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2.2 Expression and immunogenicity of the *P. falciparum* circumsporozoite protein: The role of GPI signal sequence

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SUMMARY

Previous studies have shown that the immunogenicity of rodent malaria parasite-derived circumsporozoite protein (CS) can be improved by deleting the glycosyl-phosphatidylinositol (GPI) signal sequence. To study whether GPI signal sequence deletion would also improve immunogenicity of CS derived from the major plasmodium species causing mortality in humans (*P. falciparum*), we tested different variants of the *P. falciparum* CS protein in the context of a live vector-based vaccine carrier (rAd35). We demonstrate that deletion of the GPI signal sequence from CS did not result in altered expression or secretion. In contrast, cellular localization was clearly altered, which perhaps helps explain the significant improvement of anti-CS antibody and T-cell responses observed in mice using deletion variants in the context of the rAd35 carrier. Our results show that rational design of antigens is warranted for further development of malaria vaccines.

INTRODUCTION

Previously we have reported on the potency of a human recombinant adenovirus type 35 (rAd35)-based vaccine expressing *Plasmodium yoelii* circumsporozoite (CS) antigen. We demonstrated induction of both CS-specific T-cells and antibodies in mice, as well as protection upon high-dose sporozoite challenge [1]. The rAd35 carrier was chosen based on: (i) accumulating data demonstrating that a T-cell response, which is generally induced efficiently by adenovirus [2-6], plays an important role in controlling the malaria parasite [7-9]; and (ii) adenovirus sero-prevalence studies demonstrating that human type 35 is rarely being neutralized with human sera derived from volunteers living in malaria endemic regions [1]. This contrasts with the most commonly used adenoviral vector, rAd5 [10-12].

The *Plasmodium yoelii* CS protein was chosen because vaccination with irradiated sporozoites [13-15], as well as studies in the field [16-23], have demonstrated that CS derived from human *Plasmodium spp.* represents an important immunological target. High and frequent immune responses against this protein have been established in humans.

The *P. yoelii* CS protein used in our earlier studies contained CS protein with partially deleted GPI signal sequence [24]. This followed studies in established mouse malaria models, which had shown that presence of the complete GPI signal sequence in *P. berghei* and *P. yoelii* CS protein led to impaired anti-CS immune responses [25,26].

Subsequent studies have suggested that presence of the GPI signal sequence sequentially affected total CS protein production, cellular distribution, antigen processing and secretion, leading to less effective antigen presentation [26]. The GPI signal sequence is known to serve as a recognition site for transamidases localized in the endoplasmic reticulum (ER), which upon recognition cleaves the C-terminal pro-peptide of the CS proprotein. Subsequently, a GPI anchor is provided and the CS protein is transported and expressed on the cell surface of the *Plasmodium spp.* [27-29]. However, the transamidase reaction is highly species-specific. Studies have shown that parasitic proteins with GPI signal sequence that remain non-cleaved stick in the internal cell organelles of mammalian cells [30]. For the *P. falciparum* species, which causes high mortality in humans it remains to be investigated whether presence or absence of the GPI signal sequence in *P. falciparum* CS protein influences the expression, secretion and antigen presentation.

Therefore, we designed CS proteins carrying two different GPI deletion mutants: one carrying a complete deletion of the GPI signal sequence ($\Delta 28$) and one CS protein carrying a 14 amino acid deletion, thereby deleting only the hydrophobic sequence at the C-terminus [31]. The role of the GPI signal sequence was assessed in the context of the rAd35 carrier, since this allows immediate selection of the most immunogenic *P. falciparum* CS sequence in the context of a potent vaccine. The data obtained demonstrates that deletion of the GPI

signal sequence from the *P. falciparum* results in increases in both B- and T-cell responses, as compared to full length CS protein.

MATERIAL AND METHODES

Adenovirus production and cell transduction

Replication-incompetent Ad35 vectors were generated in PER.C6/55K cells using pBR322-based adaptor plasmid pAdApt535 together with cosmid pWE.Ad35.AfIII-rITRΔE3 as previously described [32]. The adaptor plasmid contained the left portion of the Ad genomes nt 1-464 followed by transcriptional control elements and the adaptor Ad DNA region, nt 3401-4669. The circumsporozoite consensus sequence is gathered by the alignment of different available protein sequences present in the Genbank database.

All CS complete or partial sequences were used in order to identify variation between the different geographical areas and identified laboratory strains to determine the final consensus sequence. The sequence, representing the full length CS protein was optimized for high levels of expression in mammalian cells (GeneArt, Regensburg, Germany) and cloned into the expression cassette in the adaptor plasmid. The resulting pAdapt535-CS plasmid expressed full-length *Plasmodium falciparum* CS under transcriptional control of the human, full-length, immediate-early CMV promoter and the SV40 polyadenylation signal. CS GPI signal sequences deletion mutants were amplified by the polymerase chain reaction (PCR) using pAdapt535-CS plasmid as template. A PCR fragment encoding for aa 1-358 of CS, deleted for the GPI signal sequence was amplified with a Forw Falc.CS 5'-CCAAGCTTGCCACCATGATGAGG-3' (sense) and anti-sense primer Rev Falc.CS-28 5'-CCGGATCCTCAGCAGATCTTCTCTCG-3. The CS sequence partial deleted for the GPI signal (aa 1-372) was amplified with the Forw Falc.CS primer as described above and Rev.Falc.CS-14 5'-CGGATCCTCAGCTG-TTCACCACGTTG-3 respectively. The amplified PCR products were digested with the restriction enzymes HindIII and BamHI and cloned into pAdapt535 and co-transfected into PER.C6/55K[®] cells together with the cosmid pWE.Ad35.AfIII-rITRΔE3. All generated recombinant Ad35 vectors were purified by cesium chloride density centrifugation and vaccine preparations were stored at -80^o C until further use. The virus titer, expressed as the number of virus particles (vp) per milliliter, was determined by high-performance liquid chromatography. Quality control testing of virus batches included identity PCR, absence of mycoplasma, remaining cesium chloride content, bioburden and vp/plaque-forming unit ratio determination.

Cultured human lung carcinoma A549 cells (ATCC) were exposed to 2500 vp/cell rAd35 expressing Pf.CS antigens of different lengths; full length (rAd35Pf.CS FL), deletion of 14 amino acids (rAd35Pf.CS Δ14) or 28 amino acids (rAd35Pf.CS Δ28) at the carboxyl terminal, respectively. Cells were cultured in DMEM culture medium supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1x Penicillin-Streptomycin (Gibco BRL) at 37^o C and 10% CO₂. Every 24 h samples were harvested for in vitro analysis.

Western blotting detection CS and PARP protein

Human lung carcinoma A549 cells (10⁵) were transduced with 2500 vp/cell rAd35 containing Pf.CS antigens or left untreated (control). Cell lysates and corresponding supernatants were harvested and expression levels of proteins were determined by western blotting analysis. Rabbit polyclonal antibodies against Pf.CS (MRA-24, MR4/ATCC) were used in combination with goat-anti-rabbit, IgG, conjugated to horseradish peroxidase (HRP) (Southern Biotechnology Associates) to detect CS expression. Human GAPDH, detected by an anti-GAPDH antibody (Santa Cruz Biotechnology) was used as loading control. For the detection of apoptosis marker poly-(adenosine diphosphate-ribose) polymerase (PARP) ~ 116 kDa protein and ~85kDa (apoptosis-induced cleavage fragment), mouse anti-PARP antibody (Biomol. Int.) was used in combination with rabbit-anti-mouse antibody conjugated to HRP (Dako cytometry). Neuroblastoma cells (IMR32) treated with trichostatin, a deacetylase inhibitor that induces high levels of apoptosis in these cells, were included as a positive control for apoptosis detection [33].

For all blots the detection was performed by the enhanced chemiluminescence assay (ECL⁺, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Flow cytometric analysis of cell viability

The percentage of cells in low DNA content gate, used as an indication of DNA fragmentation [34] was determined by flow cytometry using propidium iodide (PI) DNA staining. Briefly, 10⁶ cells were harvested, fixed in 70% ethanol in phosphate-buffered saline (PBS), and stored at -4^o C until staining with PI. Ethanol-fixed cells were centrifuged (1 min, 2200 rpm), resuspended in 4 mg/ml RNase/PBS, 1 μg/ml PI/PBS containing 0.02% Saponin, and incubated for 20 min at 37^o C. Samples were passed through a 0.7 mm needle to reduce cell aggregation before flow cytometry. The stained cell nuclei were analyzed using FACS caliber (Becton and Dickinson) flow cytometer and WinMD1 version 2.7 software.

Indirect immunofluorescence assays

For the determination of CS specific antibodies in sera of rAd35Pf.CS vaccinated animals, NF-54 *P.falciparum* sporozoites were spotted on multi-spot slides.

Two-fold serial dilution of mice sera was made in PBS pH 7.2 (Gibco BRL) and 20 µl of each serum was incubated with the sporozoites slides for 30 min at room temperature. Excess serum was removed by washing three times for 5 min with PBS pH 7.2. A 1:10 dilution of FITC-conjugated goat anti-mouse Immunoglobulin (Becton Dickinson) was added to each spot for 30 min at room temperature. After incubation 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).

For the detection of CS protein in A549 cells, the cells were transduced with 2500 vp/cell rAd35Pf.CS FL, rAd35Pf.CS Δ14 or rAd35Pf.CS Δ28, respectively, in multi-well chambers (Labtek, Nunc) for 24 h at 37°C. Recombinant Ad35.empty (i.e. adenovector containing no transgene) infected cells were used as negative control. After incubation, cells were washed twice with PBS and fixed for 10 min with 3% paraformaldehyde in PBS (pH 7.4). Fixed samples were washed twice with PBS and cells were permeabilized with 0.1% Triton X-100 (Merck) in PBS for 5 min at room temperature. The excess of Triton X-100 was removed by washing twice with PBS and wells were blocked with PBS supplemented with 1% bovine serum albumine (PBS/1%BSA) for 30 minutes at room temperature. Cells were incubated with a primary rabbit anti-CS polyclonal antibody (MRA-24), diluted 1:250 in PBS/1%BSA. After washing, cells were incubated for 1 h with FITC conjugated goat anti-rabbit IgG (Pharmingen), 1:10 diluted in PBS. Finally after three washing steps with PBS, samples were air-dried and cover-slipped using mounting fluid (Dako Cytomation). The slides were directly analyzed using Axioplan 2 Imaging microscope (Zeiss).

CS protein ELISA

Cell lysates and supernatants of A549 cells were assessed for CS expression using an enzyme-linked immunosorbent assay (ELISA). Briefly, Maxisorp ELISA plates (Nunc) were coated with 1:6400 diluted rabbit anti Pf.CS antibody (MRA-24, MR4/ATCC) in PBS overnight at 4 °C. Plates were washed three times with PBST (PBS with 0.05% Tween-20 (Sigma)) and blocked with 2% bovine serum albumin (BSA) in PBST for 2 h at room temperature. Cell lysates and supernatants were serially diluted in blocking buffer and added to the antibody coated wells for incubation at room temperature for 1 h. The plates were washed with PBST, an appropriate dilution (1:1600) of rat anti-Pf.CS antibody (MRA-21, MR4/ATCC) was added and incubated for 1 h at room temperature. After washing the plates, peroxidase-conjugated goat anti-rat IgG (Santa Cruz biotechnology) was added to the wells and the plates were incubated for 1 hour at room temperature. An *o*-phenylenediamine dihydrochloride (OPD, Sigma) substrate was added, and the optical density at 492 nm (OD492) was determined using an ELISA reader (BioTek instruments). The amount of CS protein expressed (cell lysate) and secreted (supernatant) was quantified using a CS protein standard (MRA-50, MR4 /ATCC) with a detection range of 100 pg -1 µg CS protein/ml. Amounts of CS protein in supernatant (secreted) were calculated relative to the total amount (the sum of CS protein in the cell lysate and the supernatant) and expressed as a percentage of CS secretion. CS secretion below detection limit was arbitrarily given a relative value of 0.01 (1.0%). Non-parametric testing of the relative CS secretion values was performed using Kruskal-Wallis Tests for each time point separately, i.e. we compared the secretion between the FL, Δ14 and Δ28 groups after 24, 48, 72 and 96 hours, respectively. The kinetics of CS secretion was modeled by fitting logit-transformed relative values to a linear mixed model. Time was treated both as a repeated and a fixed effect. The appropriate covariance structure for residual terms was chosen on the basis of Akaike's Information Criterion (AIC).

Mice and Immunizations

Six- to eight-week-old female CBA (H-2K^b) mice purchased from Harlan (Zeist, the Netherlands) were vaccinated intramuscularly (i.m.) in the quadriceps by a single administration of 10⁹ vp rAd35Pf.CS FL, rAd35Pf.CS Δ14 or rAd35Pf.CS Δ28 (eight mice per group). Two or four weeks after vector administration, blood and spleen were isolated to determine vector- and CS-specific immune responses.

Determination of CS-specific cellular and humoral immune responses

The number of CS-specific, interferon gamma-secreting T cells in the spleen of immunized mice was determined with an ELISPOT assay as previously described [5]. The CD8 T-cell immunodominant *P.falciparum* CS peptide DYENDIEKKI (H-2K^b) was used for stimulation. CS-specific humoral response was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, Maxisorp ELISA plates (Nunc) were coated with 2 µg/ml of CS specific peptide (NANP)₄NVDPC in 0.05 M Carbonate buffer. Plates were washed and blocked with 200 µl PBS/1.0 % BSA/0.05% Tween-20 for 1 h at 37 °C. After washing the plates, 100 µl serially twofold-diluted sera in PBS/0.2%BSA/ 0.05%Tween-20 was added to the wells and the plates were incubated for 2 h at 37 °C. The plates were washed and incubated with 100 µl well biotin-labeled goat anti-mouse IgG biotin-conjugated antibodies

(Dako Cytomation) for 1 h at room temperature. Goat anti-mouse IgG1 and anti-mouse IgG2a biotin-conjugated antibodies (Southern biotech) were used to determine isotype of CS antibodies. Finally, the plates were washed and 100 μ l per well *o*-phenylenediamine dihydrochloride (OPD, Sigma) substrate was added to each well and incubated for 10 min at room temperature in the dark. The optical density (OD) of developed staining was measured at 492 nm. The end-point titer was determined as the last dilution at which the OD was higher than three-times background value.

Statistical analysis

Data are presented as (geo) means or medians. Statistical analyses were performed with SPSS version 12.0.1 (SPSS Software, Inc., 2004). Immune responses (logarithmically transformed) among groups of animals were assessed by one-way ANOVA post-hoc LSD. There was no correction for multiple group comparisons.

RESULTS

Generation of CS vaccines carrying GPI signal sequence deletions

To study the role of GPI signal sequence three *P. falciparum* CS sequences were generated. All three sequences (as shown schematically in Fig. 1A) contain the major domains as described for the CS protein from frequently used lab-strain 3D7 (Genbank database, accession numbers CAA33421, CAB38998, CSP_PLAFO, NP_473175).

Also, all three sequences contained the T-helper 2 region (aa 326-345), which contains of a consensus sequence representing a universal T cell epitope [35]. Finally, a mouse H2-K^k restricted T-cell epitope (DYENDIEKKI) was introduced at amino acid position 350, in all three sequences by the substitution of one single amino acid (glutamic acid for alanine), enabling read out of CD8⁺ T-cell immune response upon vaccination. The three CS coding sequences thus only differed in the GPI signal sequence, present as full length (FL), partial deleted (Δ 14aa) or complete deleted (Δ 28aa). The three sequences were cloned into recombinant replication deficient adenoviral vector type 35, and recombinant vaccines were produced to high titer ($> 1 \times 10^{11}$ virus particles per ml) on PER.C6[®] 55K cells [36].

From the resulting vaccines, coded Ad35Pf.CS FL (full length CS), Ad35Pf.CS Δ 14 (deleted hydrophobic part of the GPI signal sequence), or Ad35Pf.CS Δ 28 (GPI signal removed), DNA was isolated to determine presence of the correct insert. Hereto, PCR was performed as schematically shown in Fig. 1B with primers located in the CMV promoter region and within the pIX coding region of rAd35. This PCR thus amplifies the ~ 2 kb CS fragment. Subsequent restriction analyses using enzymes Mfe-1 and Nsp-1 (see Fig. 1B) confirmed that the constructs Ad35Pf.CS Δ 14 (531 bp) and Ad35Pf.CS Δ 28 (495 bp) have the expected deletion within the GPI signal sequence (Fig. 1C) as compared to the full length CS sequence (FL: 590 bp).

Role of GPI signal sequence in CS protein expression and secretion

To determine the influence of GPI signal sequence deletions on expression and secretion of the *P. falciparum* CS protein, the rAd35 vectors were used to transduce A549 cells with equal amounts of virus particles. Subsequent Western blot analyses (Fig. 2A) demonstrates the efficient expression in of the ~ 50 kDa CS protein for all three constructs in these human cells. However, no apparent differences could be observed between the different GPI variants of CS protein, neither in level of intracellular CS expression (cell lysate), level of secreted CS protein (supernatant), nor in efficiency of secretion as witnessed in timing of maximum secretion (optimal secretion between 48-72 hrs after transduction).

In order to exclude a possibility of potential rAd35 mediated cellular toxicity resulting in non-specific protein release, we have analyzed the influence of rAd35 vector on cell viability. Hereto, A549 cells were infected with 2500 vp/cell of Ad35Pf.CS FL, Ad35Pf.CS Δ 14 or Ad35Pf.CS Δ 28 whereas none transduced cells served as control.

Trypan blue staining, demonstrating the loss of membrane integrity, showed that a maximum decrease of 10% cell viability was observed after 96 hours irrespective of exposure to rAd35 vector (data not shown).

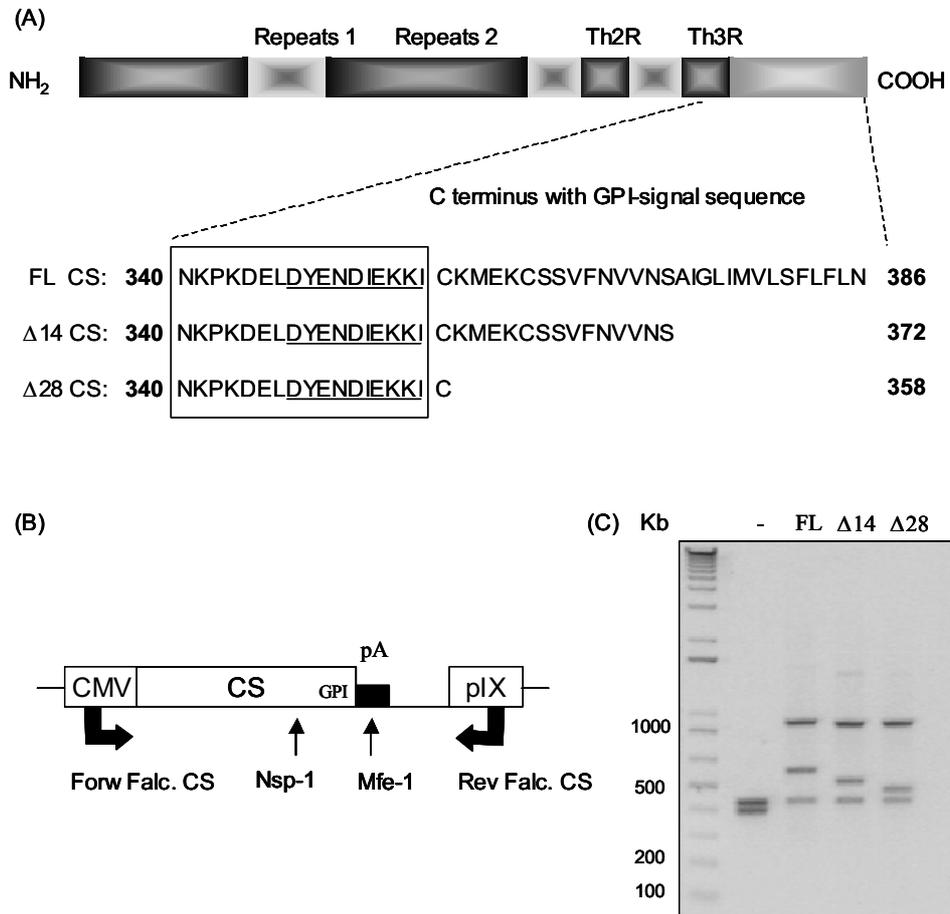


Fig. 1. (A) Schematic representation of the *Plasmodium falciparum* circumsporozoite gene. The Pf.CS gene encodes two repeat regions, 27 NANP repeats and 3 NVDP repeats respectively. Within the carboxyl-terminal part a T-helper 2 (Th2) and T-helper 3 (Th3) region are defined. The Th2 region consists of an universal epitope whereas the Th-3 (Box) region contains a H-2^K restricted dominant epitope (DYENDIEKKI) for mice (underlined). The C- terminus of the CS protein, containing the full length GPI signal sequence (CS FL) is partial represented by amino acid 340 to 386.

In CS Δ14 amino acid 373 to 386 were deleted, representing the hydrophobic part of the GPI signal sequence whereas the complete GPI signal sequence, 359 to 386 was deleted in CS Δ28. Viral DNA was isolated and the CS gene was amplified by PCR with internal primers located on the CMV promoter and pIX coding region of rAd35. (B) PCR products (indicated on the top of each lane) were subjected to Mfe-1 and Nsp-1 restriction enzyme digestion in order to detect the GPI-signal sequence deletions. In the CS deletion mutants, fragments were detected with a total length of 531 or 495 bp respectively, representing the 14 and 28 amino acids deletions of the GPI signal sequence (C).

To investigate in more detail possible cell apoptosis induced by rAd35 vector, two additional assays were performed: (i) poly-(adenosine diphosphate-ribose) polymerase (PARP) cleavage [33] and (ii) DNA content analyses through propidium-iodine staining

[34]. For PARP cleavage cells were harvested 24 h after exposure to rAd35 vector and degradation of the 116 kDa PARP protein was assessed through Western blot analysis. As shown in Fig. 2B, no significant PARP cleavage occurred after transduction with rAd35Pf.CS vectors, as indicated by the presence of intact PARP protein (116 Kd band). In contrast, the positive control demonstrated complete PARP cleavage resulting in an 85 kDa product [33]. Using propidium iodide (PI) cell staining, as a measure of the DNA content, significant (32.73%) DNA fragmentation was observed in the positive control (A549 cell treated with Mitomycin C), whereas no apoptosis was observed in cells transduced with rAd35 vectors (Fig. 2C).

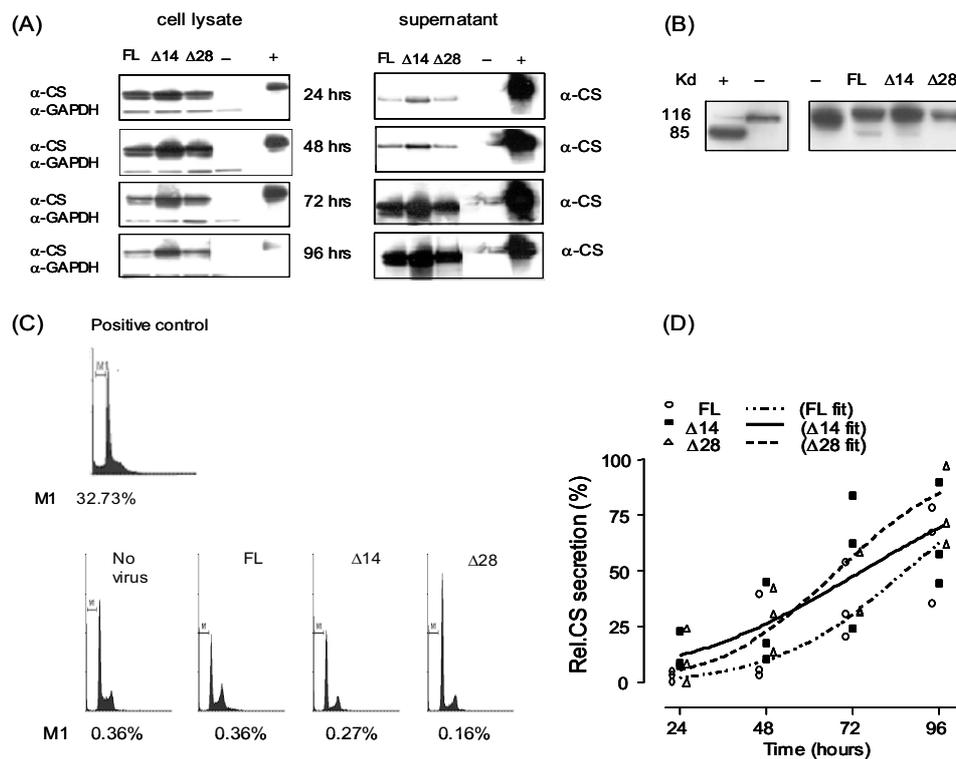


Fig. 2. Total CS protein production from A549 cells transduced with the different rAd35Pf.CS constructs. (A) Western blot analysis of cell lysates (left panel) and supernatant (right panel) of A549 cells transduced with 2500 vp/cell FL (lane 1) Δ14 (lane 2) or Δ28 (lane 3) respectively. None transduced A549 cells were used as a negative control (lane 4) and recombinant CS protein was used as positive control (lane 5). A monoclonal antibody against GAPDH was used as loading control (left panel). Cell viability. (B) A549 cell were transduced with 2500 vp/cell FL (lane 2), Δ14 (lane 3) or Δ28 (lane 4) respectively. After 24 degradation of PARP (116kDa) into an apoptosis-induced cleavage fragment of 85 kDa was detected by anti-PARP antibodies. Untreated A549 cells were used as negative control. Trichostatin-A treated neuroblastoma cells (IMR32) were used as positive control (left panel) (C). A549 cells transduced with FL, Δ14 or Δ28 were stained with Propidium Iodide, and DNA content was analyzed by flow cytometry. Marker (M1) represents the percentage of cells with low DNA content 24 hours after viral transduction. Cells treated with Mitomycin C were used as positive control. Fig. 2D. Quantification of CS production by ELISA. Quantification was performed by comparison with a standard curve of recombinant CS protein. Relative CS secretion is depicted at the Y-axis. Individual samples of three experiments are measured and the pattern of CS secretion was modeled over time and depicted as a kinetic curve.

Taken together, these data demonstrate that the viability of A549 cells was unaffected upon exposure to rAd35 vectors carrying diverse GPI deleted CS sequences and therefore the CS

secretion results obtained by Western blot are unlikely to be influenced by rAd35 mediated cellular toxicity.

In order to quantify in more detail the amount of produced and secreted CS protein we performed CS-specific ELISA, using *P. falciparum*-specific CS antibody recognising an epitope preserved in all three sequences. The average amount of expressed and secreted protein varied between 10 and 1500 μg CS protein/ml. From the data obtained, and shown in Fig. 2D as relative CS secretion, it can be calculated that the time required to achieve maximum cellular expression did not significantly differ between the tested CS protein sequences. Also, the time required to obtain 50% total CS protein secretion did not significantly differ between the FL, $\Delta 14$ or $\Delta 28$ CS proteins, which were provided in the context of rAd35 vector.

Role of GPI signal sequence in intracellular localization

Although no apparent differences could be observed between the different CS sequences regarding secretion and expression level, the intracellular localization could be different given the known role of GPI anchor sequences. Intracellular localization was investigated using immunofluorescence. Hereto, human A549 cells were analyzed 24 h after exposure to rAd35Pf.CS FL, rAd35Pf.CS $\Delta 14$ or rAd35Pf.CS $\Delta 28$, respectively. Human A549 cells transduced with rAd35Empty were used as a negative control. In contrast to cells infected with rAd35Pf.CS FL, exhibiting confined CS protein expression a entirely homogenous distribution of CS protein was found in the cytoplasm of cells transduced with rAd35 vector carrying either $\Delta 14$ or $\Delta 28$ GPI signal sequence (Fig. 3). These results indicate that deletion of GPI signal sequence can influence the intracellular localization of CS protein, which could potentially impact on CS protein processing.

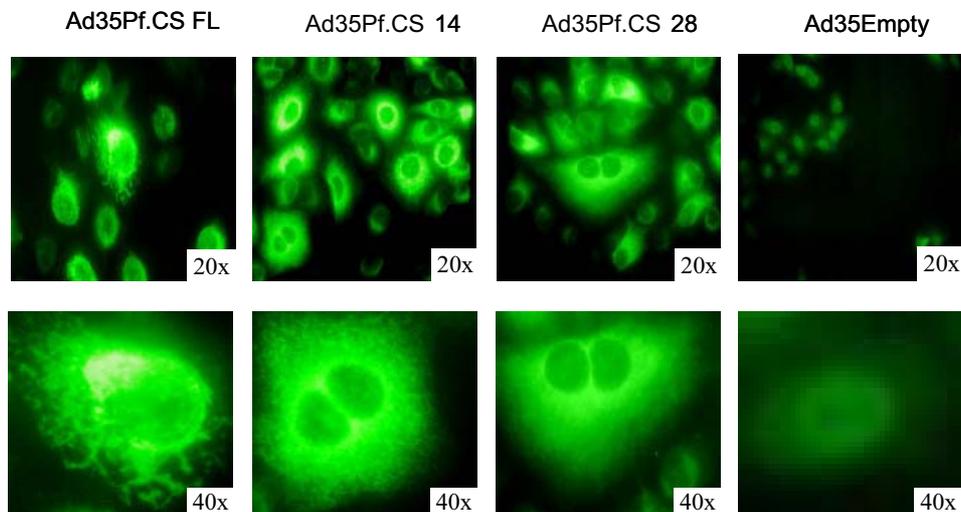


Fig. 3. Indirect immunofluorescence assay of fixed A549 cells transduced with 2500 vp/cell rAd35Pf.CS FL, $\Delta 14$ or $\Delta 28$.

Role of GPI signal sequence in *P. falciparum* CS immunogenicity

In order to investigate whether deletions of GPI signal sequences have an effect on immunogenicity, CBA mice were immunized (im) with a single optimal dose (10^9 vp) of either rAd35Pf.CS FL, rAd35Pf.CS $\Delta 14$ or rAd35Pf.CS $\Delta 28$ vaccines. Based on dose

response studies in several mice strains, including mice of H-2K^k haplotype (CBA, B10BR) the dose of 10^9 vp was chosen as most optimal (data not shown). Results obtained at week 4 post-immunization (Fig. 4A) demonstrate that vaccination with rAd35 vectors expressing either of two GPI deleted CS variants results in significantly ($p < 0.001$) higher CS-specific antibody responses as compared to mice immunized with rAd35 vector carrying the full length CS protein. No significant differences ($p > 0.05$) were observed between rAd35Pf.CS $\Delta 14$ and $\Delta 28$ CS vaccines. Immunofluorescence titers, expressed as the highest serum dilution still able to visualize sporozoites, were in agreement with ELISA data, and proved lower titer for serum derived from rAd35Pf.CS FL vector immunized mice (1:50) as compared to titers obtained with either rAd35Pf.CS $\Delta 14$ (1:200) or with rAd35-Pf.CS $\Delta 28$ (1:400) (Fig. 4A).

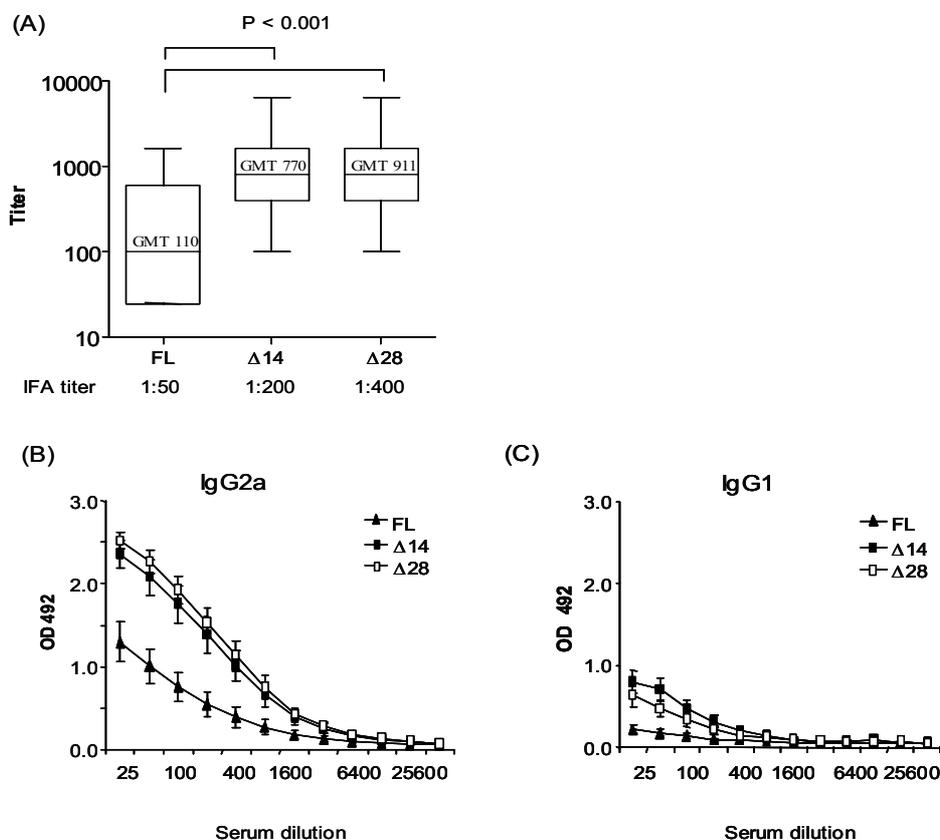


Fig. 4. Comparison between rAd35Pf.CS FL, $\Delta 14$ or $\Delta 28$ induced humoral immune responses. CBA mice were immunized with 10^9 of rAd35Pf.CS FL, $\Delta 14$ or $\Delta 28$ and blood was collected four weeks later. (A) CS-specific humoral responses were assessed in ELISA. Isotype profile of CS specific antibodies, IgG2a (B) and IgG1 (C). The results are presented as mean \pm SEM for each group ($n=8$) expressed in optical density (492 nm). Pooled data from two independent experiments are shown.

These data also demonstrate that deletion of the complete GPI anchor improves the induction of functional antibodies given the observation that antibodies raised against the truncated CS still recognize the native form of CS protein expressed on sporozoites. In view of the fact that Th1-mediated CS responses have been associated with protection against liver stage parasites, we tested whether differences could be observed in the type of antibody

responses. Therefore, we determined the isotype of anti-CS antibodies induced by the rAd35Pf.CS FL, rAd35Pf.CS Δ 14 and rAd35Pf.CS Δ 28 vaccines. Analysis of CS-specific antibodies showed that significantly higher IgG2a responses were obtained after vaccination with Δ 14 and Δ 28 containing constructs as compared to the FL variant of CS (Fig. 4B). However, since also IgG1 titers were higher for deletion variants (Fig. 4C), the ratios of IgG2a versus IgG1 were not significantly different between the three constructs (FL 3.3; Δ 14 5.2 ; and Δ 28 5.6), and all were exhibiting skewing towards Th1 response (ratio higher than 1). Thus, based on the antibody data either Δ 14 or Δ 28 seems to represent a better antigen, at least in the setting of a live viral vector, as compared to *P. falciparum* full length CS.

Next, we assessed CS specific cellular immune responses. Hereto, splenocytes were isolated two and four weeks after vaccinating CBA mice with 10^9 vp rAd35Pf.CS FL, rAd35Pf.CS Δ 14 or rAd35Pf.CS Δ 28 vaccine and CS-specific INF γ -producing CD8 $^+$ T cell responses were determined using ELISPOT. Vaccination with all three vaccines induced clearly detectable CS specific CD8 $^+$ T-cell responses (Fig. 5) while low numbers, less than 25 SFU/ 10^6 splenocytes were determined in rAd35.empty immunized mice (data not shown). Statistically significant differences between Ad35Pf.CS FL versus the deletion variants were obtained two weeks (Δ 14 $p=0.03$) and four weeks (Δ 28 $p=0.001$) post immunization, respectively. Thus, partial or complete deletion of the GPI anchor of the CS protein derived from *P. falciparum* results in a antigen that significantly improved both B- and T-cell responses in the setting of a live vectored vaccine.

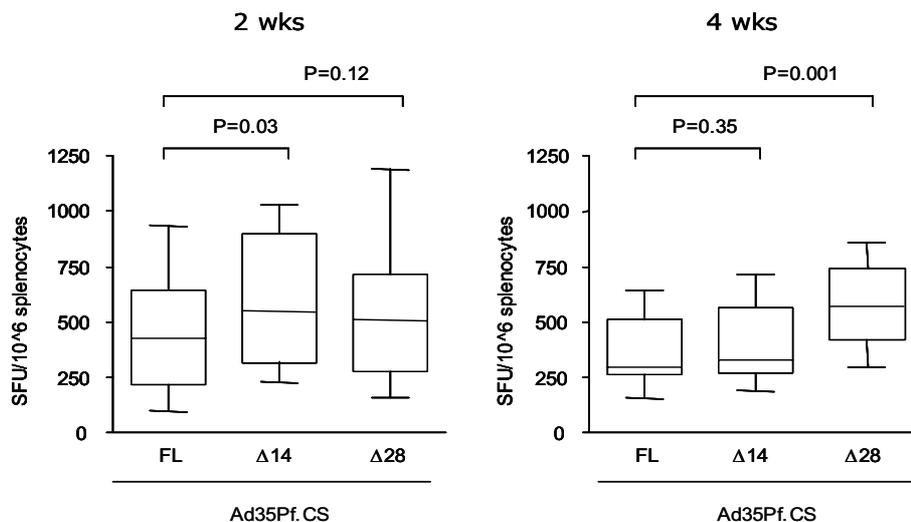


Fig. 5. (A) Splenocytes were isolated two and four weeks after immunization with 10^9 vp of rAd35Pf.CS FL, Δ 14 or Δ 28. The number of gamma interferon-secreting, CS specific CD8 $^+$ T cells was determined in an ELISPOT assay. Box plots indicate the median of SFU/ 10^6 splenocytes. Pooled data from two independent experiments are shown.

DISCUSSION

Malaria is one of the world's major public health challenges, causing more than one million deaths each year. The disease primarily affects children of the developing world [37], and it is understood to be both a disease of poverty and a cause of poverty. There are currently no effective countermeasures to control transmission of *P. falciparum*, making the development of novel vaccines and drugs to battle malaria a high research priority. Although no vaccine

is available yet, circumsporozoite protein (CS) of *P. falciparum* directly fused to the hepatitis B surface antigen, named RTS,S/AS02A, has shown excellent safety in studies and provided approximately 30-40% protection in human field trials in Africa [38]. Such studies are extremely important because they demonstrate the feasibility of effective vaccination against *P. falciparum*.

Although protection was clearly obtained upon vaccination with RTS,S/AS02A, it was short-lived. This is most likely due to a low induction of memory T-cell responses [39]. Thus, it can be envisaged that a vaccine that induces both strong B and T-cell responses could result in long-term protection after vaccination.

As vaccine carriers, recombinant replication-deficient adenoviral (rAd) vectors are safe and have elicited strong, antigen specific T-cell responses [9-12,40,41]. As such, adenoviral vectors could represent a promising novel vaccine technology to combat malaria. Another important feature contributing to the selection of adenoviral vector technology for malaria vaccination originates from the believe that it is also capable of producing the required number of doses at low cost, one of the key requirements for a vaccine to be successfully introduced among African populations. However, in our previous studies the most commonly used vector, based on human serotype 5 (rAd5), has been shown to have high sero-prevalence amongst human populations in both the developed world and developing countries [42,43]. This is particularly the case in malaria-endemic areas [1]. To circumvent the resulting pre-existing immunity, our group and others [44,45], have developed vectors based on rare human serotypes such as rAd11[46], rAd35 [32], rAd49 [47] and derivatives thereof [48]. Furthermore, we have shown that replication-incompetent vectors based on these rare human serotypes still share the advantages of rAd5 with regard to manufacturing and immunogenicity. They are not, however, hampered by rAd5 pre-existing immunity [5,8]. Based on these data we have explored the potency of the rAd35 vector as a vaccine against malaria and demonstrated that circumsporozoite (CS) protein from *Plasmodium yoelli* induces protective T-cell and antibody response in mice [1]. Going forward, we needed to generate rAd35-based vaccines carrying the CS protein from *P. falciparum*. For this purpose we first constructed model rAd35 vectors expressing different variants of CS protein to investigate the role of the GPI signal sequence in expression, secretion, localization and immunogenicity of CS derived from this major human pathogen.

The results obtained demonstrate that deletion of the GPI signal sequence from the *P. falciparum* CS protein did not impair expression or secretion, but did result in altered cellular localization and significantly improved CS-specific immunogenicity.

These results are partly in agreement with literature findings [25,26] showing that disruption of the GPI signal sequence in the rodent CS protein (*P. berghei* and *P. yoelii*) contributes to enhanced immune responses. However, contrary to these literature reports, we observed no differences in CS protein expression nor secretion of GPI signal sequence deleted CS protein, which according to the authors of the published reports partly explained the increased immunogenicity [25,26].

Results similar to our observations were reported by Moran et al. [49], who also demonstrated no differences in expression of *P. berghei* CS after deletion of GPI signal sequence as compared to full lengths CS. One possible explanation for the observed increase in immunogenicity of GPI signal sequence deleted CS, without difference in expression or secretion, could be found in the altered cellular localization of the GPI deleted CS protein. Previous studies reported retention of various proteins in distinct internal cell organelles, probably ER [50-53] when carrying uncleaved GPI signal sequences. Based on these studies, we hypothesize that deletion of the GPI signal sequence abrogates retention of the *P. falciparum* CS protein in the ER and might contribute to an altered cellular localization. However, additional studies need to be performed to further clarify localization.

Alternatively, our finding that expression is not impaired by the presence of GPI signal sequence could be influenced by the use of rAd35 as expression vehicle. This has not been used in other studies. We did not observe significant differences between the rAd35Pf.CS Δ 14 and rAd35Pf.CS Δ 28 regarding induction of CS specific response. The CS Δ 14 was designed to mimic the CS Δ 11 constructs from *P. berghei* and *P. yoelii*, which have been proven to be more immunogenic than full length CS derived from these rodent parasites. The CS Δ 28 was designed to ensure removal of the entire GPI signal sequence. Although the complete GPI signal sequence is defined within the last 28 amino acids at the C-terminus of the CS protein, our data show that removal of the last 14 amino acids, defined as the attachment and recognition site, suffices to significantly improve immune responses in mice against *P. falciparum*-derived CS. In summary, our results demonstrate that, in the context of a rAd35 vaccine carrier, removal of GPI signal sequence of *P. falciparum* CS protein does not affect the expression or secretion of the protein. Rather, it alters cellular localization, which most likely contributes to the observed increased immunogenicity.

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