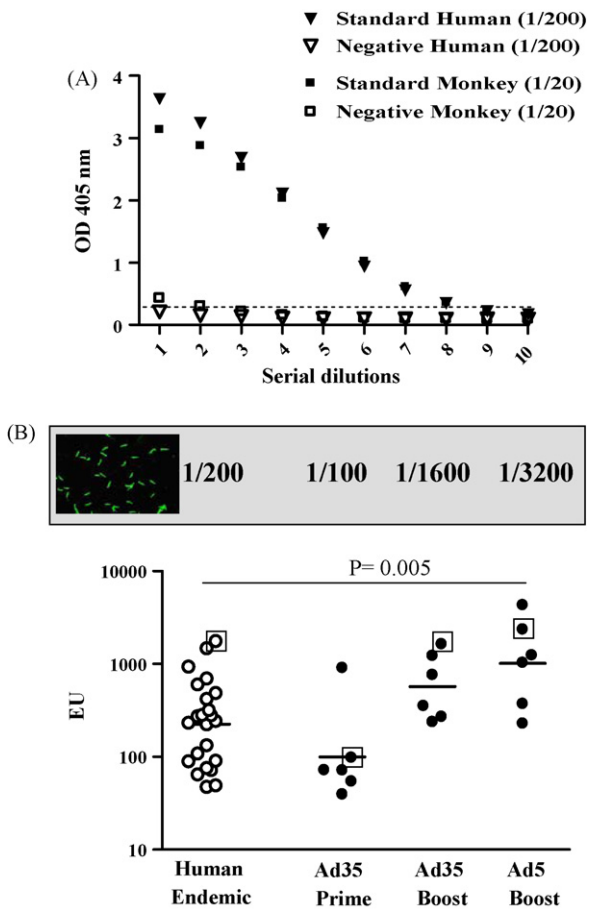

Fig. 5. Comparison of the CS-specfic antibody responses between the rhesus macaque sera samples and human samples from an endemic area. Rhesus macaque sera samples from weeks 4, 16 and 29 were compared to sera samples from Liberian malaria-immune adults using ELISA and IFA (selected sera). Bars represent the geometric means of the EU. The results from the IFA analysis are expressed as IFA titters. Rectangular indicates sera selected for the IFA. EU = ELISA Unit; *P* = statistical significance versus human sera.

associated with cellular [41] and antibody responses [42]. Recently, a CD4$^+$ T-cell epitope from *P. falciparum* CS antigen was reported to be strongly associated with protection of humans from malaria infection and disease, using a cultured ELISPOT assay [43]. However, although promising, this association has not been confirmed in other clinical trials. It is well accepted that both IFN-γ-producing CD8$^+$ and CD4$^+$ T cells are required for clearance of parasite-infected hepatocytes [44].

Immunizations with adenoviral vectors induce a high level of antigen-specific T-cell response and, in addition, different adenoviral serotypes elicit qualitatively distinct T cells [27]. This phenomenon can be explained by the differences in receptor usage, tissue tropism and different intracellular processing of distinct adenoviral vectors [45–49]. These differences lead to the activation of different type of cells of the immune system at different locations, which ultimately results in the induction of different type of immune responses. Accordingly, the combination of adenovectors of different serotypes in heterologous prime-boost vaccinations can result in the stimulation of multiple immune pathways, which can synergistically enhance the immune response to target antigens. In our study the analysis of the T-cell responses showed that immunizations with Ad35.CS efficiently induced CS-specific IFN-γ-producing T cells in both mice and rhesus macaques. Importantly, the heterologous boost with Ad5.CS induced a further substantial enhancement of the CS-specific T-cell responses which in non-human primates remained relatively stable for at least 3 months after the boost. The number of CS-specific IFN-γ-producing T cells induced with Ad35.CS/Ad5.CS at 4 weeks post-boost was comparable to the response measured at the same time point in our earlier study [10] wherein a heterologous prime-boost immunization with Ad35.CS and RTS,S was evaluated. In our current non-human primate study the maximum T-cell response and, in particular, duration of the response were significantly higher. However, it should be mentioned that in the previous study [10] only CS C-terminal 15-mer peptides were used for the *in vitro* stimulation in ELISPOT while in current study the complete CS peptide pool was used. Future studies are needed to confirm that boosting with Ad5.CS induced qualitatively different T-cell response in rhesus macaques. In mice, the heterologous Ad35.CS/Ad5.CS prime-boost regimen activated mainly antigen-specific CD8$^+$ T cells, although CD4$^+$ T cells were also induced at low frequency. The poor induction of CD4$^+$ T cells seen in our study might be due to the use of peptide stimulations (15-mers) for T-cell read-out instead of a protein stimulation, which is known to induce a more robust stimulation of CD4$^+$ T cells as compared to stimulations with peptides [50].

The combination of other type viral vectors, such as fowlpoxvirus (FP9) and modified vaccinia virus Ankara (MVA), has been evaluated in a heterologous prime-boost regimen for malaria. In a recent study, the heterologous prime-boost combination using FP9 and MVA expressing the Multiple Epitope string and Thrombospondin Related Adhesion Protein (ME-TRAP) antigen was shown to elicit T-cell responses and sterile protection in two out of five malaria-naïve volunteers upon experimental challenge [51]. The protection in one of these individual lasted for 20 months and it was associated with the persisting memory T cells. However, the same combination failed to protect children in Kenya [52,53]. A possible explanation for this outcome might be that the FP9/MVA viral vector combination, albeit inducing T-cell responses, does not efficiently induce antibody responses [51,54], which are also required for protection against the malaria pre-erythrocytic stage.

A number of epidemiological studies have reported an association between antibodies against various pre-erythrocytic antigens, such as the CS antigen, and protection from malaria infection and disease [38,39]. In addition, clinical studies with the RTS,S vaccine candidate have reported an association of CS-specific antibodies with prevention of parasitemia after malaria challenge [8,55,56]. In our study, analysis of the antibody responses using ELISA showed that immunizations with the Ad35.CS adenovector efficiently induce potent CS-specific responses. These results were consistent with our previous study showing the induction of reasonable levels of CS-specific antibodies in rhesus macaques upon two homologous immunizations with the same vector [10]. In that study heterologous boosting of the Ad35.CS-induced response with the RTS,S vaccine resulted in an enhancement of the antibody response up to 7–10-fold. This antibody level was not statistically different from the response induced by homologous immunizations with RTS,S. In the current study we demonstrate a close to 10-fold increase in the level of CS-specific antibody response induced by the heterologous Ad35.CS/Ad5.CS prime-boost regimen in rhesus macaques, as compared to the response achieved upon two immunizations with Ad35.CS. In agreement with the previous study [10], antibody titers induced with adenovector vaccines remained relatively stable and decreased for only about half log 3 months after the boost.

Epidemiological studies have demonstrated that people living in malaria endemic areas, with intense and recurrent malaria transmission, develop gradually acquired immunity against the disease [57–59]. We compared the CS-specific antibody responses elicited by the Ad35.CS/Ad5.CS heterologous prime-boost in rhesus macaques, using ELISA and IFA assays, to the responses exhibited

by malaria-immune adults living in Liberia, a malaria endemic area where transmission occurs nearly throughout the entire year [60]. Malaria in Liberia is a leading cause of morbidity and mortality and claims every year at least 21,000 deaths among the children under 5 years of age. Our results showed that the CS-specific antibody response induced by the Ad35.CS/Ad5.CS prime-boost regimen was significantly higher than the CS antibody response of the endemic human sera samples, not only in the magnitude of the response but also in the ability of the antibodies to recognize and bind the *P. falciparum* parasites. In a recent RTS,S clinical trial the antibody responses of vaccinees was assessed using IFA [8]. Results showed that the majority of the RTS,S vaccinated individuals induced titters ranging from 1600 to 6400. In our study we demonstrate the capability of the Ad35.CS/Ad5.CS heterologous prime-boost to evoke antibodies of comparable IFA titters, as two consecutive vaccinations with Ad35.CS elicited a titter of 1600 and the heterologous boost with Ad5.CS raised the titter to 3200.

Neutralizing antibodies against Ad35 are rare in humans worldwide and, if present, then in general only at a low titer [28,61–63]. Contrary, the neutralizing antibodies against Ad5 are highly prevalent, and at high titer, particularly in Africa [62,63], where the large majority of malaria cases occur. It has been demonstrated earlier that high titers of Ad5 neutralizing antibodies decrease vaccine efficacy, thus severely impeding vaccination regimens that include Ad5 vector [31,32,62]. Recently, we assessed the seroprevalence of neutralizing antibodies against different adenovectors, including Ad35 and Ad5, in pediatric populations in sub-Saharan Africa [64] and demonstrated an age dependence of the seropositivity for neutralizing antibodies to adenovectors. Virtually all infants were negative for neutralizing antibodies to Ad35 from 6 months to 2 years of age, and the seroprevalence increased only slightly (up to 2.6%) between 2 and 7 years of age, with a low geomean serum titer (GMT range 16–200). Contrary, more than 90% newborns exhibited neutralizing antibodies against Ad5 with relatively high titers (48% infants exhibited GMT >1000), due to the passive transport of maternal antibodies to infant, as indicated by the high correlation between mother and infant titers (correlation coefficient $R^2 = 0.83$). The seroprevalence of Ad5 neutralizing antibodies declined substantially to just above 10% in children between 6 months and 1 year of age, as expected for the maternal immunoglobulines. From the first year on, the seroprevalence of Ad5 neutralizing antibodies in children rose, due to encounter with natural Ad5 viral infections, and reached around 30% at the age of 2 years. Overall, these results demonstrated that, while Ad35-based vaccines can be administered to infants of any age without an issue of pre-existing immunity, for Ad5-based vaccines there is a "window" of low pre-existing immunity at age of 6 months to 1.5 years, in which Ad5-based vaccines can be administered.

In summary, we describe a promising vaccination strategy for malaria whereby the combination of two serologically distinct adenovectors, Ad35.CS and Ad5.CS, in a heterologous prime-boost regimen results in the stimulation of significant CS-specific IFN-γ producing T cells and antibodies that are capable of binding *P. falciparum* sporozoites. Importantly, this antibody response as measured in IFA is comparable to the response seen for the most advanced malaria vaccine candidate RTS,S in humans. Although there is a high prevalence of neutralizing antibodies against Ad5 in adult human population, due to encountered infections, and in the newborns, due to passively transferred maternal antibodies, the infants between 6 months and 2 years of age exhibit a low prevalence of the Ad5 neutralizing antibodies. This "window of opportunity" for application of Ad5-based vaccines offers possibilities for combining Ad35 as a priming vaccine at early age with Ad5 as a boosting vaccine at age between 6 months and 1.5 year, in a schedule that can easily be implemented into the Expanded Program of Immunization (EPI). Currently we are also evaluating the suitability of other adenoviral vectors, in particular rare adenovirus serotypes, to boost Ad35.CS-primed immunity with the aim of extending the utility of this promising vaccination approach to other populations at risk of malaria.

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