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Hepatitis B virus-like particles expressing *Plasmodium falciparum* epitopes induce complement-fixing antibodies against the circumsporozoite protein

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ABSTRACT

The repetitive structure of compact virus-like particles (VLPs) provides high density displays of antigenic sequences, which trigger key parts of the immune system. The hepatitis B virus (HBV) and human papilloma virus (HPV) vaccines exploit the assembly competence of structural proteins, which are the effective immunogenic components of the prophylactic HBV and HPV vaccines, respectively. To optimize vaccine designs and to promote immune responses against protective epitopes, the "Asp-Ala-Asp-Pro" (NANP)-repeat from the *Plasmodium falciparum* circumsporozoite protein (CSP) was expressed within the exposed, main antigenic site of the small HBV envelope protein (HBsAgS); this differs from the RTS,S vaccine, in which CSP epitopes are fused to the N-terminus of HBsAgS. The chimeric HBsAgS proteins are assembly competent, produce VLPs, and provide a high antigenic density of the NANP repeat sequence. Chimeric VLPs with four or nine NANP-repeats (NANP4 and NANP9, respectively) were expressed in mammalian cells, the HBsAgS- and CSP-specific antigenicity of the VLPs was determined, and the immunogenicity of the VLPs assessed in relation to the induction of anti-HBsAgS and anti-CSP antibody responses. The chimeric VLPs induced high anti-CSP titres in BALB/c mice independent of the number of the NANP repeats. However, the number of NANP repeats influenced the activity of vaccine-induced antibodies measured by complement fixation to CSP, one of the proposed effector mechanisms for *Plasmodium* neutralization *in vivo*. Sera from mice immunized with VLPs containing nine NANP repeats performed better in the complement fixation assay than the group with four NANP repeats. The effect of the epitope-specific density on the antibody quality may instruct VLP platform designs to optimize immunological outcomes and vaccine efficacy.

1. Introduction

Virus-like particle (VLP) subunit vaccines have the ability to induce potent immune responses against their cognate virus and can be modified for the delivery of inserted foreign antigenic sequences [1–3]. Two cancer-preventing vaccines directed against human hepatitis B virus (HBV) and human papilloma virus (HPV) are composed of viral structural proteins, which are assembled into VLPs [4–7]. VLPs display an array of antigenic sequences to the innate immune system allowing the subsequent activation of the adaptive system [1,8,9]. The ability to accept foreign inserts into the VLP structure provides the basis for delivery platforms for medically relevant sequences. The structural proteins from different viruses, such as HBV, HPV and Qβ phage have been engineered to express foreign antigens [10–12]. Notably, the hepatitis B small surface antigen (HBsAgS) has been engineered to include a polypeptide derived from the *Plasmodium falciparum*

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circumsporozoite protein to generate the RTS,S (Mosquirix™) malaria vaccine [11–14].

The HBV envelope contains three related transmembrane proteins, hepatitis B surface antigen-large (HBsAgL), -middle (HBsAgM) and -small (HBsAgS). HBsAgS subunits are the sole antigenic component of the hepatitis B vaccine [4,5,15,16]. HBsAgS is composed only of the S-domain consisting of 226 amino acids (aa); HBsAgM and -L are composed of the S-domain with additional N-terminal extensions. The expression of HBsAgS proteins in eukaryotic cells in the absence of any other viral component is sufficient for the assembly into VLPs. HBsAgS proteins assemble at the endoplasmic reticulum (ER) into secretion competent, lipid containing VLPs with a size of approximately 25 nm in diameter, and approximately 100 HBsAgS subunits per particle [17,18], fifty HBsAgS dimers were identified in mice-expressed VLPs [19]. The first stage during particle morphogenesis involves the cotranslational insertion of HBsAgS into the ER membrane forming a short luminal exposed N-terminal sequence, two transmembrane regions separated by a 57 aa cytosolic loop and a luminal external 70 aa region containing loop 1 and loop 2 regions. The external hydrophilic region contains multiple epitopes, including an immunodominant ('a'-determinant) domain that is common to all HBV genotypes. HBsAgS VLPs have a compact structure due to a large number of intra- and intermolecular disulfide bonds within and between the individual subunits [15,16,20,21]. Expressing HBsAgS protein subunits in mammalian cells allows post-translational modification of HBsAgS resulting in approximately half of the subunits being glycosylated at residue Asn146 [15,16] contrasting the yeast-derived HBsAgS VLP vaccine, in which particles remain non-glycosylated [22–24]. Glycans provide defining structures that contribute defining structures that contribute to particle uptake and processing by antigen presenting cells [25]. We have previously shown that hyperglycosylated HBsAgS VLPs with additional glycans promote an earlier and longer lasting anti-HBsAgS antibody response compared to wild type or hypoglycosylated HBsAgS VLPs [26]. The presence of glycan structures may facilitate recognition by host lectin receptors expressed by antigen-presenting cells, and therefore allow immune modulation [27].

HBsAgS VLPs have been used to provide high density displays of antigenic structures leading to potent B cell and/or T cell responses, including anti-foreign cellular and humoral immune responses [28–32]. Immunization studies using a mouse model showed that chimeric HBsAgS VLPs access MHC class I and class II pathways and present inserted, ovalbumin-derived model epitopes [9].

The RTS,S (Mosquirix™) vaccine is composed of a circumsporozoite polypeptide of 189 aa that represents the NANP-repeat and C-terminal regions, fused to the HBsAgS N-terminus, and co-expressed with wild type HBsAgS proteins in yeast allowing the assembly of non-glycosylated hybrid VLPs [11–14]. The circumsporozoite protein (CSP) is an abundant protein expressed on the *Plasmodium* sporozoite surface; CSP is critical for establishing a productive infection and is essential for hepatocyte invasion, and therefore an important immune target for the development of a pre-erythrocytic vaccine [33]. The precise mechanism by which *Plasmodium* neutralization and protective immunity occurs *in vivo* remains unclear. Anti-CSP antibodies at a sufficient titre are associated with protection against sporozoite infection supporting evidence that the humoral immune response may contribute to overcome clinical malaria [34–37]. Vaccine-induced monoclonal antibodies (mAbs) to CSP can protect against infection in mice with humanized livers [34]. It has been suggested that anti-CSP antibodies may confer protection, in part, by opsonization of sporozoites to promote phagocytosis, though published findings have not been consistent [38,39] or by blocking an invasion of human hepatoma cells [40,41]. Recent studies have shown that antibodies to CSP and the NANP epitope can promote complement fixation and activation [42]; complement activation leads to sporozoite cell death and inhibition of cell traversal and hepatocyte invasion [42,43]. Furthermore, complement-fixing antibodies to CSP were associated with protection against malaria in children [42]. Antibodies of the cytophilic IgG1 and IgG3 isotypes specific for CSP possibly contribute to the protection of individuals from infection [44]. Anti-CSP antibodies are present in populations living in malaria endemic regions and may contribute to the naturally acquired immunity of adults in these regions [45]. The RTS,S vaccine has undergone phase 3 clinical trials in young children and only showed modest protective efficacy of around 50% in the first year of follow-up and 29–36% (depending on age group) over four years when given a booster at 20 months [13,14,46–48]. Antibodies are believed to play a major role in immunity provided by RTS,S [35]. The CSP $(NANP)_n$-repeat sequence is a key target of RTS,S and other CSP-based vaccines providing protective immunity against *P. falciparum* [36]. There is currently extensive evidence indicating that antibody responses generated against the CSP NANP-repeat region may contribute to protective immunity making it an ideal candidate for vaccine development [33,34,46].

Given the modest efficacy of RTS,S and other vaccines, new or additional strategies are needed to reach the World Health Organisation objective of developing a vaccine with at least 75% efficacy [49]. Therefore, in this study we evaluated strategies to promote antibody responses against the NANP sequence. HBsAgS proteins with NANP inserts were expressed to generate VLPs, which are solely composed of chimeric HBsAgS to provide a high "NANP" antigenic density. The selected "NANP" insertion site is located in the immunodominant exposed region of the HBsAgS loop to promote B cell immune responses, rather than fusing the epitopes at the N-terminus of HBsAgS as used in the RTS,S vaccine. Comparative studies with chimeric VLPs distinguished by the number of "NANP" repeats were performed to determine the impact of epitope density on antibody titre and antibody complement fixation efficacy.

2. Material and methods

2.1. Plasmids

The parent plasmid construct has been described and expresses HBsAgS protein (genotype D, serotype *ayw*) [8]. The HBsAgS gene contains an introduced *Age*I restriction site, which allows insertions at a site corresponding to a location in the external hydrophilic loop region between amino acids 127–128 [8]. Sequences encoding for four or nine NANP-repeats were inserted into the *Age*I site for synthesizing chimeric HBsAgS proteins. The HBsAgS proteins contain an N-terminal myc-tag sequence followed by a 'GASGS' linker sequence. The NANP repeats are derived from the CSP central repeat region (Fig. 1).

2.2. Immunoprecipitation and gel electrophoresis of HBsAgS proteins

The procedure was described by Hyakumura et al. [26]. Briefly, HEK293T cells (3×10^5 cells/well) were seeded into six-well plates and transfected using the reagent PEI (Polysciences), after 2 days isotopic labeling was performed. The cell culture medium was assessed for the presence of the myc-tag and HBsAgS proteins by ELISA. Cells were incubated with 1.5 ml of methionine/cysteine-free minimal essential medium, after 45 min, 200 μCi of [^{35}S] methionine-cysteine was added, and cells were incubated for 3 h. Cells were washed in phosphate-buffered saline (PBS), and incubated for 18 h with 2 ml DMEM (Dulbecco's modified Eagle's

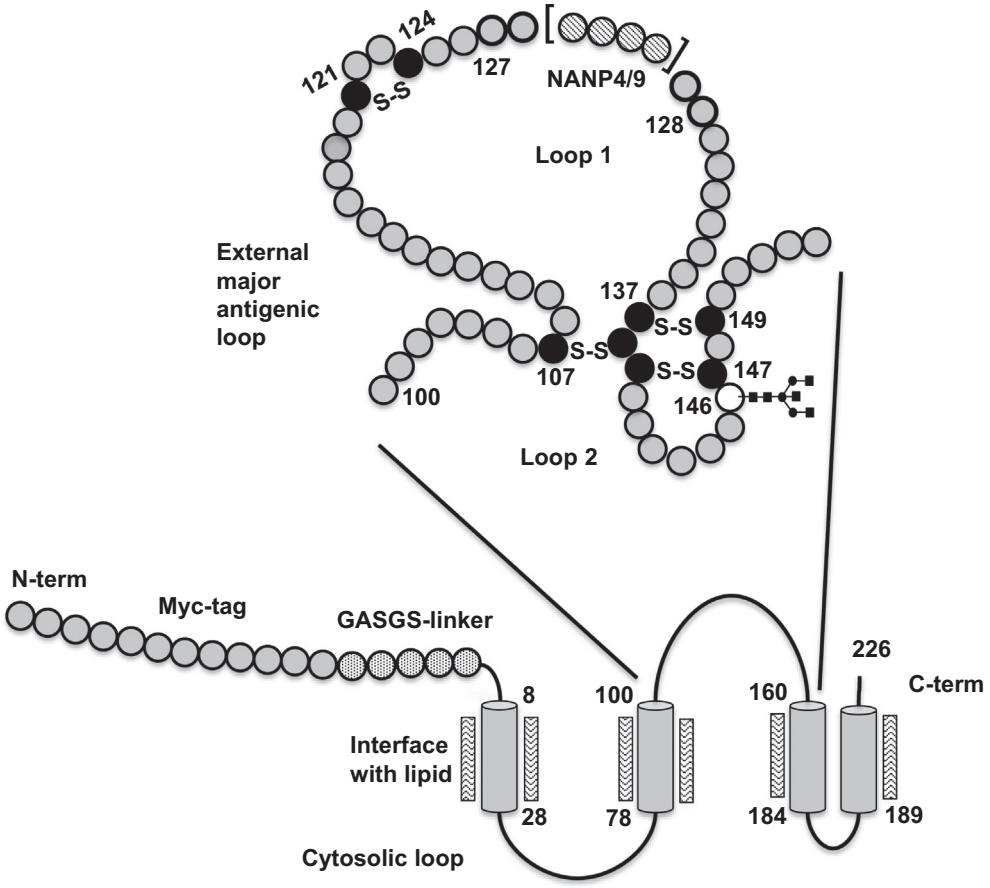

Fig. 1. Schematic representation of a chimeric HBsAgS subunit with NANP4 or NANP9 insert. The site of foreign sequence insertion using the *Age*I restriction site is located at a position corresponding to the amino acids 127–128 of HBsAgS in the loop 1 region. Figure not to scale. The N-terminal myc tag and GASGS linker precede the HBsAgS sequence.

medium) supplemented with 10% fetal calf serum (FCS). Cell culture medium was harvested and centrifuged to remove cell debris. The cells were collected and lysed in 200 μl lysis buffer (150 mM NaCl, 1%NP40, 50 mMTris HCl, pH 8) and pelleted to remove cell debris. The clarified lysate sample was collected and diluted by adding 900 μl PBS to 100 μl lysate. Iodoacetamide was added to the samples to a final concentration of 20 mM, then the samples were incubated on ice for 2 h with 25 μg polyclonal rabbit anti-HBsAg antibody (Meridian Life Science, Memphis, TN, USA), or 25 μg monoclonal mouse anti-myc antibody (9E10). Then 20 μl protein A Sepharose CL-4B (GE Healthcare, Piscataway, NJ) was added and incubated at 4 °C with rotation for 1 h. The beads were washed three times in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% v/v NP-40, 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 7.5), and once with 100 mM Tris-HCl (pH 6.8). Samples were prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by adding loading buffer (8% SDS, 0.4% w/v bromophenol blue, 40% v/v glycerol, 20% v/v 2-mercaptoethanol, 250 mMTris-HCl, pH 6.8) and boiled for 5 min. After electrophoresis, the gel was destained in 7.5% acetic acid–5% methanol and dried before exposure on a Typhoon phosphorimager (GE Healthcare).

2.3. Large scale VLP production and quantification

Suspension-adapted human embryonic kidney (HEK) 293F cells were grown in DMEM and 10% FCS in a 1 l wave bioreactor, and transfected with lipofectamine according to the manufacturer's instructions (Life Technologies). The collected tissue culture medium was centrifuged to remove any cellular debris, the medium was transferred into an ultracentrifuge tube, underlaid with a 20% sucrose cushion in STE (100 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8), and the particles pelleted by ultracentrifugation as described by Hyakumura et al. [26]. The supernatant was discarded, and the pelleted VLPs were resuspended in 200–500 μl STE. The resuspended myc-tagged wild type (wt) HBsAgS (M-HBsAgS) VLPs were quantified using the Monolisa (Bio-Rad, Hercules CA, USA) diagnostic assay according to the manufacturer's instructions. Chimeric VLPs containing the NANP4 and NANP9 inserts were quantified relative to the M-HBsAgS particles via the myc-tag. Briefly, 96 well ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with 100 μl of 500 ng/ml polyclonal rabbit anti-myc antibody (Sigma-Aldrich) and incubated overnight at 4 °C. The liquid was removed and wells were blocked with 300 μl of 5% (w/v) skim milk powder suspended in PBS for 2 h at room temperature. The blocking solution was removed and 100 μl of VLP dilutions were added and incubated for 1 h at room temperature. Plates were washed and 100 μl of 500 ng/ml mouse anti-myc monoclonal antibody (mAb) was added to each well and incubated at room temperature for 1 h. Plates were washed and 100 μl of 1 μg/ml polyclonal rabbit anti-mouse conjugated to horseradish peroxidase (HRP) was added. Plates were incubated for 1 h at room temperature. Wells were washed and 100 μl of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), H_2O_2 substrate was added, the plates were incubated in the dark for 1 h before the OD of wells was recoded at 405 nm and 490 nm. The OD recording at 490 nm was deducted from the OD recorded at 405 nm and the concentration of VLP samples was

determined based upon their relative ODs and the standard curve generated by the M-HBsAgS VLPs.

2.4. Multiplex assay

A Bio-Plex bead-based flow cytometric platform (Bio-Rad) to fingerprint HBsAg has been established to characterize the antigenic profile of the HBsAgS VLPs [26]. Briefly, the HBsAgS multiplex immunoassay comprizes panels of fluorescently tagged beads, each set conjugated to a different anti-HBsAgS envelope antibody, and a polyclonal phycoerythrin conjugated detector antibody. Seventeen mAbs were selected from a multiplex panel with specificities targeting the N-terminus, the external hydrophilic loop region, and C-terminal domain epitopes spanning amino acid residues 99–226 of HBsAgS [26,50]. VLP data were normalized to the M-HBsAgS VLP for specific analysis of the effect of the insertion of the NANP epitope repeats. The 95% confidence interval (CI) for the normal range of variation of epitope recognition from the reference backbone was established as ±0.5-fold change. A fold change <0.5 is not significant and indicates the normal variance of this assay. A gain-of-epitope recognition corresponds to positive fold change (>0.5-fold), and negative fold changes (>0.5-fold) correspond to a gain or reduction of epitope binding. A fold loss of 3 or more is considered a complete epitope knockout.

2.5. VLP preparation for electron microscopy

The collected tissue culture supernatant was spun using a benchtop centrifuge, and then the supernatant was sterile filtered with a 0.2 μm filter (Merck Millipore, Billerica, MA, USA). One liter supernatant was adjusted with 5ml 3M Tris HCl (pH8) then run through a Sephacryl 300 (Pharmacia) pre-column and anti-myc affinity column (Minileak beads, KemEnTec, Taastrup, Denmark; in house anti-myc antibody). Elutions were performed with recirculated myc-peptide. The elution volume was concentrated with Amicon Ultra 10 kDa (Merck Millipore) and run through a S200 10/30 gel filtration column to remove the excess myc-peptide. Carbon-coated 300-mesh copper grids were rendered hydrophilic by glow-discharged in nitrogen. A sample volume of 5 μl was transferred onto each grid for 10 s, the excess was removed using a Whatman 541 filter paper. The grid was washed two times with water for 1 min, stained with 2% aqueous uranyl acetate for 3 s, blotted and air-dried. All samples were examined using a Tecnai 12 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120 kV. Images were taken using a Megaview III CCD camera and AnalySIS camera control software (Olympus.)

2.6. Animals and VLP immunization procedures

BALB/c mice were used at the age of week 6. Mice were housed under pathogen-free conditions at a facility of the Monash animal research platform, and the experiments were approved by the animal ethics committee. Pre-immunization bleeds were taken, groups of seven female BALB/c mice were immunized subcutaneously at the base of the tail four times at two week intervals with 1 μg of M-HBsAgS VLPs or M-HBsAgS VLPs with NANP4 or NANP9 epitope inserts (M-HBsAgS-N4, M-HBsAgS-N9, respectively) in the presence of 2.5 nmol CpG (ODN1668, GenWorks, Australia). Serum samples were taken throughout the course of the trial. The final sample was collected two weeks after the final vaccination. The anti-HBsAgS or anti-NANP antibody responses were measured by ELISA using yeast-derived HBsAgS VLPs (serotype *ayw*; Meridian Life Science) or purified *P. falciparum* (3D7 isolate) CSP as targets, respectively. ELISA plates were coated with 100 μl of 1 μg/ml yeast derived HBsAg VLPs or 500 ng/ml CSP overnight at 4 °C. Wells were blocked with 5% skim milk powder in PBS followed by washing steps with PBS in the presence of 0.05% Tween-20. Diluted serum samples were added to the wells and detected using anti-mouse antibodies coupled to horseradish peroxidase (HRP). The plates were incubated with ABTS substrate solution, and the optical density measured at 405–490 nm. GraphPad Prism was used for statistical analysis; statistical significance of differences between groups was calculated using two-way analysis of variance (ANOVA) (a P value of <0.05 was considered significantly different).

2.7. Complement fixing assay

The complement fixing assay was performed as published with minor modifications [42,51]. Briefly, ELISA plates (MaxiSorp plates, Thermo Fisher Scientific) were coated with 500 ng/ml CSP, blocked and then sera were tested at dilutions of 1:100 and 1:250, and for extended dilutions the antisera were tested at 1:500, 1:1000 and 1:2000 dilutions. Human C1q was added to wells and C1q was detected with goat anti-C1q antibodies and anti-goat HRP. TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution was added and incubated for 1 h, a stopping solution was added to wells and the optical density was recorded at 450 nm. Recombinant CSP was expressed in *Pichia pastoris* and purified using high-performance liquid chromatography (Sanaria, Rockville, MD, USA). The protein was based on the sequence of the *Plasmodium falciparum* isolate 3D7 beginning at the amino acid residue 50 and contained 22 NANP repeats and four NVDP repeats.

3. Results

3.1. Generation of NANP-containing VLPs, and assessment of VLP secretion

For promoting B-cell responses, the CSP-derived antibody target, "NANP" was introduced into the exposed immunodominant region of the HBsAgS protein, between amino acid position 127 and 128 to allow surface exposure [8]. Four or nine repeats of the "NANP" sequence were inserted for generating a high antigenic density of the "NANP" insert. As the presence of foreign sequences in the immunodominant external region of HBsAgS may alter antigenicity and may compromise detection by anti-HBsAgS antibodies, an N-terminal myc-tag sequence was included to allow detection of VLPs independent of the HBsAgS backbone (Fig. 1). The plasmid pM-HBsAgS, which encodes the myc-tagged wt HBsAgS protein, and the plasmids pM-HBsAgS-NANP4 and pM-HBsAgS-NANP9, which encode the myc-tagged chimeric HBsAgS proteins with four or nine "NANP" repeat inserts (M-HBsAg-N4 and M-HBsAg-N9), respectively, were individually transfected into HEK293T cells. To assess the ability of the chimeric HBsAgS proteins to secrete, the cell culture supernatant was collected two days post-transfection and assessed via ELISA for the presence of HBsAgS activity and myc-tagged proteins (Fig. 2A). The cells were subsequently radiolabelled with ^{35}S-cysteine and -methionine to visualize the expressed HBsAgS proteins. The cell lysate and cell culture supernatant were harvested and the presence of HBsAgS monitored via immunoprecipitation with anti-HBsAgS or anti-myc antibodies (Fig. 2B and C). Using ELISA, detection of the chimeric M-HBsAgS-N4 and M-HBsAgS-N9 proteins with anti-myc and anti-HBsAgS antibodies was reduced compared to M-HBsAgS, indicating that the secretion efficiency of the VLPs with NANP inserts is diminished (Fig. 2A). Immunoprecipitation of VLPs with anti-myc and anti-HBsAgS antibodies and analysis via SDA-PAGE verified the presence of the correct HBsAgS proteins (Fig. 2B and C). The M-HBsAgS protein migrates at approximately 27 kDa and the glycoprotein at approximately 30 kDa. The unglycosylated

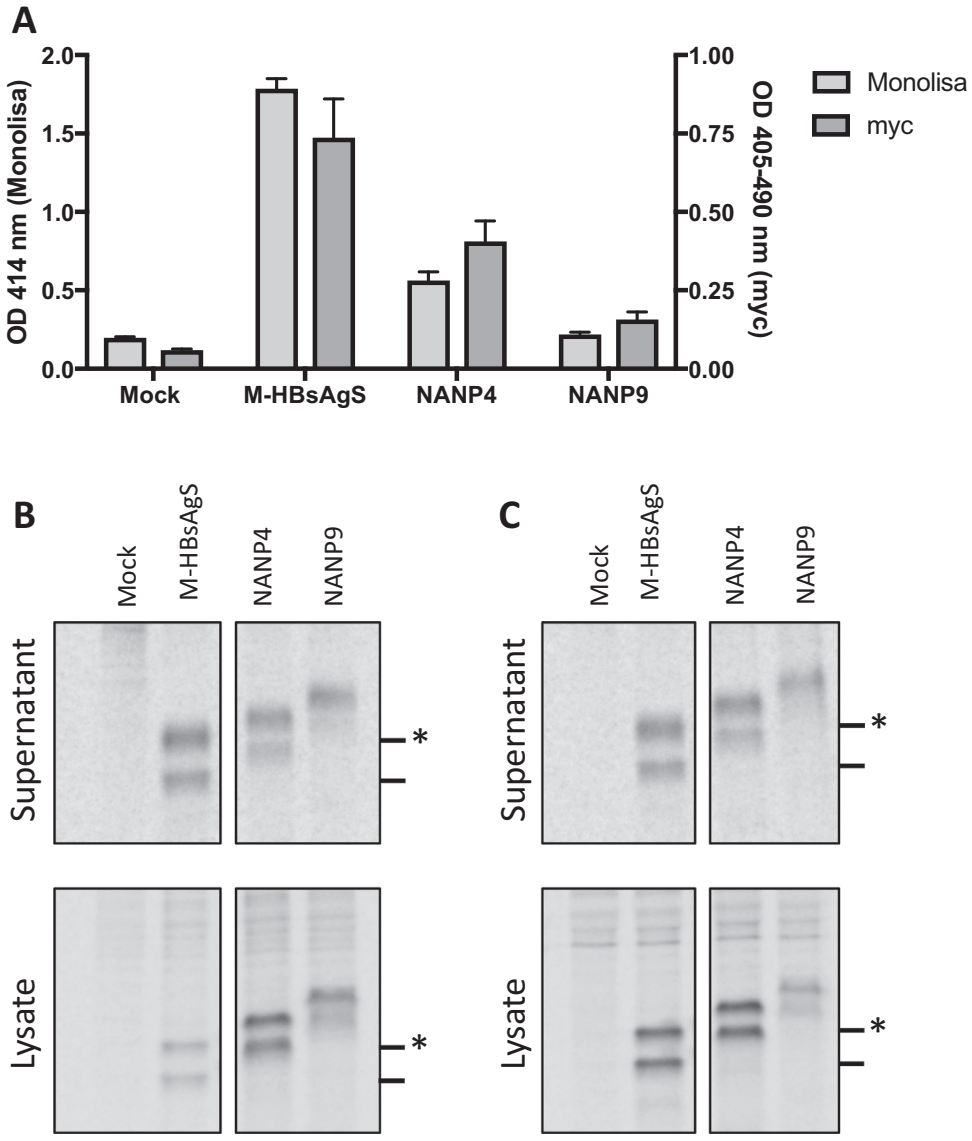

Fig. 2. Expression of chimeric HBsAgS proteins. The ability of chimeric HBsAgS proteins was assessed via HBsAgS-specific Monolisa (BioRad), an anti-myc specific ELISA (A), and immunoprecipitation of ^{35}S-labelled HBsAg proteins using anti-myc antibody (B) or anti-HBsAg antibody (C). HEK293T cells were transfected with plasmids expressing the proteins M-HBsAgS, M-HBsAgS-N4 (NANP4), and M-HBsAgS-N9 (NANP9). As control, cells were transfected with vector in the absence of insert (Mock). Forty-eight hours post-transfection cell culture supernatant was collected and assessed for HBsAgS and myc activity (A). The HBsAg ELISA (Monolisa) is recorded at OD 414 nm, the myc-specific ELISA is recorded as OD 405–490 nm, graphed mean ± SEM. Assay performed three times in duplicate (A). The cells were then labelled with ^{35}S-cysteine and -methionine and incubated for 18 h. Supernatant and lysates were immunoprecipitated with mouse anti-myc (B) or rabbit anti-HBsAg (C) antibodies. Proteins were denatured and separated by 15% SDS-PAGE, gels were dried and viewed on a phosphorimager. The M-HBsAgS protein (-) and the glycoprotein (-*) are indicted with molecular weights of 27 kDa and 30 kDa, respectively. The chimeric proteins M-HBsAgS-N4 and -N9 have molecular weights of 29 kDa and 31 kDa (unglycosylated) or 32 kDa and 34 kDa (glycosylated), respectively.

M-HBsAgS-N4 and -N9 proteins showed the expected relative molecular weights of 29 kDa and 31 kDa, respectively each being slightly larger than M-HBsAgS due to the inserted NANP4 and NANP9 epitope repeats (Fig. 2B and C). In contrast to the M-HBsAgS and M-HBsAgS-N4 proteins, the M-HBsAgS-N9 VLPs seem to be preferentially glycosylated as indicated by a stronger band intensity of the glycosylated HBsAgS subunit at 34 kDa versus the non-glycosylated HBsAgS subunit at 31 kDa (Fig. 2B and C). The insertion of the extended NANP9 insert compared to NANP4 may cause an enhanced exposure of the HBsAgS glycosylation site (Asn146). To verify that the chimeric M-HBsAgS-N4 and M-HBSAg-N9 proteins have the ability to assemble into particulate structures, the samples were analyzed by a sucrose gradient density centrifugation. A myc-tag specific ELISA identified myc-tagged wt and chimeric HBsAgS proteins at a density of approximately 1.15 g/cm^3 (data not shown), which is consistent with published data obtained with patient derived VLPs [52]. To confirm assembly competence of M-HBsAgS-N4 and M-HBsAgS-N9 proteins and particle formation, the VLPs were affinity purified from cell culture supernatants via the myc-tag, negatively stained with uranyl acetate, and then visualized by electron microscopy (Fig. 3).

3.2. Determination of antigenicity

To analyse whether the insertion of NANP-epitopes modulate HBsAgS-specific antigenicity, the chimeric VLPs were assessed by a Bioplex assay using 17 monoclonal anti-HBsAgS antibodies (Fig. 4A). The M-HBsAgS VLPs have been standardized to the reference genotype D *ayw* HBsAgS VLP and all chimeric VLPs have been normalized to the M-HBsAgS VLPs via the myc tag. The Bioplex

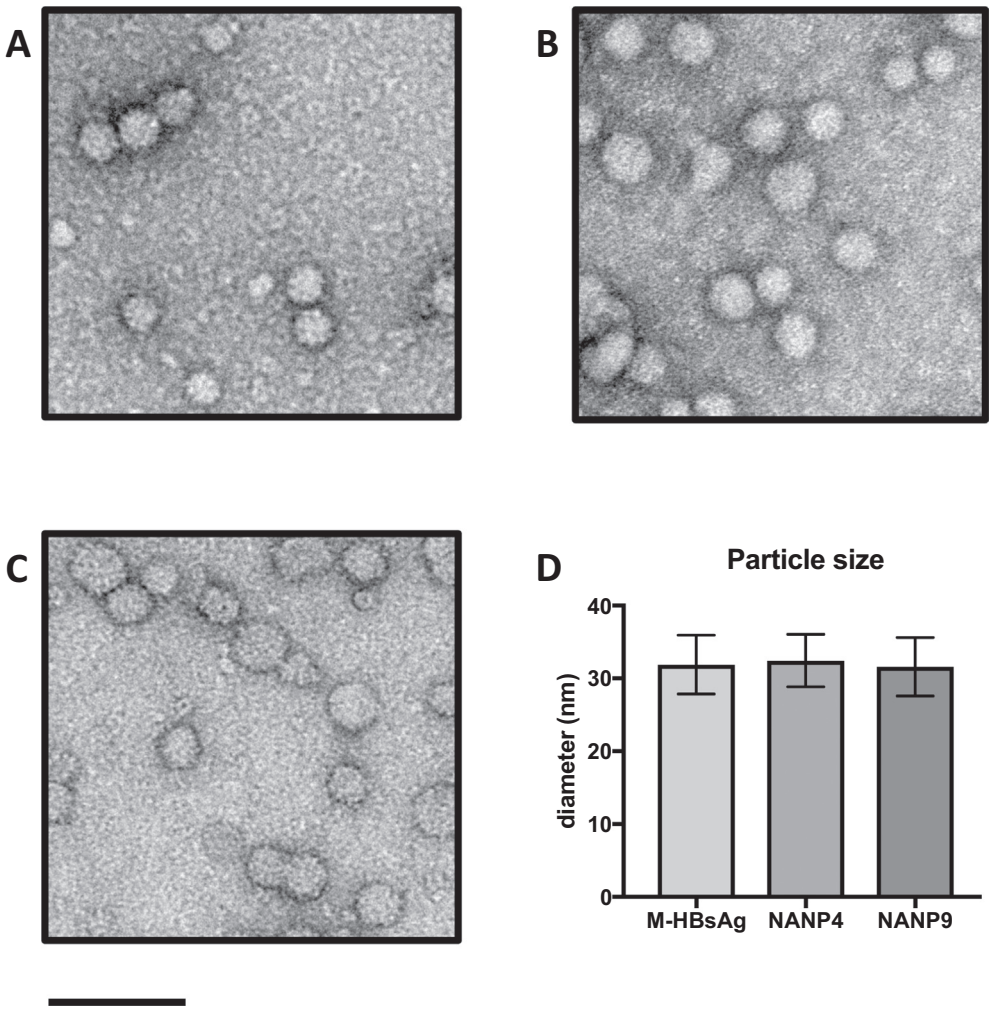

Fig. 3. Assessment of VLP formation via transmission electron microscopy (TEM). HEK293F cells were transfected with plasmid expressing M-HBsAgS VLPs (A), M-HBsAgS-N4 (NANP4) VLPs (B) and M-HBsAgS-N9 (NANP9) VLPs (C). Cell culture supernatant was collected seven days post transfection, and filtered through a 0.2 μm filter, then undergoing affinity purification using a myc-affinity column. Samples were concentrated and applied to copper grids, counterstained with 2% uranyl acid and viewed on a Tecnai 12 TEM. Scale bar 50 nm. The size of the particles was determined using ImageJ, graphed mean particle size ± SEM.

assay is primarily composed of antibodies that are directed against the major external loop of HBsAgS but also includes antibodies specific for the C-terminal region of HBsAgS [26]. Three of the 17 analyzed antibodies, mAb3, mAb10, and mAb18 retain the ability to recognize the chimeric NANP-HBsAgS VLPs to an extent equivalent to the recognition of myc-tagged wt HBsAgS (M-HBsAgS) VLPs (Fig. 4A). The antibodies recognize different antigenic sequences within HBsAgS in proximity to the NANP insertion site proximal to loop 1 (mAb10), a conformational epitope within the antigenic 'a'-determinant (mAb18), or at the C-terminal end of the external loop region (mAb3) indicating that HBsAgS-specific antigenic structures have been retained. However, 14 of 17 tested mAbs specific for the loop 1 and 2 regions, C-terminal region, and conformational epitopes have lost their ability to bind to the chimeric VLPs indicating an extensive loss of HBsAgS-specific antigenicity. Similar to the finding that antigenic structures have been retained at different HBsAgS sites, loss of HBsAgS-specific antigenicity is not limited to antigenic sites adjacent to the NANP-insertion (Figs. 1 and 4A). The antibodies mAb8 (loop 2) and mAb9 (conformational) detect M-HBsAgS-N4 VLPs within the normal range, but failed to detect the M-HBsAgS-N9 VLPs. The enhanced antigenic loss in the presence of the NANP9 insert indicates that the insertion length affects the HBsAgS-specific antigenicity possibly due to increased structural constraints (Fig. 4A). To demonstrate the accessibility of the NANP repeat sequences in the context of the M-HBsAgS-N4 and -N9 VLPs, and to show whether NANP-inserts are presented in the correct conformation, mouse anti-myc antibodies were used to capture the VLPs; VLPs were subsequently detected using rabbit anti-myc or rabbit anti-CSP antibodies (Fig. 4B). Similar amounts of the M-HBsAgS, M-HBsAgS-N4 and -9 VLPs were captured and detected via the anti-myc antibodies. Anti-CSP antibodies detected both the M-HBsAgS-N4 and -N9 VLPs at 1:5000 and 1:10000 dilutions, but not M-HBsAgS, as expected. The detection of the chimeric M-HBsAgS-N4 and -N9 VLPs with rabbit anti-CSP antibodies indicated that the NANP epitopes within the chimeric VLPs are accessible and retained their native conformation (Fig. 4B). In summary, HBsAgS proteins with NANP4 and NANP9 inserts have a reduced HBsAgS-antigenicity, but have retained the ability to assemble into particulate structures displaying foreign epitopes at a high antigenic density.

3.3. Immunogenicity of chimeric particles

To evaluate the immunogenicity of the chimeric VLPs, mice were immunized with M-HBsAgS or the chimeric M-HBsAgS-N4 and -N9 VLPs. The antibody specificities of the generated sera were assessed using yeast-derived wt HBsAgS VLPs and *P. falciparum* (3D7) CSP as targets. Mice were immunized four times with 1 μg

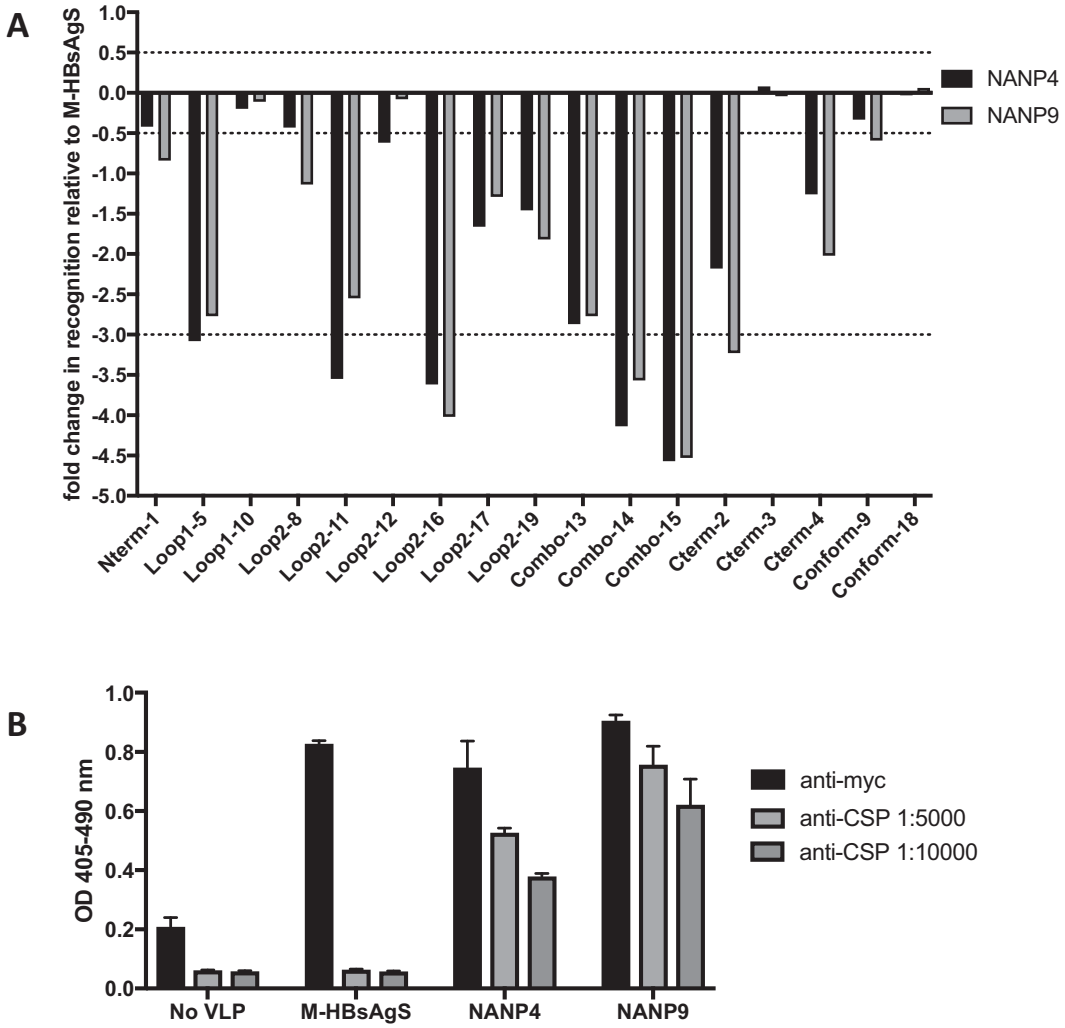

Fig. 4. Assessment of antigenicity. To determine the HBsAgS-specific antigenicity, M-HBsAgS and chimeric M-HBsAgS-N4 and HBsAgS-N9 VLPs were tested via Bioplex for HBsAg-specific antigenicity using a panel of 17 monoclonal anti-HBsAgS antibodies (mAb) (A). VLPs are normalized against M-HBsAgS. Dotted lines indicate the normal range (±0.5) and a complete knockout of epitope (-3). Variances outside of 0.5 to −0.5 indicate a gain or loss of epitope recognition, respectively. Nterm; the N-terminal region of the external loop of HBsAg (position 100–159), Combo; combination of loop 1 and loop 2, Conform; conformational epitope. The labels along the x axis indicate the name of the mAb used. For instance, loop1-5 refers to mAb 5, which recognizes an epitope in the loop 1 region of HBsAgS. To determine the circumsporozoite protein (CSP) specific antigenicity, partially purified VLPs were assayed against rabbit anti-CSP immune serum (B). Enzyme linked immunosorbent assay (ELISA) was performed with M-HBsAgS, M-HBsAg-N4 (NANP4) and M-HBsAgS-N9 (NANP9) VLPs as targets to assess detection by anti-myc antibody and anti-CSP antibodies. To capture the myc-tagged VLPs, the ELISA plates were coated with mouse anti-myc mAb, then incubated with the M-HBsAgS, M-HBsAg-N4 or M-HBsAgS-N9 VLPs, including a sample derived from a mock-transfection control (no VLPs present). VLPs were then detected with rabbit antibodies against either myc (anti-myc, loading control) or CSP at two dilutions 1:5000 or 1:10000. For detection, horseradish peroxidase (HRP) conjugated anti-rabbit antibodies and ABTS-H_2O_2 substrate solution were used. Graphed are the ODs 405–490 nm of three independent replicates performed in duplicate, graphed mean and SEM.

VLP in the presence of CpG adjuvant to promote IgG2a/b-specific immune responses. After the final immunization, all mice from the M-HBsAgS VLP immunization group generated antibodies capable of detecting HBsAgS VLPs between 1:500 and 1:4000 dilutions, but did not develop an anti-CSP antibody response (Fig. 5A and B). Importantly, the M-HBsAgS-N4 and -N9 VLPs generated both anti-CSP and anti-HBsAgS antibodies. Summarized endpoint anti-HBsAgS titres demonstrate that there is no significant difference between the M-HBsAgS VLP control group and the chimeric HBsAgS-N4 and -N9 VLP groups (Fig. 5A) ($p = 0.50$ and $p = 0.17$, respectively) in spite of the partial loss of HBsAgS-specific antigenicity demonstrated by the Bioplex assay (Fig. 4A). Similarly, in spite of the different number of NANP repeats, there is no significant difference in the anti-CSP antibody titres induced by immunizations with M-HBsAgS-N4 and M-HBsAgS-N9 VLPs ($p = 0.24$) (Fig. 5B). The VLP immunizations were performed in the presence of the CpG adjuvant, which is known to produce Th1-biased immune responses producing predominately IgG2a/b antibodies in mice [53,54]. The subsequently generated mouse IgG2a and IgG2b antibodies should enable complement fixation [55,56], which is known to be important for *Plasmodium* neutralization [42]. The generated antisera were tested by an ELISA against yeast-derived wt VLPs and visualized using isotype specific secondary antibodies. Immunizations with M-HBsAgS, M-HBsAgS-N4 and -N9 VLPs resulted in the responses with IgG2a and IgG2b dominance but also IgG1 was detected, and minor levels of IgM antibodies (Fig. 5C).

3.4. Functional activity of vaccine-induced antibodies

While high antibody titres to the NANP epitope correlate with protection, antibody fixation and activation of complement on the sporozoite surface is thought to be an additional mechanism of immunity [42]. Therefore, the ability of mouse antibodies to

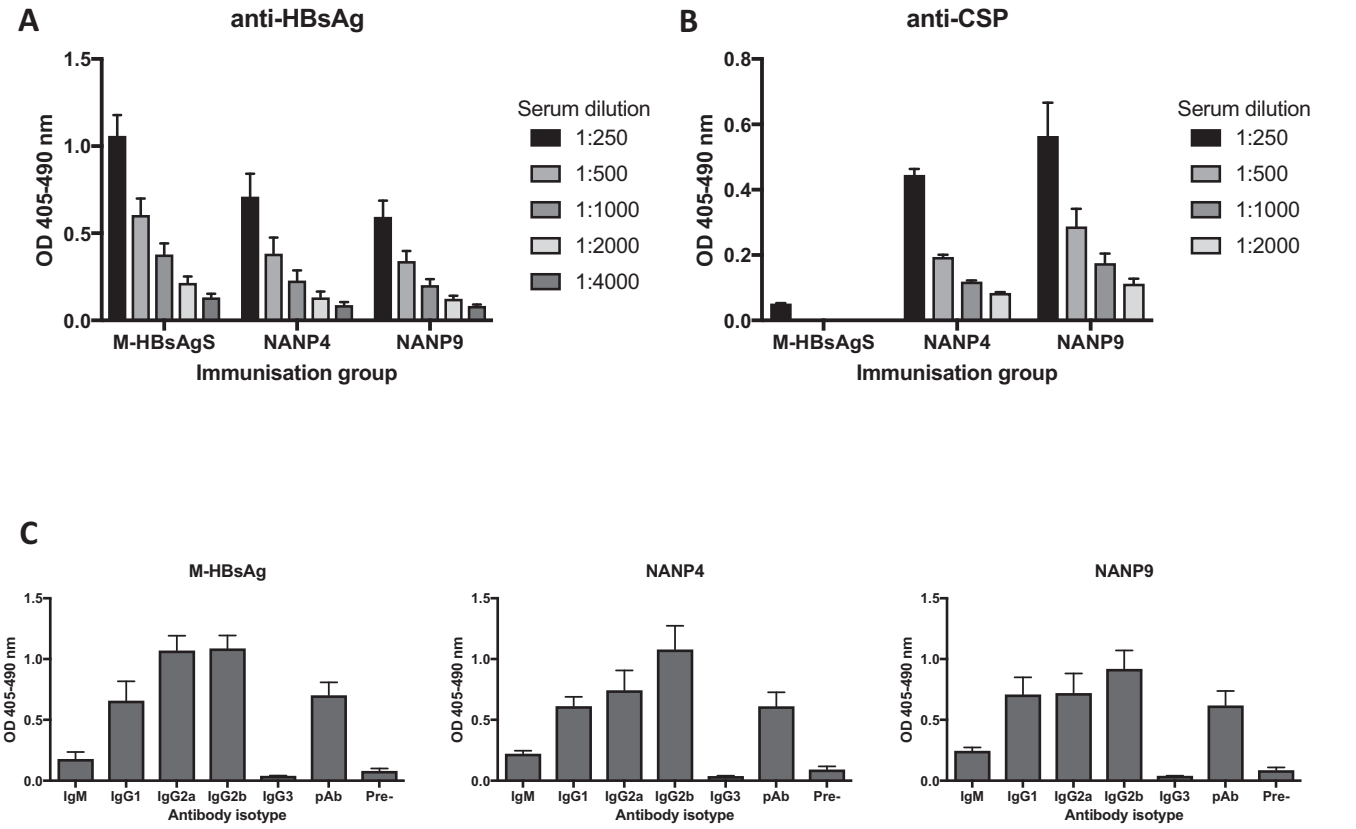

Fig. 5. Summarized endpoint titres and antibody isotypes. For the generation of antibodies, three groups of 7 mice were immunized four times at two-week intervals with 1 μg of M-HBsAgS, M-HBsAgS-N4 (NANP4) and -9 (NANP9) VLPs in the presence of 2.5 nmol CpG. Sera were collected two weeks post final immunization. Serum samples were assessed for the presence of anti-HBsAgS (A) and anti-circumsporozoite protein (CSP) (B) activity. Serum samples were also assessed for antibody isotypes using IgM, IgG1, IgG2a, IgG2b, IgG3 specific secondary antibodies, or a polyclonal anti-IgG (pAb). Sera were tested at a dilution of 1:250 using yeast-derived wild type VLPs as the target. Pre-immune serum (Pre-) represents the negative control. Graphed mean and SEM (n = 7), assay performed in duplicate.

fix the human complement component C1q, representing the first step in the classical pathway, was evaluated as an indirect measure of antibody functionality. To assess the ability of mouse sera to fix human C1q, assays were performed on full-length *P. falciparum* CSP. Antisera specific for the M-HBsAgS VLPs were used as a negative control, anti-M-HBsAgS-N4 and -N9 sera were tested for the ability to fix C1q to CSP (Fig. 6A and B). Sera generated by immunization with M-HBsAgS-N4 and -N9 VLPs fixed complement at 1:100 and 1:250 dilutions in contrast to sera derived from mice immunized with the control M-HBsAgS VLPs (Fig. 6A and B). The sera from mice immunized with M-HBsAgS-N9 appeared to have saturated the assay at both a 1:100 and 1:250 dilutions; as such an extended dilution series was performed to observe a dose-dependence in the ability to fix complement (Fig. 6C). The extended dilution of the anti-CSP antibodies for the anti-M-HBsAgS-N9 sera indicated that anti-CSP antibodies generated by vaccination with M-HBsAgS-N9 VLPs promote effective antigen-specific complement deposition at a dilution up to 1:2000. The number of the NANP repeats seems to impact on the functionality of the generated anti-CSP antibodies. The *in vitro* complement fixation assay is a good indicator of *in vivo* function against the parasite and indicates that the M-HBsAgS-N9 VLP is a promising vaccine candidate.

4. Discussion

Modern technologies allow the development of rationally-designed immunogens with the aim to generate protective immune responses against complex pathogens, such as *Plasmodium* ssp [1,3,11,12]. VLPs represent a modular system assembled from multiple subunits, which may allow the delivery of relevant antigenic sequences inserted in targeted locations to promote the preferred type of immune response [1,28,30,32]. VLPs are the antigenic components of the pre-erythrocytic RTS,S vaccine composed of wt HBsAgS subunits and chimeric HBsAgS proteins with N-terminal, CSP polypeptide-specific extensions [11,12]. To generate VLPs capable of eliciting responses against CSP, the relevant NANP-repeat sequence was expressed within the exposed and immunodominant HBsAgS loop region [8,50]. In contrast to the RTS,S vaccine, the generated M-HBsAgS-N4 and -N9 VLPs were exclusively composed of the chimeric proteins in the absence of wt HBsAgS subunits. Combining these particles with suitable adjuvanting agents, the VLPs with a high NANP-specific antigenic density may facilitate the induction of appropriate antibody isotypes and the persistence of high anti-CSP titres.

For the generation of chimeric VLP immunogens, the modified subunits must retain assembly competence. HBsAgS activity was detected in the cell culture supernatant, which is indicative of VLP assembly (Fig. 2). This was then further confirmed by density analyses, and visualization by EM (Fig. 3). Antigenicity studies with anti-HBsAgS specific mAbs demonstrated that the NANP insertions are associated with loss of HBsAgS-specific antigenic recognition (Fig. 4A). The introduction of NANP insertions, in a size dependent manner, possibly modifies the structural folding of the VLP subunit and/or alters the display of the HBsAgS antigenic loop domains, thereby selectively occluding or disrupting HBsAg epitopes within the external loop domain. Epitope loss is not restricted to neighbouring sequences to the insertion site in the loop 1 of the external

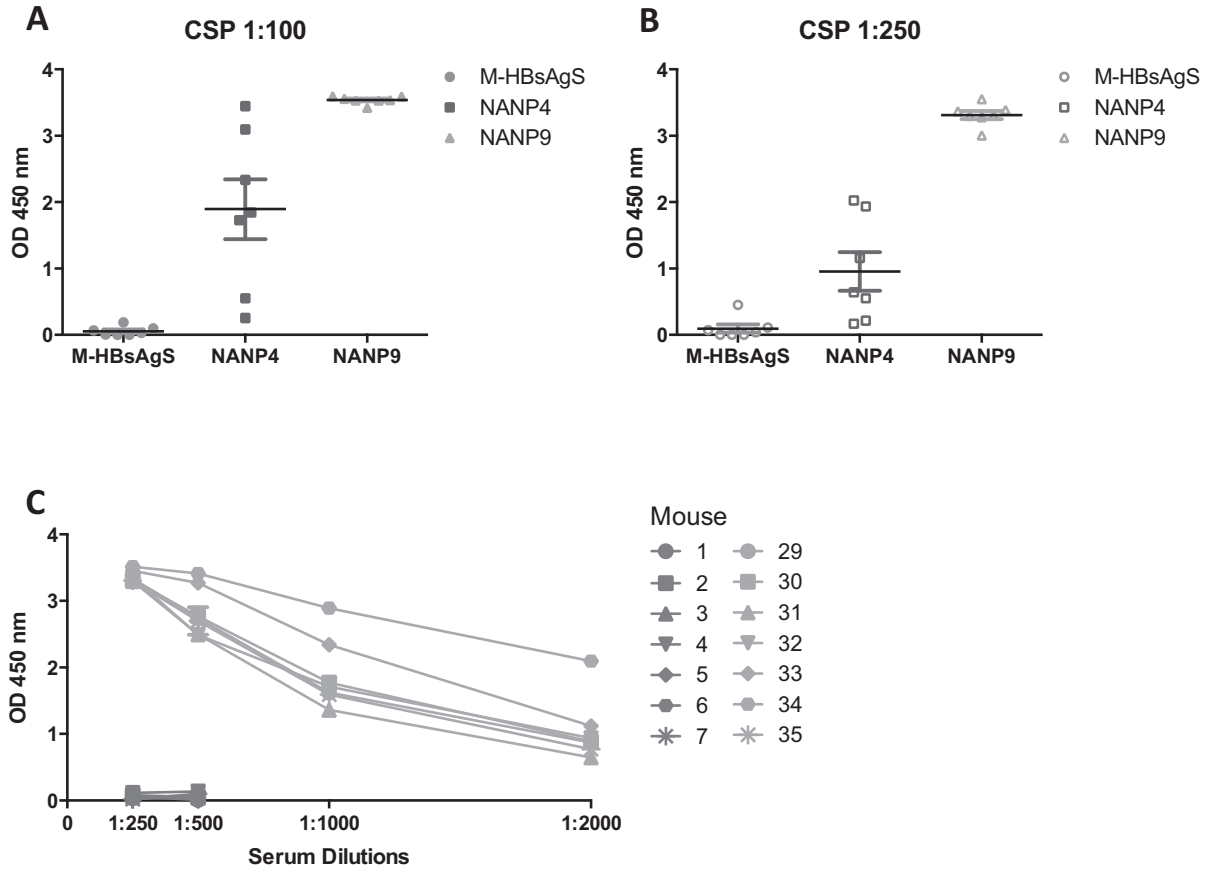

Fig. 6. Complement fixation activity of antibodies. For the generation of antibodies three groups of 7 mice were immunized four times at two-week intervals wit 1 μg M-HBsAgS, or chimeric HBsAgS VLPs with NANP4 or NANP9 inserts in the presence of 2.5 nmol CpG. The ability of the mouse sera to fix the human complement component C1q was assessed against *Plasmodium falciparum* 3D7 circumsporozoite protein (CSP) at a dilution of 1:100 (A) or 1:250 (B). The antibodies generated against the M-HBsAgS-N9 VLPs (mice 29–35) underwent extended dilutions between 1:250 and 1:2000. Sera from mice immunized with M-HBsAgS VLPs (1–7) served as control at dilutions 1:250 and 1:500 (C). Each sample was assayed in duplicate; the mean of each group is graphed ± SEM at OD_{450nm}.

hydrophilic region. For instance, mAb2 and mAb4, which are specific to epitopes in the HBsAgS C-terminal region were unable to detect the chimeric VLPs. The inability of the mAb2/mAb4 antibodies to recognize the HBsAgS epitopes is possibly due to conformational changes, or alternatively the presence of inserted foreign sequences prevents access to the corresponding epitopes. The use of polyclonal anti-HBsAgS sera confirmed that the chimeric VLPs retained HBsAgS-specific antigenicity in spite of epitope loss, and provides further evidence for the structural integrity of the HBsAgS backbone. The data are consistent with the outcomes achieved using chimeric VLPs containing hepatitis C virus-specific inserts, the partial loss of HBsAgS-specific antigenicity, and the ability of the chimeric HBsAgS proteins to be glycosylated is retained [8,28]. Importantly, the inserted NANP4 and NANP9 sequences were detected using anti-CSP antibodies (Fig. 4B). The NANP4 and NANP9 sequences are exposed as expected due to the insertion into the external hydrophilic HBsAgS loop region, and presented in the correct conformation. This is consistent with the immunization studies, which demonstrated that the anti-M-HBsAgS-N4 and -N9 VLP antibodies detect CSP.

Comparative immunogenicity studies with M-HBsAgS-N4 and M-HBsAgS-N9 VLPs demonstrated that the chimeric VLPs induce both anti-HBsAgS and anti-NANP specific antibodies (Figs. 5A, B and 6). The ability of the chimeric HBsAgS VLPs to induce anti-HBsAg antibodies is consistent with the finding that the chimeric VLPs have retained some HBsAgS-specific antigenic structures (Figs. 2C and 4A). Due to the partial HBsAgS-specific epitope loss indicated by the BioPlex assay, the breadth of the anti-HBsAgS antibody repertoire induced by the chimeric NANP-containing VLPs is possibly reduced compared to the profile induced by M-HBsAgS VLPs.

There is no significant difference in the anti-HBsAgS or anti-CSP antibody titres induced by immunizations with M-HBsAgS-N4 and M-HBsAgS-N9 VLPs, and no significant difference in the antibody isotype bias (Fig. 5). HBsAgS VLPs when co-administered with CpG oligonucleotides generate strong Th1 type responses with a primarily IgG2a isotype [53]. In mice, the IgG2a/b isotype antibodies are cytophilic, fix C1q and can activate the complement cascade [57]. The antibodies generated by immunizations with the M-HBsAgS VLPs and the chimeric VLPs with the NANP repeats in the presence of CpG induced substantial levels of IgG2a/b antibodies against HBsAgS VLPs (Fig. 5C). Surprisingly, when assessed for the ability to fix C1q to CSP, the sera from the M-HBsAgS-N9 immunization group performed more effectively than the sera from the mice immunized with M-HBsAgS-N4 (Fig. 6). The data suggest that an increased number of NANP repeats provided by the M-HBsAgS-N9 VLPs may induce antibodies with higher binding affinity to the CSP to allow efficient complement fixation outcomes.

Current and past research suggests that the development of a highly efficacious malaria vaccine may require the inclusion of multiple protein targets [58,59]. Here we established the successful generation of chimeric VLPs containing CSP-derived sequences with the antigenicity and immunogenicity of the CSP sequences being retained. This has established an approach that could be used for generating VLPs incorporating other candidate vaccine

antigens. Combining multiple different chimeric VLP types into a single vaccine may allow for the synergistic development of a more effective protective response against *Plasmodium* to prevent clinical malaria.

5. Conclusions

We have developed chimeric VLPs with the important, antigenic NANP sequence displayed in an exposed, immunodominant loop structure. We could demonstrate that the chimeric VLP subunits, M-HBsAgS-N4 and M-HBsAgS-N9 were able to form VLPs, and induced anti-NANP antibodies. Depending on the repeat number, functionally distinguished antibodies could be produced. VLPs with NANP9 repeats induced antibodies with the ability to activate complement, which could contribute to the inactivation of invading sporozoites. The ability to express the chimeric VLPs in mammalian cell lines allows additional manipulations of the HBsAgS backbone to further enhance immunogenicity [21,26]. This provides additional optimization strategies to enhance immunogenicity and longevity of immune responses, in combination with efforts to optimize adjuvanting compounds or immunization schedules. Further, the platform may be adapted for the insertion of additional *Plasmodium* target sequences to enable the development of a polyvalent vaccine.

6. Declaration of interests/Conflict of interest statement

The authors of the manuscript do not have a conflict of interest.

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References

[1] Yan D, Wei YQ, Guo HC, Sun SQ. The application of virus-like particles as vaccines and biological vehicles. Appl Microbiol Biotechnol 2015;99:10415–32.
[2] Ungaro F, Conte C, Quaglia F, Tornesello ML, Buonaguro FM, Buonaguro L. VLPs and particle strategies for cancer vaccines. Expert Rev Vaccines 2013;12:1173–93.
[3] Pushko P, Pumpens P, Grens E. Development of virus-like particle technology from small highly symmetric to large complex virus-like particle structures. Intervirology 2013;56:141–65.
[4] Zuckerman JN. Protective efficacy, immunotherapeutic potential, and safety of hepatitis B vaccines. J Med Virology 2006;78:169–77.
[5] Jilg W, Lorbeer B, Schmidt M, Wilske B, Zoulek G, Deinhardt F. Clinical evaluation of a recombinant hepatitis B vaccine. Lancet 1984;2(8413):1174–5.
[6] Harro CD, Pang YYS, Roden RBS, Hildesheim A, Wang Z, Reynolds MJ, et al. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. J Natl Cancer Inst 2001;93:284–92.
[7] Garland SM, Kjaer SK, Munoz N, Block SL, Brown DR, DiNubile MJ, et al. Impact and effectiveness of the quadrivalent human papillomavirus vaccine: a systematic review of 10 years of real-world experience. Clin Infect Dis 2016;63:519–27.
[8] Netter HJ, Macnaughton TB, Woo WP, Tindle R, Gowans EJ. Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes. J Virol 2001;75:2130–41.
[9] Moffat JM, Cheong WS, Villadangos JA, Mintern JD, Netter HJ. Hepatitis B virus-like particles access major histocompatibility class I and II presentation pathways in primary dendritic cells. Vaccine 2013;31:2310–6.
[10] Jennings GT, Bachmann MF. Immunodrugs: therapeutic VLP-based vaccines for chronic diseases. Ann Rev Pharmacol Toxicol 2009;49:303–26.
[11] Rutgers T, Gordon D, Gathoye AM, Hollingdale M, Hockmeyer W, Rosenberg M, et al. Hepatitis B surface antigen as carrier matrix for the repetitive epitope of the circumsporozoite protein of *Plasmodium falciparum*. Biotechnology 1988;6:1065–70.
[12] Gordon DM, McGovern TW, Krzych U, Cohen JC, Schneider I, LaChance R, et al. Safety, immunogenicity, and efficacy of a recombinantly produced *Plasmodium falciparum* circumsporozoite protein-hepatitis B surface antigen subunit vaccine. J Infect Dis 1995;171:1576–85.
[13] Olotu A, Fegan G, Wambua J, Nyangweso G, Leach A, Lievens M, et al. Seven-year efficacy of RTS, S/AS01 malaria vaccine among young African children. New Engl J Med 2016;374:2519–29.
[14] RTS,S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet*, 386, 2015, 31–45.
[15] Seeger C, Zoulim F, Mason WS. Hepadnaviruses. In: Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Wolters Kluver, Lippincott Williams & Wilkins; 2013. p. 2185–221.
[16] Bruss V. Hepatitis B virus morphogenesis. World J Gastroenterol 2007;13:65–73.
[17] Peterson DL. The structure of hepatitis B surface antigen and its antigenic sites. BioEssays 1987;6:258–62.
[18] Gavilanes F, Gonzalez-Ros JM, Peterson DL. Structure of hepatitis B surface antigen. J Biol Chem 1982;257:7770–7.
[19] Gilbert RJC, Beales L, Blond D, Simon MN, Lin BY, Chisari FV, et al. Hepatitis B small surface antigen particles are octahedral. Proc Natl Acad Sci USA 2005;102:14783–8.
[20] Mangold CM, Unckell F, Werr M, Streeck RE. Analysis of intermolecular disulfide bonds and free sulfhydryl groups in hepatitis B surface antigen particles. Arch Virol 1997;142:2257–67.
[21] Cheong WS, Hyakumura M, Yuen L, Warner N, Locarnini S, Netter HJ. Modulation of the immunogenicity of virus-like particles composed of mutant hepatitis B virus envelope subunits. Antiviral Res 2012;93:209–18.
[22] Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 1982;298:347–50.
[23] Janowicz ZA, Melber K, Merckelbach A, Jacobs E, Harford N, Comberbach M, et al. Simultaneous expression of the S and L surface antigens of hepatitis B, and formation of mixed particles in the methylotropic yeast, *Hansenula polymorpha*. Yeast 1991;7:431–43.
[24] Cregg JM, Tschopp JF, Stillman C, Siegel R, Akong M, Craig WS, et al. High-level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast, *Pichia pastoris*. Biotechnology 1987;5:479–85.
[25] Al-Barwani F, Young SL, Baird MA, Larsen DS, Ward VK. Mannosylation of virus-like particles enhances internalization by antigen presenting cells. PLoS ONE 2014;9:e104523.
[26] Hyakumura M, Walsh R, Thaysen-Andersen M, Kingston NJ, La M, Lu L, et al. Modification of asparagine-linked glycan density for the design of hepatitis B virus virus-like particles with enhanced immunogenicity. J Virol 2015;89:11312–22.
[27] Johannssen T, Lepenies B. Glycan-based cell targeting to modulate immune responses. Trends Biotech 2017;35:334–46.
[28] Vietheer PTK, Boo I, Drummer HE, Netter HJ. Immunizations with chimeric hepatitis B virus-like particles to induce potential anti-hepatitis C virus neutralizing antibodies. Antiviral Therapy 2007;12:477–87.
[29] Beaumont E, Patient R, Hourioux C, Dimier-Poisson I, Roingeard P. Chimeric hepatitis B virus/hepatitis C virus envelope proteins elicit broadly neutralizing antibodies and constitute a potential bivalent prophylactic vaccine. Hepatology 2013;57:1303–13.
[30] Cheong WS, Reiseger J, Turner SJ, Boyd R, Netter HJ. Chimeric virus-like particles for the delivery of an inserted conserved influenza A-specific CTL epitope. Antiviral Res 2009;81:113–22.
[31] Phogat S, Svehla K, Tang M, Spadaccini A, Muller J, Mascola J, et al. Analysis of the human immunodeficiency virus type 1 gp41 membrane proximal external region arrayed on hepatitis B surface antigen particles. Virology 2008;373:72–84.
[32] Delpeyroux F, Chenciner N, Lim A, Malpièce Y, Blondel B, Crainic R, et al. A poliovirus neutralization epitope expressed on hybrid hepatitis B surface antigen particles. Science 1986;233:472–5.
[33] Casares S, Brumeanu T-D, Richie TL. The RTS, S malaria vaccine. Vaccine 2010;28:4880–94.
[34] Foquet L, Hermsen CC, van Gemert GJ, Van Braeckel E, Weening KE, Sauerwein R, et al. Vaccine-induced monoclonal antibodies targeting circumsporozoite protein prevent *Plasmodium falciparum* infection. J Clin Invest 2014;124:140–4.
[35] White MT, Bejon P, Olotu A, Griffin JT, Bojang K, Lusingu J, et al. A combined analysis of immunogenicity, antibody kinetics and vaccine efficacy from phase 2 trials of the RTS,S malaria vaccine. BMC Med 2014;12:117.
[36] Chappel JA, Rogers WO, Hoffman SL, Kang AS. Molecular dissection of the human antibody response to the structural repeat epitope of *Plasmodium falciparum* sporozoite from a protected donor. Malaria J 2004;3:28.
[37] Cohen S, McGregor IA, Carrington S. Gamma-globulin and acquired immunity to human malaria. Nature 1961;192:733–7.
[38] Schwenk R, Asher LV, Chalom I, Lanar D, Sun P, White K, et al. Opsonization by antigen-specific antibodies as a mechanism of protective immunity induced

by *Plasmodium falciparum* circumsporozoite protein-based vaccine. Parasite Immunol 2003;25:17–25.
[39] Chaudhury S, Ockenhouse CF, Regules JA, Dutta S, Wallqvist A, Jongert E, et al. The biological function of antibodies induced by the RTS, S/AS01 malaria vaccine candidate is determined by their fine specificity. Malaria J 2016;15:301.
[40] Ballou WR, Rothbard J, Wirtz RA, Gordon DM, Williams JS, Gore RW, et al. Immunogenicity of synthetic peptides from circumsporozoite protein of *Plasmodium falciparum*. Science 1985;228:996–9.
[41] Young JF, Hockmeyer WT, Gross M, Ballou WR, Wirtz RA, Trosper JH, et al. Expression of *Plasmodium falciparum* circumsporozoite proteins in Escherichia coli for potential use in a human malaria vaccine. Science 1985;228:958–62.
[42] Kurtovic L, Behet MC, Feng G, Reiling L, Chelimo K, Dent AE, et al. Human antibodies activate complement against *Plasmodium falciparum* sporozoites, and are associated with protection against malaria in children. BMC Med 2018;16:61.
[43] Behet MC, Kurtovic L, van Gemert GJ, Haukes CM, Siebelink-Stoter R, Graumans W, et al. The complement system contributes to functional antibody-mediated responses induced by immunization with *Plasmodium falciparum* malaria sporozoites. Infect Immun 2018;86. https://doi.org/10.1128/IAI.00920-17.
[44] John CC, Moormann AM, Pregibon DC, Odada Sumba P, Mchugh MM, Narum DL, et al. Correlation of high levels of antibodies to multiple pre-erythrocytic Plasmodium falciparum antigens and protection from infection. Am J Trop Med Hyg 2005;73:222–8.
[45] Del Giudice G, Lambert P-H, Mendis K, Pessi A, Tanner M. Antibody responses to *Plasmodium falciparum* and *P. vivax* sporozoites in areas with stable and unstable malaria. Bull World Health Org 1990;68(Suppl):191–6.
[46] Campo JJ, Sacarlal J, Aponte JJ, Aide P, Nhabomba AJ, Dobaño C, et al. Duration of vaccine efficacy against malaria: 5th year of follow-up in children vaccinated with RTS,S/AS02 in Mozambique. Vaccine 2014;32:2209–16.
[47] Greenwood BM, Targett GAT. Malaria vaccines and the new malaria agenda. Clin Microbiol Infect 2011;17:1600–7.
[48] Regules JA, Cicatelli SB, Bennett JW, Paolino KM, Twomey PS, Moon JE, et al. Fractional third and fourth dose of RTS, S/AS01 malaria candidate vaccine: a phase 2a controlled human malaria parasite infection and immunogenicity study. J Infect Dis 2016;214:762–71.
[49] Malaria Vaccine Funders Group. Malaria Vaccine Technology Roadmap; 2013.
[50] Stirk HJ, Thornton JM, Howard CR. A topological model for hepatitis B surface antigen. Intervirology 1992;33:148–58.
[51] Boyle MJ, Reiling L, Feng G, Langer C, Osier FH, Aspeling-Jones H, et al. Human antibodies fix complement to inhibit *Plasmodium falciparum* invasion of erythrocytes and are associated with protection against malaria. Immunity 2015;42:580–90.
[52] Kim CY, Tilles JG. Purification and biophysical characterization of hepatitis B antigen. J Clin Invest 1973;52:1176–86.
[53] Lin L, Gerth AJ, Peng SL. CpG DNA redirects class-switching towards "Th1-like" Ig isotype production via TLR9 and MyD88. Eur J Immunol 2004;34:1483–7.
[54] Davis HL, Weeranta R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–6.
[55] Seino J, Eveleigh P, Warnaar S, van Haarlem LJM, van Es LA, Daha MR. Activation of human complement by mouse and mouse/human chimeric monoclonal antibodies. Clin Exp Immunol 1993;94:291–6.
[56] Koolwijk P, Boot JHA, Griep R, Bast BJEG. Binding of the human complement subcomponent C1q to hybrid mouse monoclonal antibodies. Mol Immunol 1991;28:567–76.
[57] Carroll RC, Rubinstein E, Worthington RE, Boucheix C. Extensive C1q-complement initiated lysis of human platelets by IgG subclass murine monoclonal antibodies to the CD9 antigen. Thromb Res 1990;59:831–9.
[58] Boes A, Spiegel H, Voepel N, Edgue G, Beiss V, Kapelski S, et al. Analysis of a multi-component multi-stage malaria vaccine candidate - tackling the cocktail challenge. PLoS ONE 2015;10:e0131456.
[59] Osier FH, Mackinnon MJ, Crosnier C, Fegan G, Kamuyu G, Wanaguru M, et al. New antigens for a multicomponent blood-stage malaria vaccine. Sci Transl Med 2014;6. 247ra102.