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Supplementary Information for

Optimization of Plasmodium Falciparum Circumsporozoite Protein Repeat Vaccine using the Tobacco Mosaic Virus Platform

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Supplemental Methods

Ethics: Animal procedures were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition. All procedures were reviewed and approved by the Walter Reed Army Institute of Research's Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Structure Prediction: TMV-NPNA structures were generated by comparative modeling using Rosetta Protein Structure Prediction Server; (http://robetta.bakerlab.org) (1, 2). Images were generated using PyMOL software (Schrodinger, New York NY).

Expression and purification of GST fusion proteins: Recombinant proteins representing the N-terminal, Repeat and C-terminal regions of *P. falciparum* CSP were designed **(*SI Appendix,* Fig S2A)** and cloned into pGEX-His expression vector (GE health Care Life Sciences, Chicago IL). GST fusion proteins were purified on Glutathione-Sepharose column as per manufacturer's instructions **(*SI Appendix,* Fig S2B)**. The identity and correct folding of proteins were confirmed by ELISA using mouse mabs against the C-terminal and repeat regions. Briefly, plates were coated with GST fusion proteins and mouse mabs were titrated to determine binding specificity. Abs against C-term (mAbs:1B10, 1E3, 1F8, 2F12) recognized the C-term GST fusion protein and Repeat Abs (mAbs:3C2, 3D9, 5B12, 8D7) recognized the Repeat GST fusion protein **(*SI Appendix,* Fig S2C)** (3).

Inhibition of Liver Stage Development Assay (ILSDA): The NF54 strain of *Plasmodium falciparum* (Pf) sporozoites (obtained from salivary gland dissections) were mixed with a positive control anti-CSP monoclonal NFS1 or the test sera (4). All sera were mixed with the sporozoites at room temperature for 20 minutes and this sporozoite-Ab mix was added to wells containing cryopreserved human hepatocytes (BioIVT, Baltimore MD). Incubation was carried out at 37°C for 3 hours to allow sporozoites to infect hepatocytes. The hepatocytes were washed with fresh culture media to remove non-invaded sporozoites and incubated at 37°C for 96 hours. The RNA from the cells was purified and used in a quantitative real-time PCR to determine Pf 18S rRNA levels. Percent inhibition was calculated against a pre-immune negative control. Mouse ILSDA was performed on pooled sera at 1:80. Rhesus ILSDA was performed on individual sera tested at 1:200, 1:300 or 1:80 dilution and percentage inhibition was determined against a pre-immune control.

Detection of CSP on Sporozoite Surface by Indirect Immunofluorescence Assay (IFA): NF54 strain *P. falciparum* sporozoites were spotted onto slides and

fixed in 100% methanol and air dried. Wells were blocked with 1% goat serum for 30 minutes at room temperature. Serum samples were diluted 1:1600 in 1% goat serum and added to wells for 1 hour. Serum was aspirated and slides were washed with PBS-Tween-20. FITC-conjugated secondary Ab was then added at a dilution of 1:5000 and incubated for 1 hour at room temperature in the dark. Secondary Ab was aspirated off, slides were washed with PBS, and allowed to air dry. A small drop of anti-fade was added to each well prior to imaging by fluorescent microscopy under 40X power.

Production and purification of NPNA fused TMV particles: Genes for the TMV particles were optimized for high level expression in *E. coli*. The genes were cloned in an expression plasmid pD451-SR (Atum, Newark, CA) and transformed into BL21 (DE3) cells. The cells were grown in 1 L culture in shake flasks, and induced at an OD_{600} of 0.6 to 0.8 using 0.1 mM IPTG at 37^{o} C. After 2 hr induction the cells were harvested by centrifugation and stored at -80°C. A typical batch started with 2 g of *E.coli* paste suspended in 100 mL of 20 mM Tris, 20 mM sodium chloride pH 9.0 (Buffer A). The sample was kept on ice, a protease inhibitor cocktail was added and then microfluidized followed by centrifugation at 26,000 x g for 30 min. The pellet was solubilized in 20 mM Tris, 20 mM sodium chloride, 7M urea pH 9.0 (Buffer B) and centrifuged at 26,000 x g for 30 min. Supernatant was loaded onto 6 mL Ni-NTA column equilibrated in Buffer B. The column was washed with 60 mL of Buffer B, and 70 mL of Buffer B containing 20 mM imidazole. Protein was eluted with 250 mM imidazole in Buffer B and peak fractions diluted 10X with Buffer B. This diluted protein was passed through a 3 mL Q-Sepharose column equilibrated in Buffer B and the flow-through was diluted to OD ~0.1 with Buffer B and 100 mL dialyzed against 4.5 L of Buffer A containing 0.05% β-mercaptoethanol overnight at 4^{o} C. The following day dialysis buffer was exchanged with 4.5 L of 20 mM sodium phosphate dibasic, 20 mM sodium chloride pH 7.4 (Buffer C). After 8 hrs, the dialysis buffer was exchanged with 4.5 L of fresh Buffer C, and dialysis was continued for the overnight. Protein was filtered through 0.22µm filter, concentrated on a Amicon Ultra Centrifugal Filter (MilliporeSigma) to 15 mL. Protein purity was confirmed by SDS-PAGE and Coomassie blue staining. A western blot was conducted using a repeat-specific monoclonal Ab 2A10 obtained from the MR4 repository (https://www.beiresources.org/About/MR4.aspx). Vaccine proteins were stored at -80°C until further use.

Negative Stain Electron Microscopy: TMV VLPs were diluted to concentrations between 0.1 mg/mL to 0.5 mg/ mL in 20 mM sodium phosphate dibasic, 20 mM sodium chloride pH 7.4. 3 µl of diluted TMV sample was then applied onto Carbon-Formvar 300 Mesh copper grids (EM sciences) for 5 minutes and excess liquid was blotted away with bibulous paper. Grids were then stained with 3 µl of 1-2% uranyl acetate for 10 seconds before wicking of excess liquid with bibulous paper. Grids were imaged on at 40,000X.

Expression of mAbs 317, 580 and CIS43: The heavy and light chain sequences of mAb 317 derived from PDB 6AXL (5), mAb 580 sequence derived from PBD 5BK3 (6) and mAb CIS43 sequence derived from PDB 6B5M (7) were grafted onto the human IgG1 sequence. Synthetic genes were synthesized expressed using HEK293.SUS cells. Expression was carried out at 10 mL scale and after expression the culture supernatant was passed over Protein A to capture the IgG. Eluted IgG were immediately neutralized and buffer exchanged into PBS. After dialysis the IgG concentrations were quantified by OD_{280} and quality was determined using SDS-PAGE under reduced and non-reduced conditions. Aggregation of products was determined using SEC-HPLC on a 300 Å pore size column using PBS as the running buffer. IgG were concentrated and snap frozen in liquid nitrogen.

Monoclonal Ab ELISA: TMV-NPNAx3, -x4, -x5, -x7, -x10, -x20, and FL-CSP (100 ng/well) were coated (Thermo Scientific, Rochester, NY, USA) overnight at 4°C and all subsequent incubations were at room temperature (RT). Plates were washed three times with PBS containing 0.05% Tween-20 (wash buffer) and blocked 1.5 hour with PBS containing 0.5% casein and 1% Tween-20. Plates were washed three times and incubated for 2 hours with either mAb 317, CIS43 or mAb 580 at a starting concentration of 1000 ng/ mL serially diluted 3-fold down the plate in PBS containing 0.5% casein and 0.05% Tween-20 (dilution buffer). Plates were washed three times and 1:4000 dilution of goat anti-human IgG-HRP secondary Ab was incubated for 1 hour. Plates were then washed a final four times before being developed for 1 hour with ABTS 2-component substrate (KPL) and stopped by adding SDS to a final 2% concentration. Absorbance was read at 415 nm (OD_{415}). Potency was determined as the concentration (ng/mL) of mAb required where OD_{415}=1.000 was achieved using Gen5^{TM} 4-parameter nonlinear regression (BioTek, Winooski, VT, USA).

TMV ELISA: ELISA plates (Immulon 2HB) were coated overnight at 4°C with 100 ng/well of TMV-NPNAx3 or TMV-NPNAx20. Plates were washed three times with PBS containing 0.5% Tween-20 (wash buffer) and blocked for 1.5 hours with PBS containing 0.5% casein and 1% Tween-20. Polyclonal sera from individual mice immunized with TMV-NPNAx3, -x4, -x5, -x7, -x10, -x20, or FL-CSP in Addavax adjuvant were plated at 1:8000 dilution and incubated for 2 hrs. Likewise, sera from mice immunized with TMV-Loop, TMV-NT, TMV-CT were plated at 1:8000. Rest of the ELISA was similar to that described above. OD_{415} after 1 hr was determined and the ratio of the ODs on NPNAx3 / NPNAx20 plate antigen were plotted.

Formulation and Immunization of mice: Female C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were immunized intramuscularly (IM) with 50 µl of the vaccines alternating injection in right and left rear thighs at 0, 3, and 6 weeks. Animals were bled at three week intervals after the first and second immunizations and the day before challenge. The GST fusion protein experiment involved vaccinating mice (n=10) three times at three week interval with 10 µg subunit

protein or 10 µg of a nearly full-length *P. falciparum* CS protein (FL-CSP (3)) using AFLQ adjuvant. All other vaccinations were done using 1.25 or 2.5 µg protein in AddaVax^{TM} adjuvant (Invivogen, San Diego, CA) or ALFQ adjuvant as previously described (8)..

Malaria challenge: Protective efficacy of vaccines was assessed using transgenic *P. berghei* sporozoites expressing a functional full-length *P. falciparum* CSP gene. Animals were challenged 15 days after the last immunization with 100 µL intravenous (IV) injection of 3000 sporozoites into the caudal vein, as described by Porter *et al.* (9). Blood-stage parasitemia was detected by microscopy of Giemsa-stained thin blood smear. Animals were considered protected if parasitemia was not detected during a two-week observation period immediately following challenge.

Mouse IgG titer and subclass ELISA: $(NANP)_6$ peptide (20 ng/well) or FL-CSP (200 ng/well) were coated on Immulon 2HB 96-well flat bottom microtiter plates and ELISA to detect total IgG or IgG subclasses was carried out as described previously (8).

Mouse avidity ELISA: ELISA plates (Immulon 2HB) were coated overnight at 4°C with 100 ng/well of $(NANP)_6$ peptide. Plates were washed three times with PBS containing 0.5% Tween-20 (wash buffer) and blocked for 1.5 hours with PBS containing 0.5% casein and 1% Tween-20. Samples of mouse polyclonal serum were plated at 1:6400 and 1:12800 dilution and incubated for 2 hrs. To test for avidity, 50 µl of 2M sodium thiocyanate (NaSCN) or PBS (control) was added to wells for 15 min, followed by washing with wash buffer. Bound Abs were detected by incubation with goat anti-mouse secondary Ab conjugated to HRP (1:4000). Plates were then washed, developed, and read as described in the monoclonal Ab ELISA section. Percentage OD_{415} in the corresponding NaSCN/PBS wells was used to determine the avidity index.

Rhesus Macaques: Eighteen adult rhesus macaques of Indian or Chinese origin were housed at WRAIR animal facility and used under the IACUC-approved protocol. Rhesus were colony bred (Alice, Texas) and their ages ranged from 8-11 years old. They were divided into 3 groups (n=6). All monkeys were seronegative for macacine herpesvirus 1, measles, simian retrovirus, simian immunodeficiency virus (SIV), simian T cell leukemia virus, and tuberculin skin test. Additionally, all rhesus were pre-screened for any preexisting Abs against CSP and the immune-stimulator MPL. Animals were pair-housed, fed a commercial diet (Lab Diet 5038, Purina Mills International), provided water ad libitum, and diet was supplemented with a variety of fresh fruits and vegetables. Environmental enrichment was provided in accordance with WRAIR Veterinary Service Program standard operating procedures. Animal cages were cleaned daily and sanitized bimonthly. Automatic lighting on a 12:12 hour cycle.

Vaccine Formulation and Immunization of Rhesus: Groups of 6 or 4 animals were given 3 vaccinations of TMV-NPNAx5 (20 or 40 µg) or soluble CSP (20 µg) along with 1 mL ALFQ that contained 100 µg QS21 and 200 µg 3D-PHAD™ per dose (8). Antigen with the adjuvant (1:1 volume) was mixed on a slow rotary platform for 1 hour prior to vaccination. All rhesus macaques were sedated using Ketamine 11 mg/kg and Acepromazine 0.55 mg/kg, given intramuscularly (IM). Once sedated the vaccine injection site was shaved, cleaned and disinfected with 70% isopropyl alcohol. The vaccines was injected IM in the hind limb, alternating between left and right thigh for each injection. Monkeys were monitored for any signs of local and systemic toxicity as described (10). Sera was collected before vaccination (pre-immune) and at various time points after vaccination for analysis.

Rhesus IgG titer and avidity ELISA: ELISA titer and avidity against the $(NPNA)_6$ peptide was measured by the International Malaria Serology Laboratory, WRAIR, using a human $(NANP)_6$ repeat ELISA protocol described previously (10, 11).

Statistical Analysis: ELISA data were log transformed and multiple comparisons were made by ANOVA with p-values corrected by Tukey's method (GraphPad Prism software, La Jolla, CA). Statistically significant difference in group means was indicated in figures as **** ($p < 0.0001$), *** ($p < 0.001$), ** ($p< 0.01$), or * ($p < 0.05$). All comparisons between two groups were made using a 2-tailed T-test. Correlation was determined using a non-parametric Spearman correlation test and protection outcomes were compared using Fisher's Exact Test.

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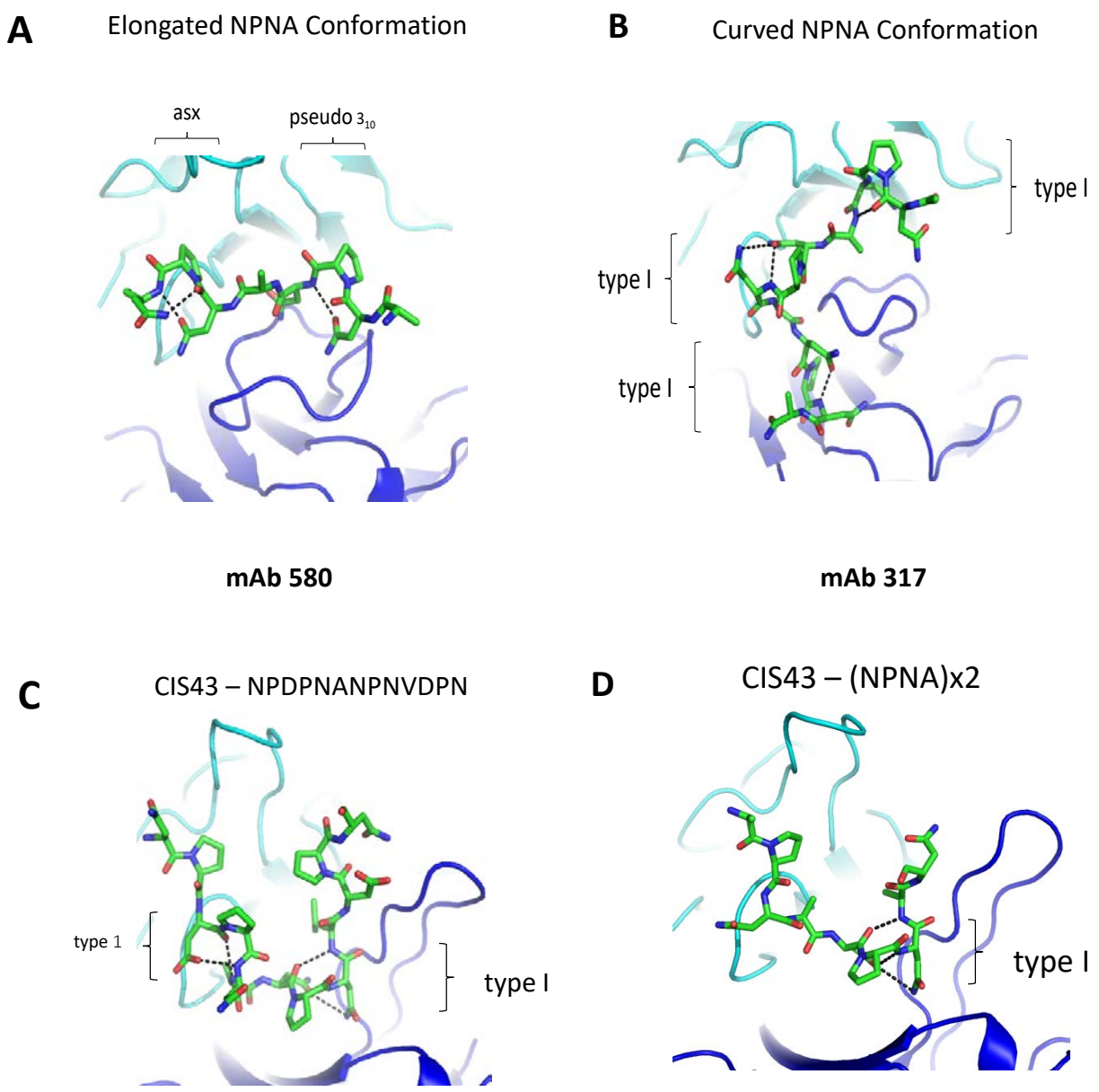


<u>Figure S1</u> – Structures of epitopes for mAbs 580, 317 and CIS43: A) Heavy and light chain structures of mAb 580 sequence co-crystallized with NPNAx2 derived from PBD 6AZM (6); **B)** mAb 317 derived from PDB 6AXL (5) co-crystalized with NPNAx3 peptide; **C, D)** mAb CIS43 derived from PBD 6B5M co-crystallized with the junctional or the NPNAx2 peptide (7). The mAb 580 binds to an elongated conformation and mAb 317 binds to a curved conformation of the NPNA repeat epitope. Ig heavy chain, dark blue; light chain, light blue; NPNA peptide, green.

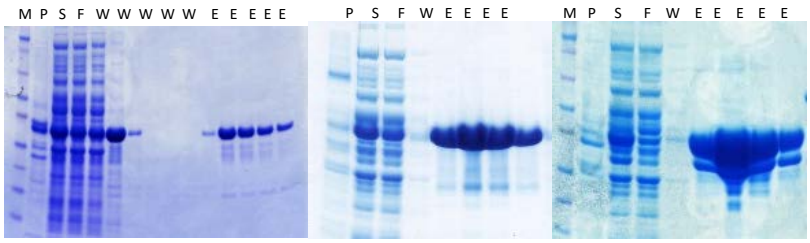
A: Sequence Boundaries

NT-CSP (Y_{26}-P_{104}):
YGSSSNTRVLNELNYDNAGTNLYNELEMNYYGKQENWYSLKKNSRSLGENDDGNNEDNEKLRKPKHKKLK
QPADGNPN.

Repeat-CSP (L_{94}-N_{284}):
LKQPADGNPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANPNANP
NANPNANPNANPNANPNKNNQGNGQGHN.

CT-CSP ($K_{274}L_{387}$):
KNNQGNGQGHNMPNDPNRNVDENANANSAVKNNNNEEPSDKHIKEYLNKIQNSLSTEWSPCSVTCGNGI
QVRIKPGSANKPKDELDYANDIEKKICKMEKCSSVFNVVNSSIGL.

B: Protein Profiles



C: mAb ELISA

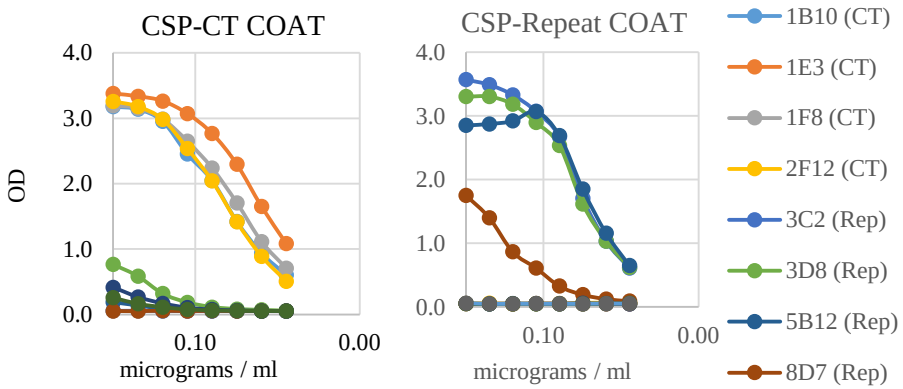


Figure S2 – GST-fusion proteins and their characterization using mouse mAbs. A) GST fusion proteins with the given sequence boundaries were expressed in *E. coli*. Shaded residues were common between two proteins. **B)** Coomassie blue stained SDS-PAGE gels showing the purity of GST protein eluted from the GST-sepharose columns. M, marker; P, pellet; S, supernatant; F, flow-through; W, wash; E, elution. **C)** Repeat-CSP and CT-CSP proteins coated on an ELISA plate were tested for reactivity to mouse monoclonal Abs against the C-terminal cysteine rich region (mAbs against FL-CSP antigen labelled as CT: 1B10, 1E3, 1F8, 2F12) or repeat region (mAbs against the NPANx5 antigen labelled as Rep: 3C2, 3D9, 5B12, 8D7). MAb concentration plotted against OD_{415}. Data shows GST fusion proteins reacting to the appropriate region-specific mAbs.

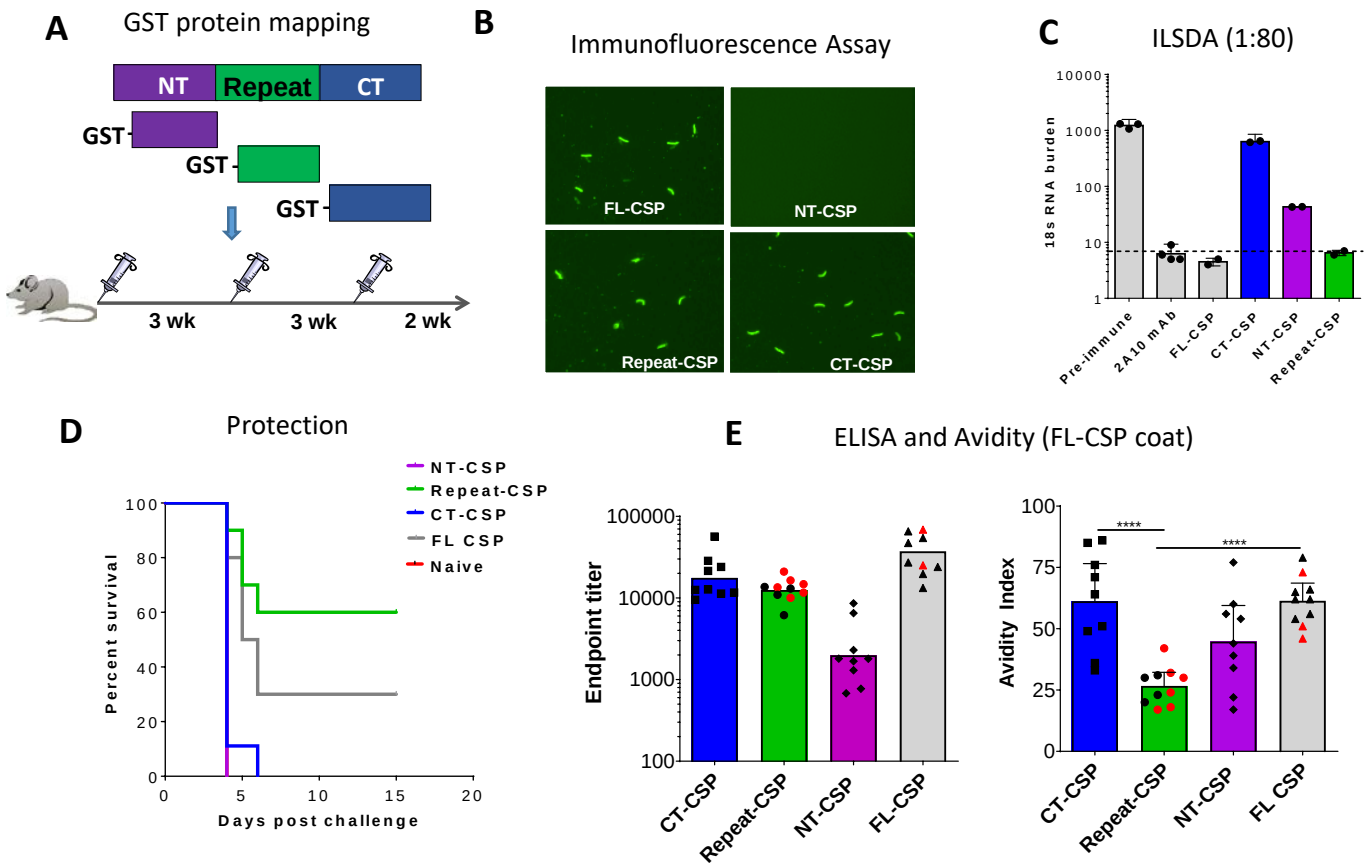


Figure S3 - Mapping inhibitory regions of CSP using mouse transgenic parasite challenge model. A) Cartoon representation of GST fusion proteins and outline of the C57Bl/6 mouse study. Three doses of 10 µg GST fusion NT-CSP, Repeat-CSP, CT-CSP and FL-CSP formulated in ALFQ (50 µl) were administered IM at 3 week intervals (n=10). At 2 weeks after third immunization sera were assayed and mice challenged with transgenic *P. berghei* expressing the full-length PfCSP gene (9). **B)** Immune-fluorescence assay (at 1:1600 serum pool dilution) show reactivity to NF54 strain of *P. falciparum* sporozoites. **C)** Inhibition of Liver Stage Development Activity (ILSDA) performed in duplicate on pooled sera using NF54 strain *P. falciparum* sporozoites and primary human hepatocytes (1:80 serum dilution). Parasite burden estimated by 18S rRNA qPCR. A repeat specific mAb 2A10 (10 µg/mL) was used as the positive control. Dotted line indicates 2 log reduction of parasite burden compared to the pre-immune serum pool. **D)** Survival curves of mice vaccinated with NT-CSP, Repeat-CSP, CT-CSP or the FL-CSP and challenged with transgenic parasites. Protected mice had no blood-stage infection until 15 days post challenge. **E)** FL CSP coated ELISA and avidity. Titer was defined as the serum dilution needed to reach OD=1 and avidity index was the ratio of ELISA titer using sodium thiocynate or with PBS wash. ELISA titer is geometric mean and avidity is Mean±SE; red dots are protected and black dots are unprotected mice. These results showed that Abs to FL-CSP, Repeat-CSP, and CT-CSP reacted with *P. falciparum* sporozoite surface while NT-CSP Abs showed no reactivity. ILSDA showed that anti-Repeat and FL-CSP along with a positive control mAb 2A10 (anti-repeat) exhibited >2 log-fold reduction in *P. falciparum* 18S rRNA burden in human hepatocytes. Transgenic parasite challenge resulted in 100% infection in naïve control mice by day 4 and protection was observed only in the Repeat-CSP and FL-CSP vaccinated mice (60 and 30% respectively). Thus despite the induction of high titer and avid Abs, no anti-parasitic effects were observed if a vaccine lacked the NPNA repeat region.

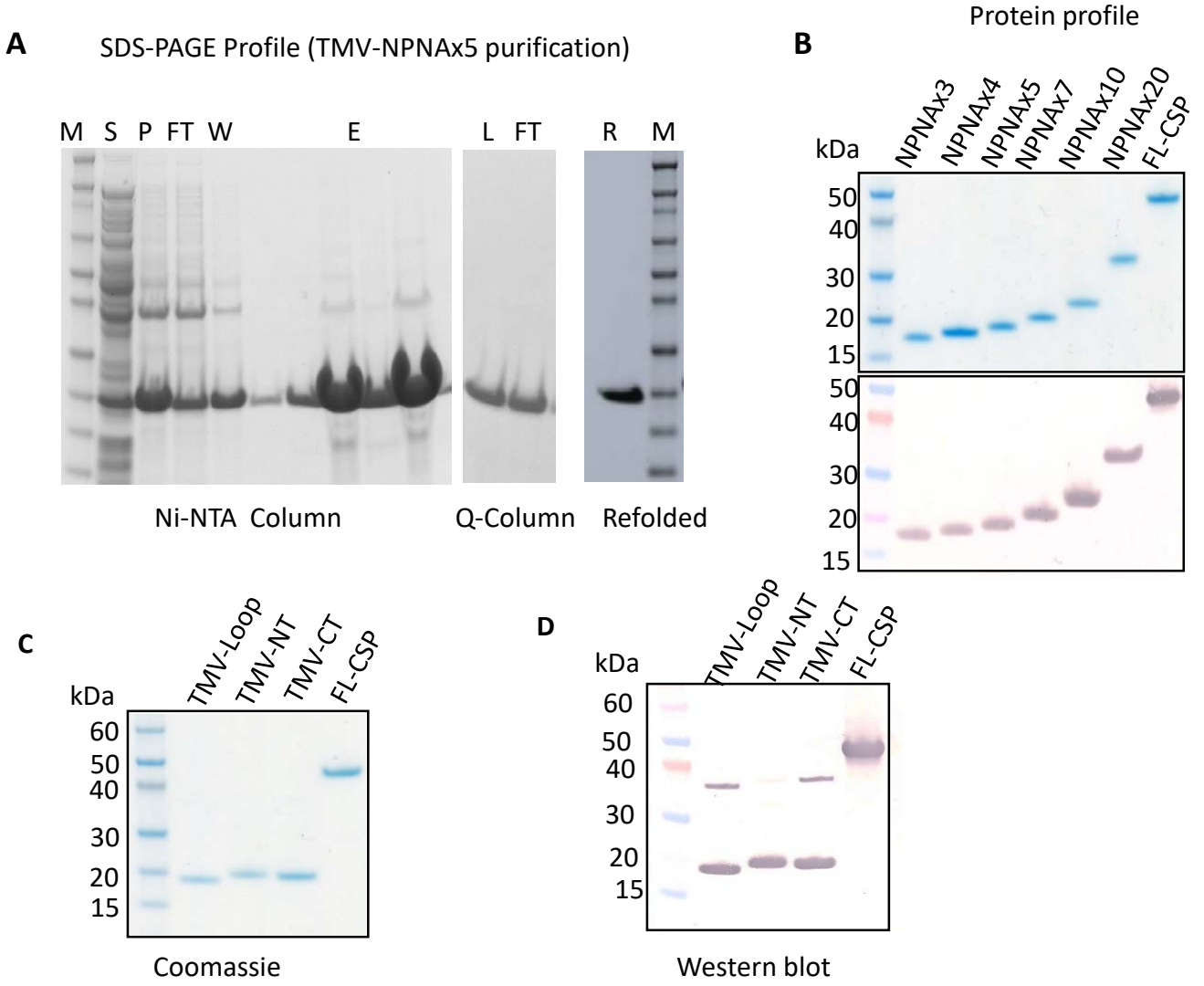


Figure S4 - Coomassie blue stained SDS-PAGE and Western Blots: A) Purification of a representative TMV vaccine (NPNAx5). Ni-NTA Chromatography: Soluble fraction (S), urea solubilized pellet (P); flow-through of Ni column (FT); wash (W) and Ni-Elution fractions (E). Q-Sepharose chromatography: Column load (L); flow-thorough (FT). Refolded final product (R) used to vaccinate mice. Molecular weight marker (M). **B)** Coomassie blue stained gel of NPNAx3 to x20 products (top) and Western blot (bottom) with mouse mAb 2A10. **C, D)** Coomassie blue and mAb 2A10 western blots for TMV-Loop, TMV-NT and TMV-CT proteins. FL-CSP run as the positive control on gels.

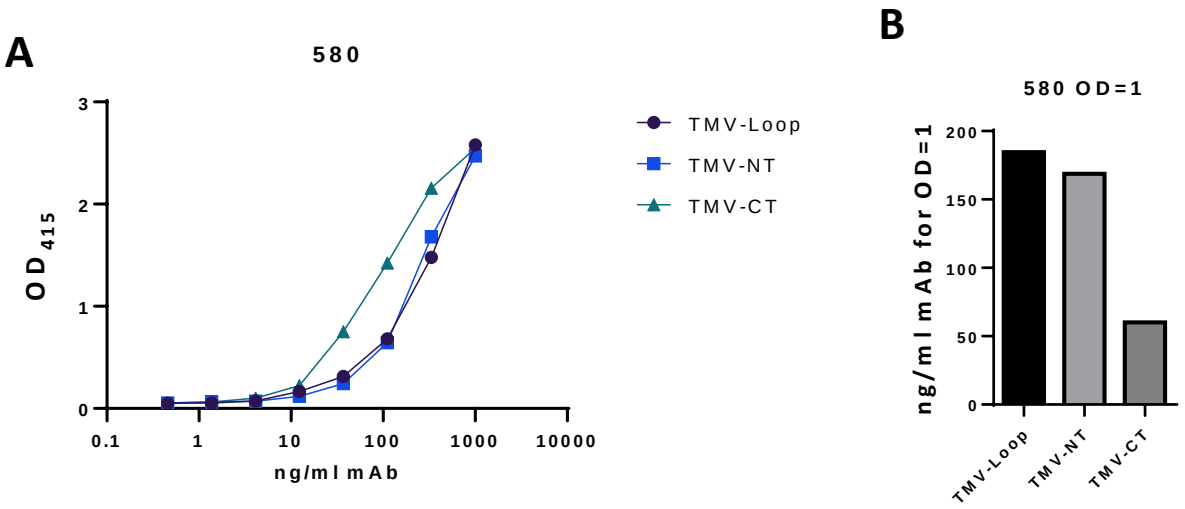
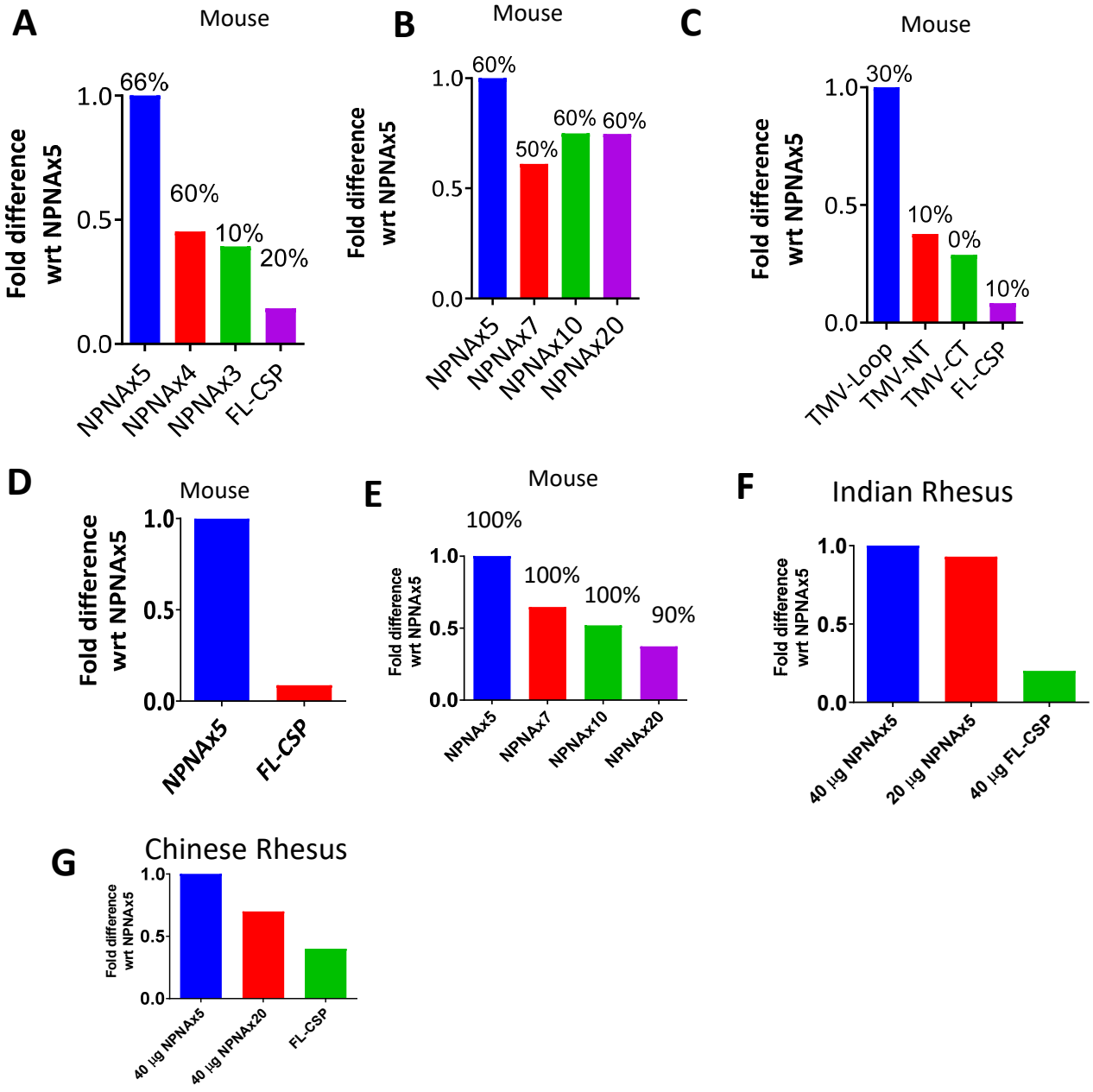


Figure S5 - ELISA titration curve and potency of mAb 580: TMV antigens expressing NPNAx5 displayed on the Loop, NT or CT were coated at 100 ng/mL. **A)** mAb ELISA curves; **B)** Relative potency (OD=1 mAb concentration).



<u>Figure S6</u> – Fold difference in NPNA repeat titers with respect to (wrt) the highest titer group: At 2 weeks post third dose the geometric mean titer of each group was normalized to the NPNAx5 group titer for each study described in the manuscript. The percentage above each bar indicates protection in the mouse model. **A)** Addavax adjuvanted mice in Fig 4A; **B)** Addavax adjuvanted mice in Fig 4D; **C)** Addavax adjuvanted mice in Fig 4G; **D)** ALFQ adjvuanted mice in Fig 5A; **E)** ALFQ adjuvanted mice in Fig 5E. **F)** ALFQ adjuvanted Indian rhesus in Fig 6A; **G)** ALFQ adjuvanted Chinese rhesus in **Fig 6F**.

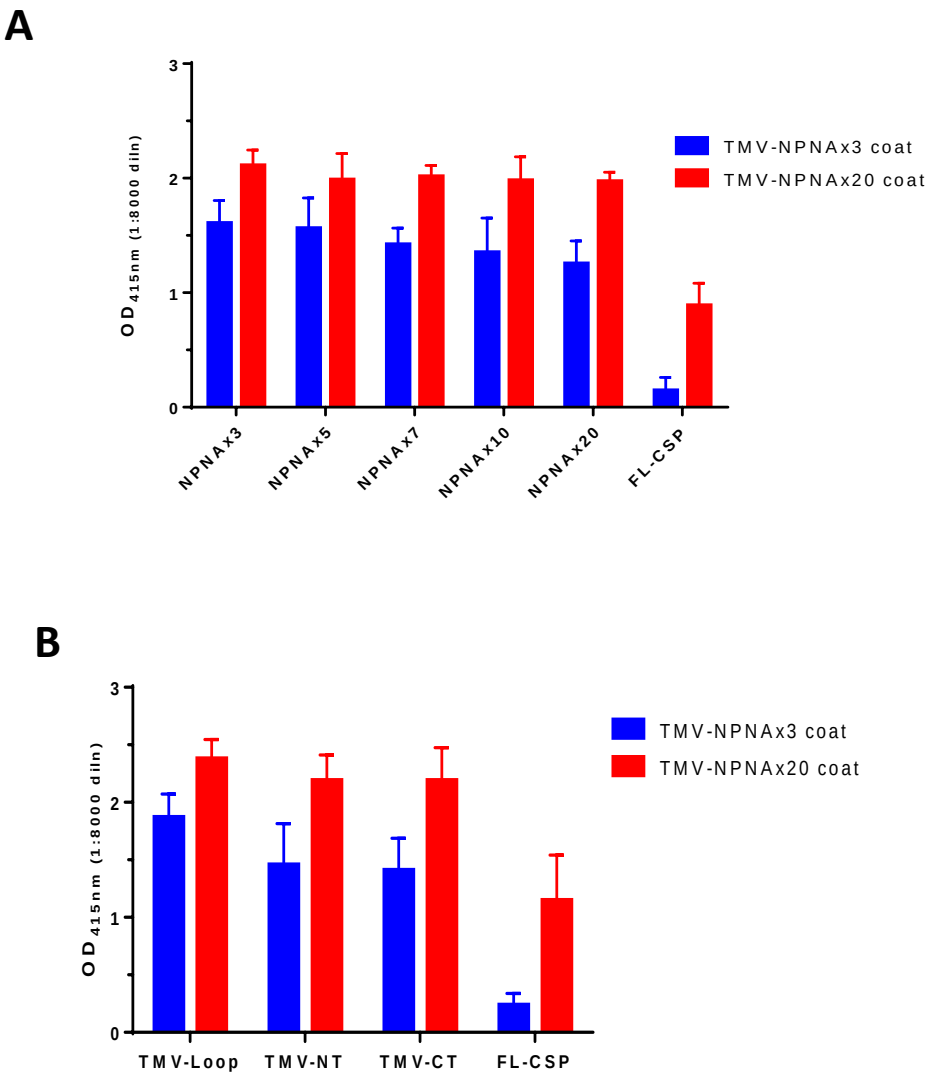


Figure S7— ELISA reactivity of mouse sera against TMV-NPNAx3 or TMV-NPNAx20 coated antigens: A, B) An ELISA was conducted to determine the relative reactivity (OD_{415}) to the two TMV constructs for mouse serum (n=9) in the Addavax adjuvanted groups.

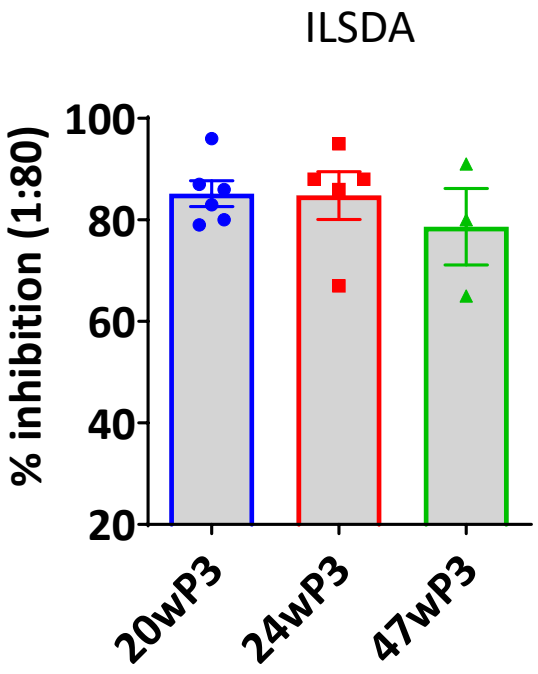


Figure S8 – ILSDA assay: An ILSDA at 1:80 serum dilution performed on individual Indian-origin rhesus macaque sera from the 20 µg NPNAx5 vaccine group, collected at weeks 20, 24 and 47 post third dose (wP3).