Supplementary information

A Malaria Vaccine Adjuvant Based on Recombinant Antigen Binding to Liposomes

Wei-Chiao Huang, Bingbing Deng, Cuiyan Lin, Kevin A. Carter, Jumin Geng, Aida Razi, Xuedan He, Upendra Chitgupi, Jasmin Federizon, Boyang Sun, Carole A. Long, Joaquin Ortega, Sheetij Dutta, C. Richter King, Kazutoyo Miura, Shwu-Maan Lee, and Jonathan F. Lovell

Additional Supplementary Methods

Antibodies for flow cytometry: For immune cell recruitment in lymph nodes, the following antibodies were obtained from Biolegend: CD11c_APC Cy7 (Clone: N418; Cat. # 117323; Lot B237078), CD3 PerCP/Cy5.5 (Clone: 17A2; Cat. # 100217; Lot B233419), I-A/I-E Alex Fluor 700 (Clone: M5/114.15.2; Cat. # 107621; Lot B24168), F4/80 Pacific Blue (Clone: BM8; Cat. # 123123; Lot B217177), Ly-6G PE (Clone: 1A8; Cat. # 127607; Lot B235376), Ly-6C (Clone: HK1.4; Cat. # 128021: Lot B221000), CD11b PE/Cy7 (Clone: M1/70; Cat. # 101215; Lot B249267). For antigen uptake into draining lymph node immune cells, the following antibodies were obtained from Biolegend: I-A/I-E Pacific Blue (Clone: M5/114.15.2; Cat. # 107619; Lot: B252426), CD11c APC (Clone: N418; Cat. # 117310; Lot: B253461), F4/80 PE (Clone: BM8; Cat. # 123109; Lot: B251636) were used. For GC B cells staining, the following antibodies against GL7 Pacific Blue (Clone: GL7; Cat. # 144613; Lot: B244647), CD95 PE (Clone: SA367H8; Cat. # 152607; Lot: B239352), B220 APC (Clone: RA3-6B2; Cat. # 103211; Lot: B205878) were used. For assessing Tfh cells, the following antibodies were obtained from Biolegend: CXCR5 APC (Clone: L138D7 Cat. # 145505; Lot B243491), PD-1 PE (Clone: 29F.1A12; Cat. # 135205; Lot: B251877), Alexa Fluor 488 CD4 (Clone: GK1.5; Cat. # 100425; Lot: B238433). For assessing long-lived plasma cells, the following antibodies were obtained from Biolegend: B220 APC (Clone: RA3-6B2; Cat. # 103211; Lot: B205878), CD138 PE (Clone: 281-2; Cat. # 142503; Lot: B246402). For BMDC activation, the following antibodies were obtained from Biolegend: CD11c FITC (Clone: N418; Cat. # 117313; Lot: B230157), CD80 APC (Clone: 16-10A1; Cat. # 104713; Lot: B248973), CD40 Pacific Blue (Clone: 3/23; Cat. # 124625; Lot: B242315), I-A/I-E PE (Clone: M5/114.15.2; Cat. # 107607; B221907)

Liposome preparation and characterization: CoPoP/PHAD liposomes were prepared with a [DPPC: Chol: PHAD: CoPoP] mass ratio of 4:2:1:1; CoPoP liposomes used a [DPPC:Chol:CoPoP] ratio of [4:2:1], NiNTA/PHAD liposomes used a [DPPC:Chol:PHAD:NiNTA] ratio of [4:2:1:1], DOPC/PHAD/QS21 liposomes used a [DOPC:Chol:PHAD:QS21] ratio of [20:5:1:1], CoPoP/PHAD/QS21 liposomes used a [DPPC:Chol:PHAD:CoPoP:QS21] ratio of [4:2:1:1:1], CoPoP/3D6A liposomes used a [DPPC:Chol:3D6A:CoPoP] ratio of [4:2:2:1], CoPoP liposomes used for acute toxicity studies had a molar ratio of 95:5 CoPoP:PEG-lipid. For liposome preparation, ethanol and PBS were preheated in a water bath at 60 ˚C. Dry lipids were weighed and dissolved in 1 mL ethanol at 60 ˚C for 10 min. During this incubation, samples were sonicated briefly for 5 seconds in a water bath sonicator to break up large particles. After 10 min of incubation, 4 mL of PBS was added to the liposome solution and incubated for another 10 min.

Supplementary information

Liposomes were passed through a 0.45 μm filter before extrusion. A nitrogen pressurized liposome extruder (Northern Lipids) was preheated to 60˚C and washed with distilled water. The liposome solution (1 mg/mL PHAD) was extruded 15 times at 60 °C with a pressure of near 200 PSI through a membrane stack sequentially comprising 200, 100 and 80 nm filters. The extruded liposomes were then dialyzed in 500 mL of PBS for 4-6 hr at 4 °C. The buffer was changed and further dialyzed overnight at 4 °C. The liposomes were diluted to 320 μg/mL PHAD in PBS and then passed through a 0.2 μm sterile filter in a biological safety cabinet. For liposomes containing QS21, QS21 (1 mg/mL) was added to the liposomes after liposome formation at an equal mass ratio as PHAD. Liposome size and polydispersity index were determined by dynamic light scattering in a NanoBrook 90 plus PALS instrument after samples were diluted 200-fold in PBS.

Assessment of protein binding with microcentrifugal filtration: To determine quantify protein binding, 120 μL of Pfs25 or lysozyme (80 μg/mL) was mixed with 120 μL of liposomes at varying PHAD concentrations in PBS for 3 hr at room temperature. Samples were placed in a 100 kDa cutoff microcentrifugal filtration tube pre-rinsed with PBS and centrifuged at 1200 g for 60 min. The flow through was collected, and absorbance at 655 nm was measured to ensure no detection of CoPoP signal from the liposomes (indicating a compromised membrane). The flow through was analyzed with micro BCA assay to detect the amount of unbound proteins. Pfs25 binding to CoPoP/PHAD liposomes was determined by measuring the BCA absorbance at 562 following manufacturer's protocol, using equation **1**:

$$\% \text{ Pfs25 binding} = (1-OD562_{\text{ filtered CoPoP/PHAD+Pfs25}}/\ OD562_{\text{ filtered Pfs25}}) \times 100\% \quad \textbf{(1)}$$

Lysozyme binding was assessed in the same manner. For binding kinetics, 120 μL of Pfs25 (80 μg/mL) was mixed with 120 μL of liposomes (320 μg/mL PHAD) in PBS at room temperature for varying durations and assessed with the microcentrifugal filtration assay. For Pfs25 binding capacity during storage, CoPoP/PHAD liposomes were stored at 4 ºC and aliquots were periodically removed and assessed for binding by combining 120 μL of Pfs25 (80 μg/mL) with 120 μL of liposomes (320 μg/mL PHAD) in PBS for 3 hr at room temperature followed by microcentrifugal filtration analysis. For binding stability of liposomes prebound with Pfs25, Pfs25 was mixed CoPoP/PHAD liposomes (4:1 mass ratio of PHAD:Pfs25) in PBS, and stored as indicated at 4 ºC prior to assessment of protein binding.

PAGE analysis of Pfs25 binding: For native PAGE, loading dye was prepared containing 50 % glycerol, Tris-HCl (0.25 M, pH 6.8) and 0.25% bromophenol blue. Loading dye was combined with the incubated samples and loaded into the gel. A Tris/Glycine buffer was used to run the gels (Biorad, Cat. # 1610734) For denaturing PAGE, the loading dye comprised 0.5 M dithiothreitol, 50 % of Glycerol, 10% sodium dodecyl sulfate (SDS), Tris-HCl (0.25 M, pH 6.8) and 0.25% Bromophenol blue. A denaturing Tris/Glycine/SDS running buffer was used (Biorad, Cat. # 1610732). For both native and denaturing PAGE, the samples were subjected to electrophoresis at 200 mV for 35 min. The gel was stained with Coomassie blue staining buffer (0.1% Coomassie Brilliant Blue G-250, 50% methanol and 10% acetic acid) for 30 min and destained with destaining buffer (40 % methanol, 10% of acetic acid in deionized water) with overnight shaking at room temperature.

Supplementary information

Cryo-electron microscopy: Holey carbon grids (c-flat CF-2/2-2C-T) were washed by submerging them into chloroform overnight. CoPoP/PHAD liposomes (320 µg/mL PHAD) were diluted with equal volumes (20 µL) of Pfs25 (80 µg/mL) in PBS or PBS. Reaction mixtures were then incubated at room temperature for 3 hrs. Grids were glow discharged at 5 mA for 15 seconds immediately before the application of the sample. 4 µL of the solution containing liposomes (1.28 µg/mL total lipid) and Pfs25 (40 µg/mL) were deposited on the electron microscopy grid. Vitrification was performed in a Vitrobot (ThermoFisher) by blotting the grids once for 3 seconds and blot force +1 before they were plunged into liquid ethane. Temperature and relative humidity during the vitrification process were maintained at 25 °C and 100%, respectively. The grid was loaded into the Tecnai F20 electron microscope operated at 200kV using a Gatan 626 single tilt cryo-holder. Images were collected using a defocus of -2.7 µm in a CCD camera at a nominal magnification of 50,000X, which produced images with a calibrated pixel size of 2.21Å. Images were collected with a total dose of 25 e-/$Å^2$.

Circular dichroism: CD spectra were recorded from 190–260 nm with a 0.1 nm data pitch using a Jasco J-815 spectropolarimeter and 1 mm path length. The cuvette chamber temperature was maintained at 20 °C. All the samples were in PBS. Spectra were acquired at 1 nm band width, 4 second response time, and a scan speed of 100 nm/min. The results were calculated after subtracting a PBS baseline spectra and are reported as mean residue. Double helical and beta strands were analyzed using the CDSSTR program on the Dichroweb online server.

Immunoprecipitation: Protein G magnetic beads (Bioclone, Cat. # MAG 102) were washed with PBS and re-suspended with a solution of anti-Pfs25 monoclonal antibody 4B7 (MRA-28), anti-Pfs25 monoclonal antibody 1245, anti-Pfs25 monoclonal antibody 1269, anti-Pfs48/45 monoclonal antibody 3E12 or anti-gp41 monoclonal antibody 2F5 (62.5 µg/mL antibody). Beads were incubated for 2 hr at room temperature with gentle pipetting of the mixture every 20 min. Following antibody-binding to the beads, free antibody was removed by placing the beads on a magnetic separator and washing. CoPoP/PHAD liposomes or PoP/PHAD liposomes were incubated with Pfs25 antigens in the conventional manner (PHAD: Pfs25= 4:1 mass ratio). The beads were mixed with CoPoP/PHAD liposomes or PoP/PHAD liposomes for 4 hr at room temperature. The mixture was pipetted every 20 min. The sample was then washed with PBS 5 times. Then, to lyse the immunoprecipitated liposomes for analytical measurement, 450 µL of 1% Triton X-100 in PBS was added to the beads. The beads themselves were then removed from the solution using a magnetic separator and the solution fluorescence (420 nm excitation, 670 nm emission) of the CoPoP and PoP was measured in microplate reader (TECAN Safire). CoPoP/PHAD or PoP/PHAD liposomes were directly mixed with 1% Triton X-100 as a control to calculate the percentage of CoPoP/PHAD and PoP/PHAD capture with equation **2**:

$$\% \text{ Liposomes captured} = [Fl_{\text{lysed bead supernatant}} / Fl_{\text{liposomes}}] \times 100 \ \mathbf{\%} \qquad \mathbf{(2)}$$

where Fl stands for fluorescence intensity.

Labeling Pfs25 with fluorescent dyes

Pfs25 was labeled with Cyanine 750 succinimidyl ester (Cy7-SE, Biotium # 90049). Labeling was carried out with a Cy7-SE to Pfs25 molar ratio of 7:1. Cy7-SE was first dissolve in 100 mM sodium

bicarbonate buffer (pH 10) and mixed with Pfs25 and incubated with shaking for 1 hr at room temperature. Free dye was removed by dialysis against PBS. Pfs25 was labeled with oyster488 tetrafluorphenylester (oyster-488, OY-488-T). Labeling was carried out with oyster-488 to Pfs25 molar ratio of 10:1. 150 µg of Pfs25 was dialyzed into 100 mM sodium bicarbonate buffer (pH 9) for 4-6 hr at 4 °C twice, and then labeled with oyster-488. Free dye was removed by dialysis against PBS.

Serum stability of labeled Pfs25 25 µL of Pfs25-Cy7 (80 µg/mL) or Pfs25-488 was incubated with CoPoP/PHAD liposomes or PoP/PHAD liposomes for 3 hr. This resulted in fluorescence quenching of the dye with CoPoP/PHAD liposomes, but not PoP/PHAD liposomes, due to energy transfer phenomenon from the dye to the porphyrin. Human serum was added into the mixture to a final volume of 20 %. The mixture was incubated at 37 ºC, and Pfs25-oyster488 binding was assessed on day 7. The binding to liposomes was calculated by equation **3**:

$$\text{Pfs25-488 binding \%} = [1-Fl_{\text{incubated sampele}}/\ Fl_{\text{fresh prepared Pfs25-oyster488 in PBS}}] \times 100\ \% \quad \textbf{(3)}$$

The fluorescence of Pfs25-488 was read with 485 nm excitation and 525 nm emission in a microplate reader (TECAN Safire). Fluorescence could be recovered by treating SNAP with 0.1% Triton X-100 plus 100 µg/ml proteinase K at 50 ºC for 30 min.

Adult bovine serum (Pel-Freeze # 37225-5) was added into the mixture to a final volume of 10 %. The mixture was incubated at 37 ºC, and Pfs25-Cy7 binding was assessed on day 7 and day 14. The binding to liposomes was calculated by equation **4**:

$$\text{Pfs25-Cy7 binding\%} = [1-Fl_{\text{incubated sampele}}/\ Fl_{\text{fresh prepared Pfs25-cy7 in PBS}}] \times 100\ \textbf{\%} \quad \textbf{(4)}$$

The fluorescence of Pfs25-Cy7 was read with 755 nm excitation and 785 nm emission in microplate reader.

*Murine immunizations***:** Murine studies were carried out in accord with the University at Buffalo IACUC (Protocol BME05044). 8-week-old female CD-1 mice received intramuscular (IM) injections on days 0 and 21 containing different doses of Pfs25s combined with CoPoP/PHAD or other adjuvants and were sacrificed on day 42 and serum was collected.

To assess immunization with all Pfs25, 25 µL of Pfs25 (80 µg/mL) was mixed with liposomes (320 µg/mL PHAD or equivalent) for 3 hr at room temperature followed by subsequent dilution to the desired amount of antigen, so the ratio of Pfs25 and liposomes is constant. For dosing with 100 ng Pfs25, samples were then diluted 20-fold, by combining 30 µL of liposome/Pfs25 solution with 570 µL of PBS. For the preparation of ISA720 samples, 15 µL of Pfs25 (80 µg/mL) was combined with 165 µL of PBS, and then combined with 420 µL of ISA 720. The sample was placed on a benchtop vortex at the maximum speed for 60 min. For the preparation of Alhydrogel, 15 µL of Pfs25 was added with 285 µL of 2 % Alhydrogel, then mixed with 300 µL of PBS, and incubated at room temperature for 1 hr prior to injection. Each mouse was vaccinated with 50 µL in the quadriceps.

For dose de-escalation with 100, 30 or 10 ng Pfs25: 25 µL of Pfs25 (80 µg/mL) was combined with 25 µL liposomes (320 µg/mL PHAD) and incubated for 3 hrs. For mice receiving 100 ng of Pfs25, 30 µL of the solution was diluted with 570 µL of PBS. For mice receiving 30 ng of Pfs25,

9 μL of the solution was diluted with 591 μL of PBS. For the mice receiving 10 ng of Pfs25, the solution was diluted 10-fold with PBS prior to additional dilution by combining 30 μL of the solution with 570 μL of PBS. Each mouse received a 50 μL intramuscular injection.

For dose de-escalation with 20, 4 or 0.8 ng Pfs25: Mice were vaccinated with Pfs25 following incubation with CoPoP/PHAD or CoPoP/PHAD/QS21 liposomes. 25 μL of Pfs25 (80 μg/mL) was mix with 25 μL of liposomes (320 μg/mL PHAD for 3 hr at room temperature. After incubation, samples were diluted 10-fold in with PBS. For 20 ng Pfs25 dosing, 60 μL of the diluted liposomes were combined with 540 μL of PBS. For mice 4 ng Pfs25 dosing, 12 μL of diluted liposomes were diluted with 588 μL PBS. For 0.8 ng dosing, 4.8 μL of the diluted liposomes were mixed with 1195 μL PBS.

To assess varying density: For standard density, 25 μL of Pfs25 (80 μg/mL) was first mixed with 25 μL of CoPoP/PHAD (320 μg/mL PHAD) for 3 hr at room temperature. For 5 % Pfs25 density, 25 μL Pfs25 was mixed with 25 μL of CoPoP/PHAD (320 μg/mL PHAD) and an additional 475 μL of CoPoP liposomes for 3 hr. To generate a control with high density but with the same total lipid dose as the low density sample, 25 μL of Pfs25 (80 μg/mL) was mixed with 25 μL of CoPoP/PHAD (320 μg/mL PHAD) for 3 hr at room temperature, and then 475 μL of CoPoP liposomes was added prior to injection. All groups were diluted to a final volume of 1 mL PBS and then 20 μL from each sample was diluted with 180 μL of PBS in order to treat each mouse with 10 ng/50 μL.

For antibody durability study, mice received 100 ng Pfs25 with CoPoP/PHAD liposomes (400 ng PHAD) by intramuscular injection on day 0 and boost on day 42. Serum was collected every ~4 weeks. Final serum collection occurred on day 250.

For comparison with additional adjuvants**:** Mice received 100 ng Pfs25 with different types of adjuvants including CoPoP/PHAD liposomes. In all cases, intramuscular injections of 50 μL per mouse were used. For CoPoP/PHAD, NiNTA/PHAD and DOPC/PHAD/QS21 liposomes, 25 μL Pfs25 (80 μg/mL) was mixed with 25 μL of liposomes (320 μg/mL of PHAD) for 3 hr at room temperature. 12.5 μL of the liposomal solution was mixed with 237.5 μL of PBS to generate the solution used for immunization. For the preparation of Addavax, Adjuphos and Alum samples, 6.25 μL Pfs25 (80 μg/mL) was mixed with 118.75 μL PBS, and 125 μL adjuvant was then added. For the preparation of TiterMax and SAS, 6.25 μL of Pfs25 (80 μg/mL) was diluted with 118.75 μL PBS, and 125 μL of adjuvant was mixed with the antigen solution, followed by high speed vortexing for 30 min. For the CFA/IFA adjuvants, mice were primed with CFA and boosted with IFA. 6.25 μL of Pfs25 (80 μg/mL) was mixed with 118.75 μL of PBS, and 125 μL of adjuvant, followed by vortexing for 30 min. For local reactogenicity, mice were injected with 50 μL sample containing 100 ng of Pfs25 combined with various types of adjuvant into their left footpads. 50 μL PBS was injected into the right footpads as a control. The following adjuvants were mixed with PBS and vortexed at maximum speed for 40 minutes: Montanide ISA720: PBS (3:7 volume ratio), CFA: PBS (1:1 volume ratio) and TiterMax: PBS (1:1 volume ratio). Quil-A, Alum, Sigma Adjuvant Sytsem, and Addavax were mixed with PBS at 1:1 volume ratio. Liposomes were prepared by mixing Pfs25 with CoPoP/PHAD liposomes for 3 hr in the standard protocol. Thickness of the footpad was measured by caliper 24 hr after footpad injection and swelling was calculated using equation **5**:

Supplementary information

$$\text{Swelling (mm)} = [\text{Thickness}_{\text{left footpad}} - \text{Thickness}_{\text{right foorpad}}] \quad \textbf{(5)}$$

Standard Membrane-Feeding Assay (SMFA): 200 µL of freshly collected serum was sent to the Laboratory of Malaria and Vector Research at NIAID to perform ELISAs and SMFA. Briefly, post-immune sera from all mice (n=10) from each group was pooled, and the total IgG was purified. The mixture of test IgG (0.75 mg/ml) and a mature gametocyte culture of *P. falciparum* (NF54 strain) was fed to *Anopheles stephensi* (Nijmegen strain) mosquitoes through a membrane feeding apparatus. After 8 days, the midgut of the mosquites were dissected to enumerate the oocysts.

Multiplexed and long-term antibody duration data ELISA results (**Fig 4a, Fig 6e, Fig S19**) were assessed at University at Buffalo. Anti-Pfs25 IgG titer was assessed by ELISA in 96-well plates. 1 µg/mL of plant-derived Pfs25 in coating buffer (3.03g Na_2CO_3; 6 g $NaHCO_3$ in 1L distilled water, pH 9.6) were coated on the plate for 2 hr at 37°C. Wells were washed and blocked with 2% BSA in PBS containing 0.1% Tween-20 (PBS-T) for 2 hr at 37°C. Mouse sera (diluted in PBS-T containing 1 % BSA) were incubated in the wells, followed by washing with PBS-T. Goat anti-mouse IgG-HRP (Cat. # A00160) was added. Wells were washed again with PBS-T before addition of tetramethylbenzidine solution (Cat 97064-344). Titers were defined as the reciprocal serum dilution at which the absorbance at 450 nm exceeded background by greater than 0.5 absorbance units. ELISA results at University at Buffalo were normalized by a constant factor to show good agreement with NIAID results (data not shown).

Mosquitos, parasite strains and cultures: Mosquitos and parasites were maintained at Johns Hopkins parasitology core. Anopheles mosquitoes were maintained on a 10% sugar solution at 27 °C and 95 % humidity with a 12-hr light/dark cycle. The *P. falciparum* strain NF54 was cultured at 5–10% parasitemia in human O+ erythrocytes at 5% haematocrit in RPMI 1640 medium supplemented with glutamine, HEPES and hypoxanthine. Gametocytes were cultured at 5 % haematocrit and 1% parasitemia, and were maintained for 17 days with daily media changes. Gametocyte cultures supplemented to 40% hematocrit containing fresh O+ human serum were fed to the mosquito. Infected mosquitoes were maintained at 27 °C and 75% humidity fed with 20% w/v dextrose.

Gametocytes and schizont slide preparation: Blood sample containing the NF54 parasites were spotted on a multispot slides (ThermoFisher Cat. # 9991090) and smeared. 2.5 µL of blood containing ~10^7 cells/mL was applied. The slide was air dried for 1 hr at room temperature, and then fixed with ice cold methanol/acetone (1:1 v/v/) at -20 °C for 15 min. The slides were air dried for 15 min and then stored at -80 °C until IFA staining.

Sporozoites slide preparation: Approximately 200 female *Anopheles stephensi* mosquitoes were fed through an artificial membrane on a blood culture containing *P. falciparum* NF54 gametocytes. After 2-3 weeks, mosquitos were killed by spraying with 70% ethanol and placed in RPMI culture medium. Salivary glands were dissected under a dissection microscope. Approximately 100 salivary glands were combined in a 1.5 mL microcentrifuge tube, and 400 µL of medium were added and a 29 G needle were used to homogenize the glands. The number of sporozoites was counted using a hemocytometer under a microscope. Approximately 5,000 sporozoites were

present per well in the multi-spot slide. The slides were air-dried and fixed and stored as described above.

Ookinete slide preparation: Approximately 200 female *Anopheles stephensi* mosquitoes were fed through an artificial membrane on a blood culture containing *P. falciparum* NF54 gametocytes. After 8-10 days, midguts were dissected under a dissection microscope. ~100 midguts were combined into a 1.5 mL microcentrifuge tube and homogenized by pipetting up and down 10 times. 2 µL of the mixture was smeared on each well of the multi-well slides. The slides were air-dried and fixed and stored as described above.

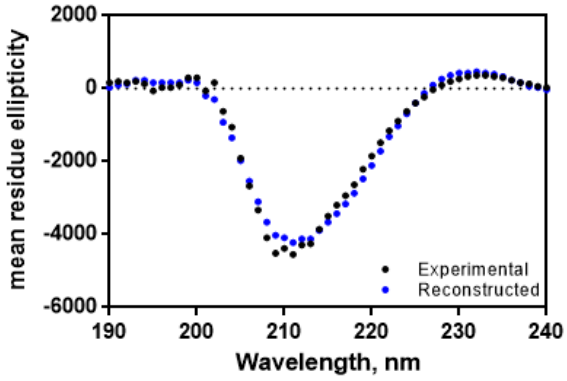

Fig S1 Circular dichroisim of his-tagged Pfs25. Reconstructed data is based on the following content: 0% Helix-1, 2% Helix-2, 29% Strand-1, 15% Strand-2, and 21% turns. The crystal structure of Pfs25 in 6B0E reported a structure of 3 % helix, 37% strand, and 17% turns. Representative spectra shown from 3 independent experiments.

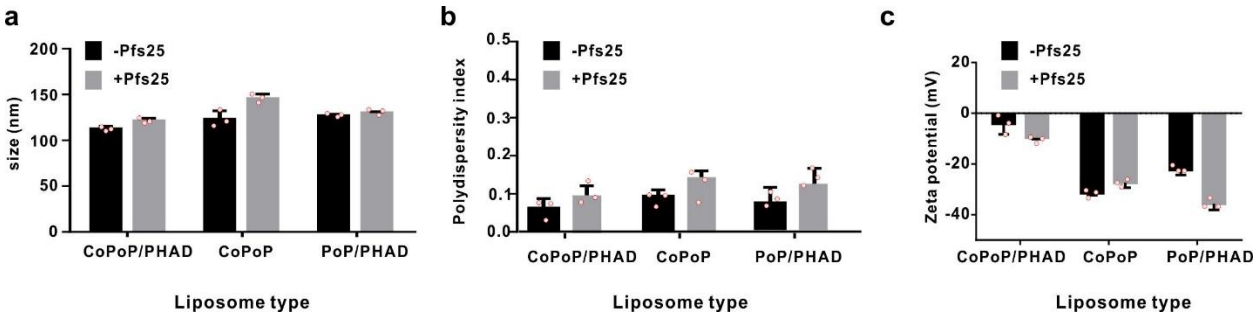

Fig S2 Hydrodynamic diameter(a), polydispersity(b) and zeta potential(c), of various liposomes used in this study. Liposomes were assessed using dynamic light scattering, with or without incubation with Pfs25. Values show mean +/- std. dev. for n = 3 independent experiments.

.

Supplementary information

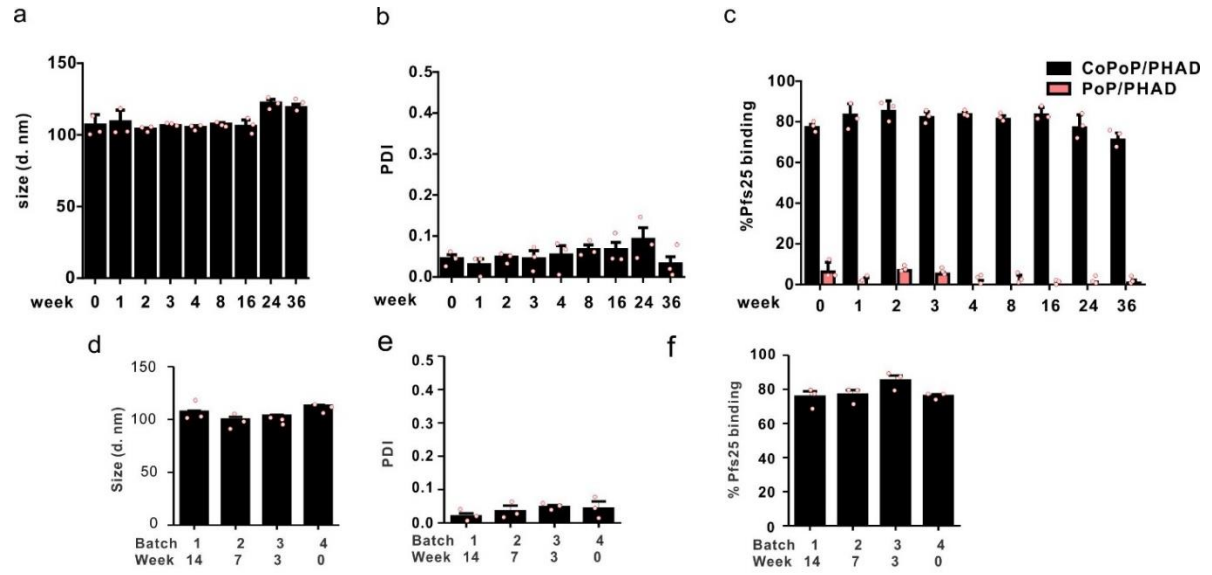

Fig S3. Storage stability of CoPoP/PHAD liposomes. CoPoP/PHAD liposomes were periodically sampled to measure: **a,** size; **b,** Polydispersity index; and **c,** Pfs25 binding capacity based on microcentrifugal filtration. Four separately prepared batches of CoPoP/PHAD liposomes were prepared individually according to standard operating procedure and the **d,** size, **e,** polydispersity index and **f,** Pfs25 binding capacity were measured. Data show mean +/- standard deviation for n=3 triplicate measurements. Bar graphs in **a, b, c,** show mean +/- std. dev. for n=3 separately prepared batches of liposomes. Bar graphs in **d, e, f**, show mean +/- std. dev. for the indicated batches for n=3 measurements per batch.

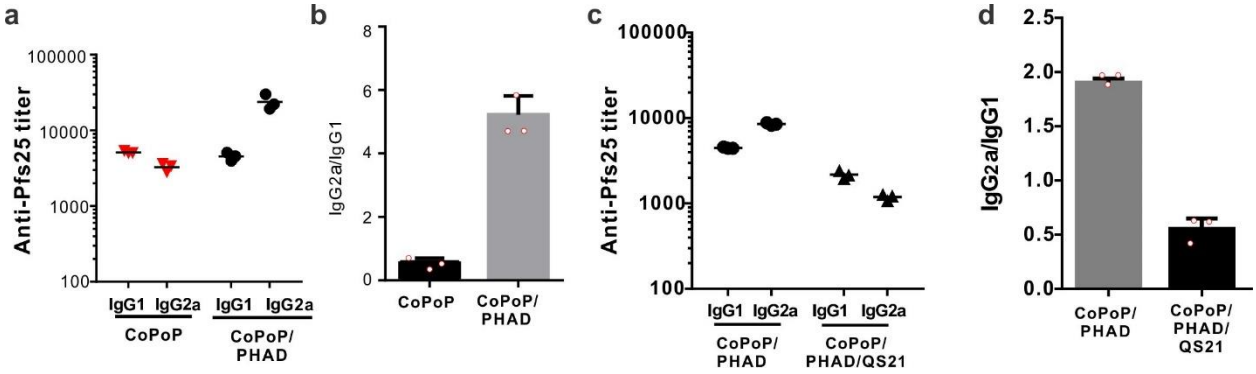

Fig S4. IgG Isotype ratios of various formulations. **a,** Anti-Pfs25 IgG titer and **b,** IgG2a/IgG1 ratios were measured in mice vaccinated with 100 ng Pfs25 incubated with CoPoP liposomes or CoPoP/PHAD liposomes. **c,** Anti-Pfs25 IgG titer and **d,** IgG2a/IgG1 ratio were measured in mice immunized with 20 ng Pfs25 with CoPoP/PHAD liposomes or CoPoP/PHAD/QS21 aggregated liposomes. For **a** and **c**, lines show geometric means for n=3 mice per group. For **b** and **d**, bars show mean +/- std. dev. for n = 3 mice per group.

Supplementary information

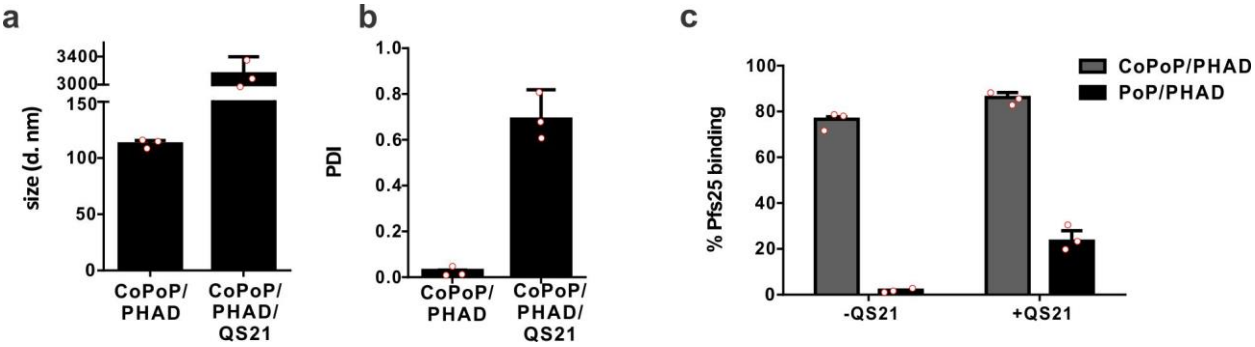

Fig S5. Effect of QS21 on CoPoP/PHAD liposomes. a, Size and **b,** polydispersity were measured by light scattering. **c,** Binding of Pfs25 to CoPoP/PHAD with or without QS21 was assessed with microcentrifugal filtration. Values show mean +/- std. dev. for n=3 independent experiments.

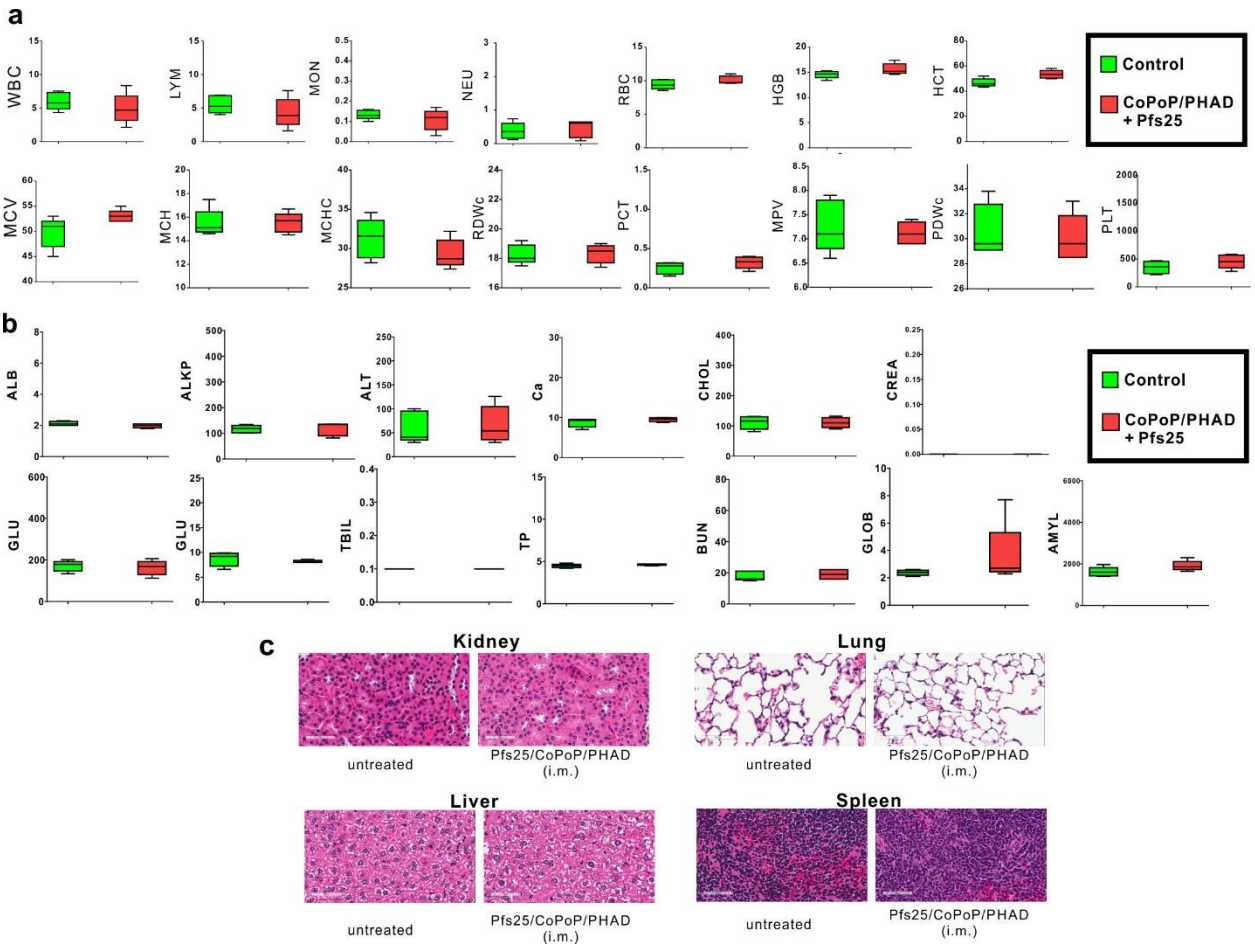

Fig S6. Tolerability of high doses of CoPoP and CoPoP/PHAD/Pfs25 Mice were treated with "CoPoP/PHAD+Pfs25" (20 µg Pfs25, 80 µg PHAD and 80 µg CoPoP particleized in conventional manner). Values show mean +/- std. dev for n=5 mice per group. No statistically significant differences were observed in any groups ($p<0.05$, based on a two-sided student's T-test). **a,** Complete blood count parameters are as follows for red blood cells: RBC (red blood cell count),

HCT (hematocrit); HGB (hemoglobin); MCV (mean cell volume); MCH (mean cell hemoglobin), MCHC (mean cell hemoglobin concentration) and RDW (red cell distribution width); white blood cell parameters are as follows: WBC (white blood cells), NEU (neutrophils), LYM (lymphocytes), MONO(monocytes); platelets parameters are as follows: PLT (platelet) and PCT (platelet crit), MPV (mean platelet volume) and PDW (platelet distribution width). **b,** Serum markers with their general description are as follows. Kidney function markers are as follows: BUN (blood urea nitrogen), CREA (creatinine), PHOS (phosphorus), Ca^{+} (calcium), liver function are as follows: ALT (alanine aminotransferase), ALKP (alkaline phosphatase), ALB (albumin), TBIL (total bilirubin), pancreas function is as follows: AMYL (amylase), Protein TP (total protein), ALB (albumin), GLOB (globulin) other chemistry GLU (glucose), CHOL (cholesterol). **c,** Embedded hematoxylin and eosin stained slices of indicated organs. Scale bar, 50 µm. For **a** and **b**, the line in the box represent the median and the whiskers issuing from the box extend to the group minimum and maximum value. The length of the box represents the interquartile range.

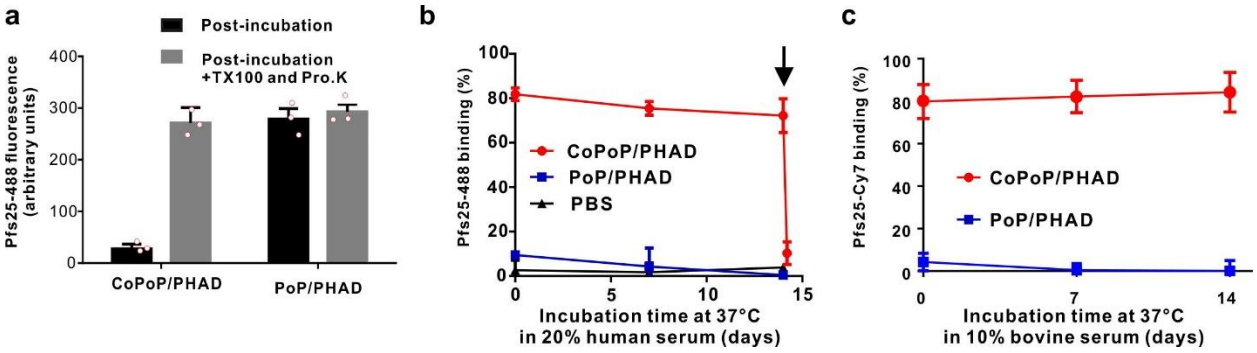

Fig S7. A fluorescence quenching assay for assessing antigen-liposome stability in serum. a, Fluorescently labeled Pfs25-488 is quenched upon incubation with CoPoP/PHAD liposomes, but not PoP/PHAD liposomes, due to energy transfer from the dye to the bilayer porphyrin when the antigen binds the liposomes. Addition of 0.1% Triton X-100 with 100 µg/mL of proteinase K leads to dissociation of the protein from the liposomes and fluorescence recovery of the oyster-488 fluorophore used to label Pfs25. **b,** Pfs25-488 binding stability in 20 % human serum. As a control, where indicated by the arrow, detergent and protease K were added to ensure the accuracy of the fluorescence reporter system. **c,** Cy-7 Pfs25 binding to CoPoP/PHAD liposomes was stable in 10 % adult bovine serum. Bar graph shows mean +/- std. dev. for n=3 independent experiments.

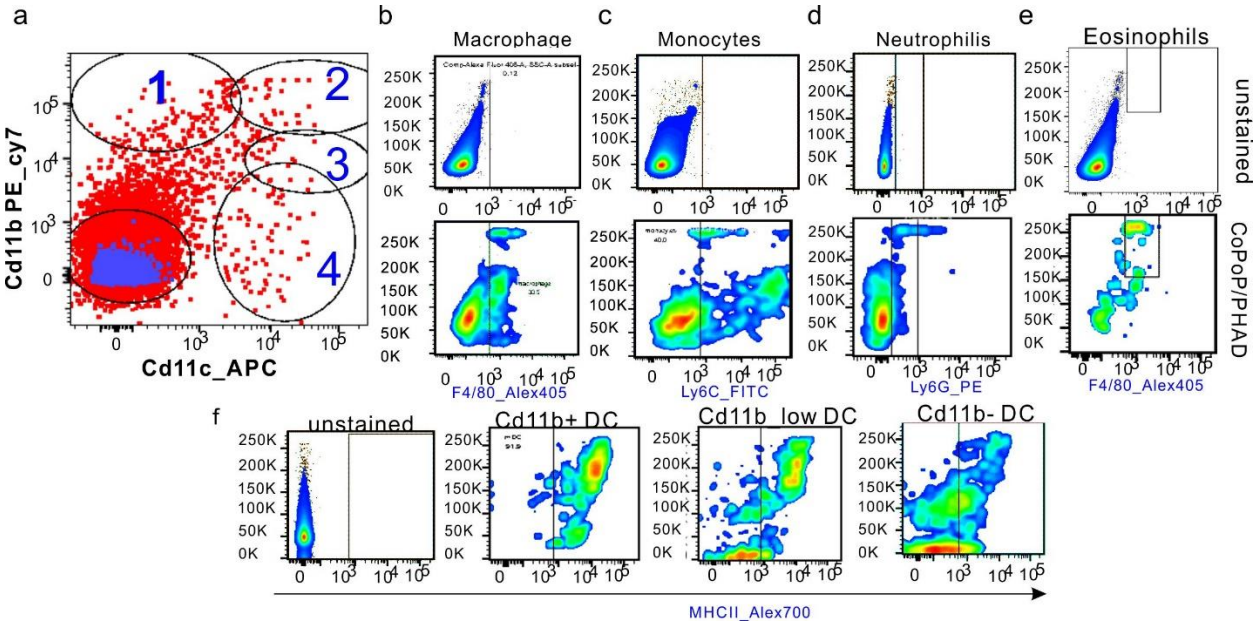

Fig S8 Recruitment of immune cells in the draining lymph node. a, Dot plot of lymph node cells collected 48 hr after CoPoP/PHAD liposomes injection. x-y axis refers to CD11c_APC-CD11b_PE cy7. Region 1 includes macrophages, infiltrating monocytes, neutrophils and eosinophils. Region 2 represents mDC, region 3 represents $CD11b^{low}$ DC and region 4 represents $CD11b^{-}$ DC. The gating for (**b**) macrophage, (**c**) monocytes, (**d**) neutrophils and (**e**) eosinophils is shown. (**f**) Left to right represents the gating for unstained, mDC, $CD11b^{low}$ DC and $CD11b^{-}$ DC. Representative plots are shown from experiments with n=5 mice.

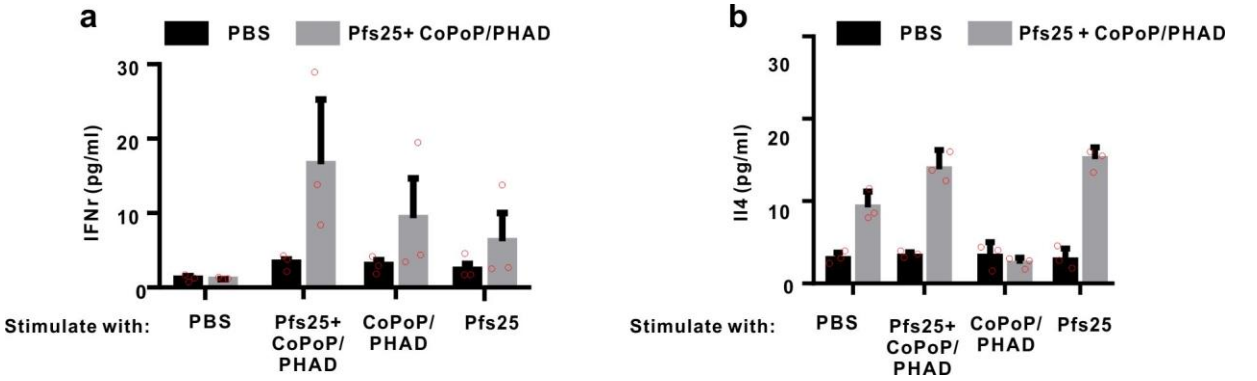

Fig S9. Splenocyte study in mice immunized with Pfs25 and CoPoP/PHAD. Mice were primed on day 0 and boosted on day 21 with 100 ng Pfs25 and CoPoP/PHAD liposomes. 4 weeks later, mice were then sacrificed, splenocytes were isolated and incubated with indicated samples. **a,** Interferon-gamma and **b,** IL-4 secretion was then assessed with ELISA. Bar graph shows mean +/- std. dev. for n=3 mice.

Supplementary information

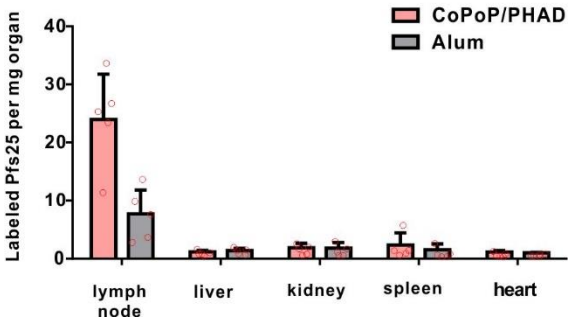

Fig S10 Biodistribution of Pfs25 following intramuscular administration. Pfs25 was fluorescently labeled with Oyster-488 TFP ester, incubated with indicated adjuvants and injected intramuscularly (0.5 µg). 24 hr later, indicated organs and all lymph nodes were removed, weighed, homogenized and assessed for Pfs25 distribution based on fluorometric standards. Bar graph shows mean +/- std. dev. for n=5 mice.

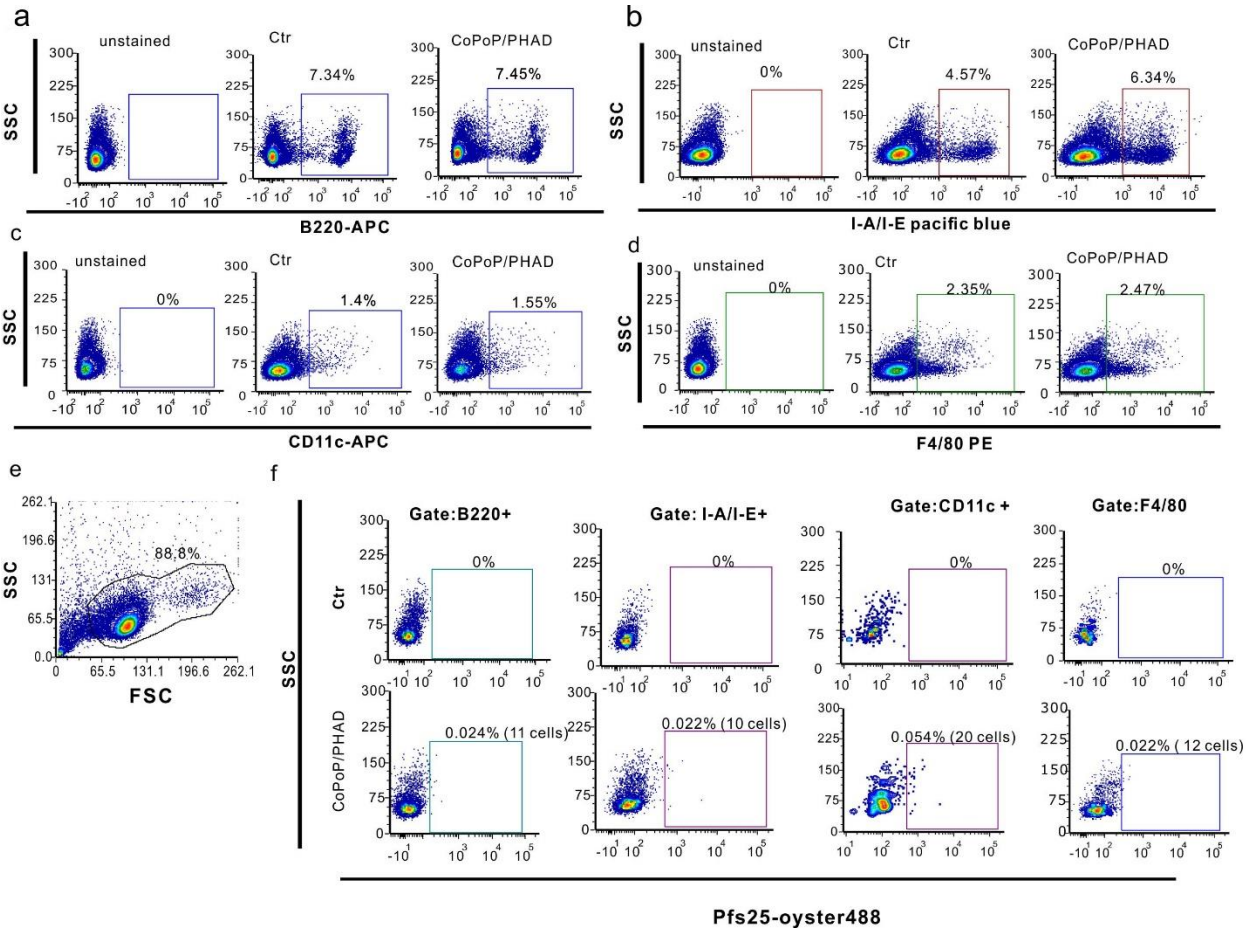

Fig S11 Gating for APC cells uptake Pfs25 in lymph nodes. Cell were first gated by SSC-FSC then **a,** B220, **b,** I-A/I-E positive, **c,** CD11c and **d,** F4/80 positive cells were gated compared to unstained cells. And followed by **f,** gating the antigen positive cells by Oyster488 labeled Pfs25. Representative plots are shown from experiments with n=5 mice.

Supplementary information

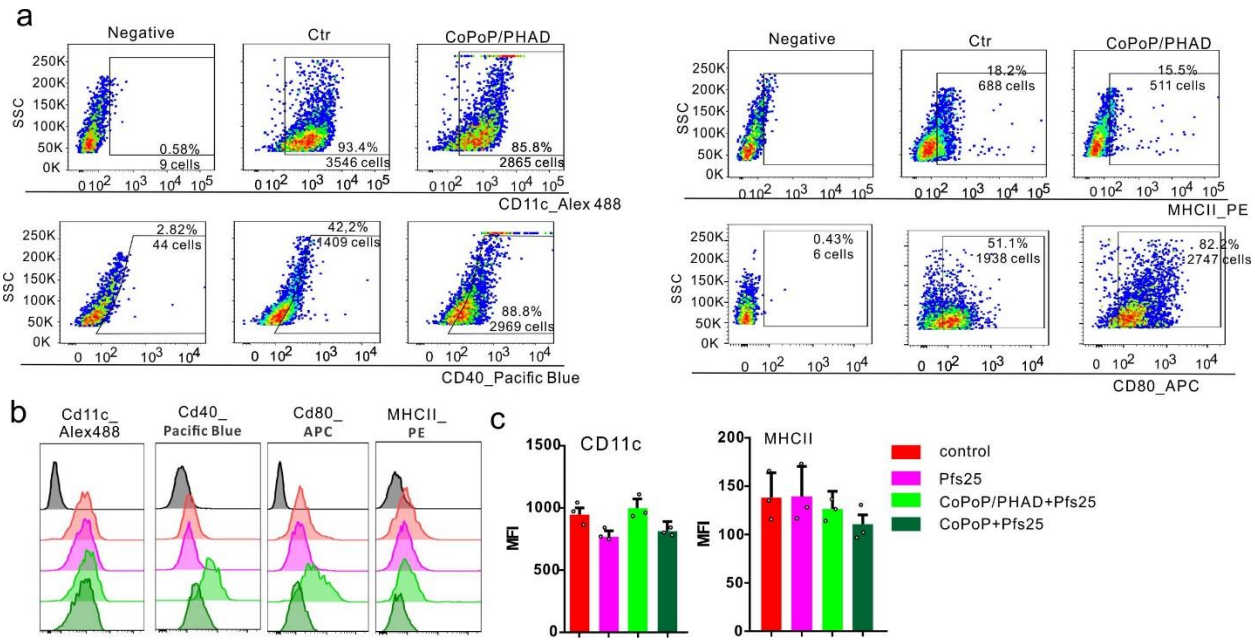

Fig S12 Gating for BMDC cell activation. a, SSC-surface marker of CD11c, CD80, CD40 and MHCII. **b,** Histogram of each panel. **c,** Mean fluorescence intensity of CD11c (left) and MHCII. Plots in **a** and **b** are representative of 3 biologically independent experiments. Bar graphs in **c** show mean +/- std. dev. for those n=3 experiments.

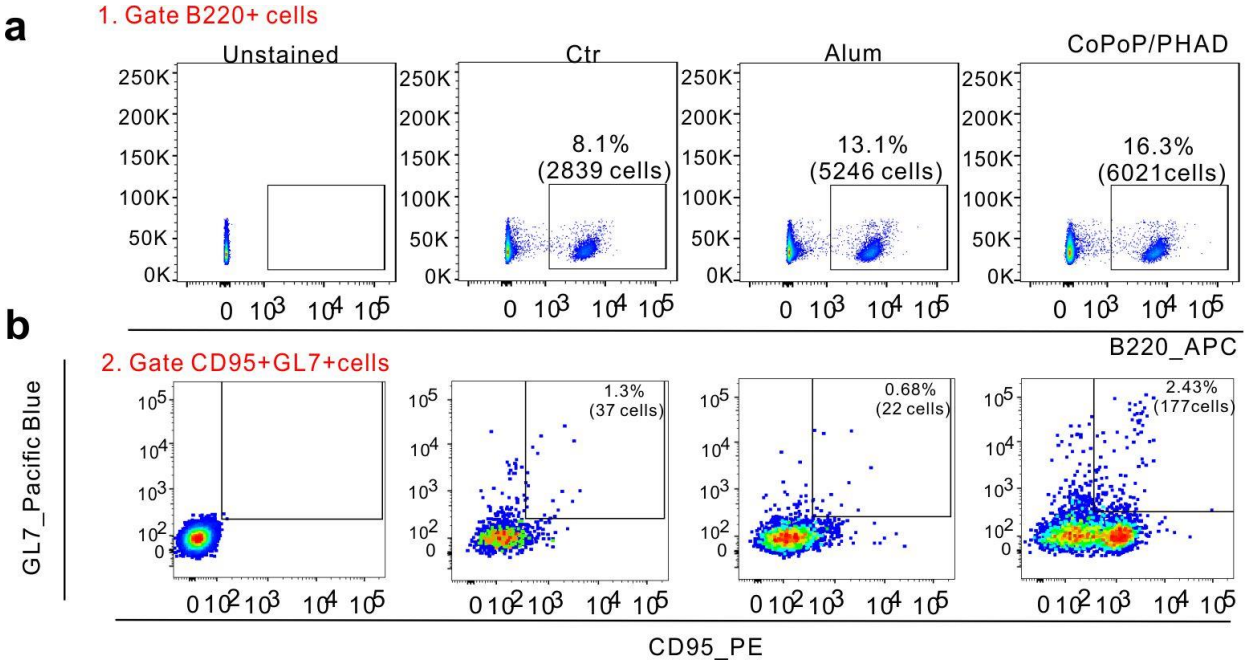

Fig S13 Gating for germinal center B cells in draining lymph nodes a week following immunization with Pfs25 with Alum or CoPoP/PHAD. a, Dot plot showing SSC - B220_APC (x-y axis). The cells were gated for $B220^+$ cells. **b,** $B220^+$ cells were then gated for **$GL7^+CD95^+$.** Representative plots are shown from experiments with n=5 mice.

Supplementary information

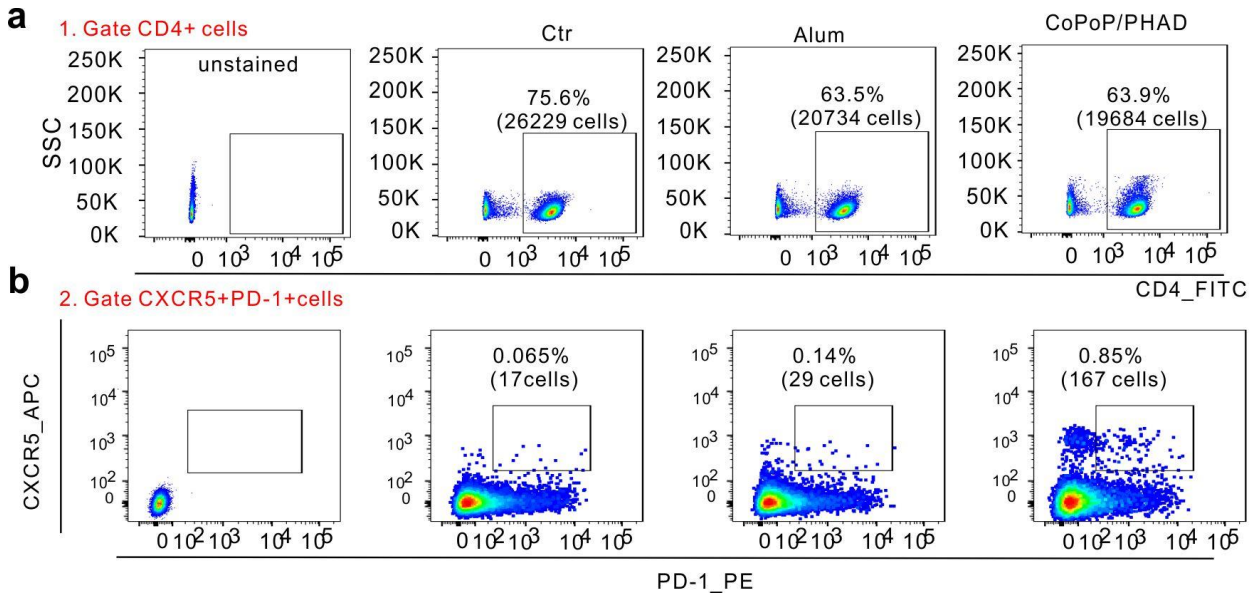

Fig S14 Gating for Tfh cells in draining lymph nodes a week following immunization with Pfs25 with Alum or CoPoP/PHAD. a, Dot plot of SSC - CD4_FITC (x-y axis); cells were gated for $CD4^+$. **b,** $CD4^+$ cells were then gated for $CXCR5^+PD\text{-}1^+$. Representative plots are shown from experiments with n=5 mice.

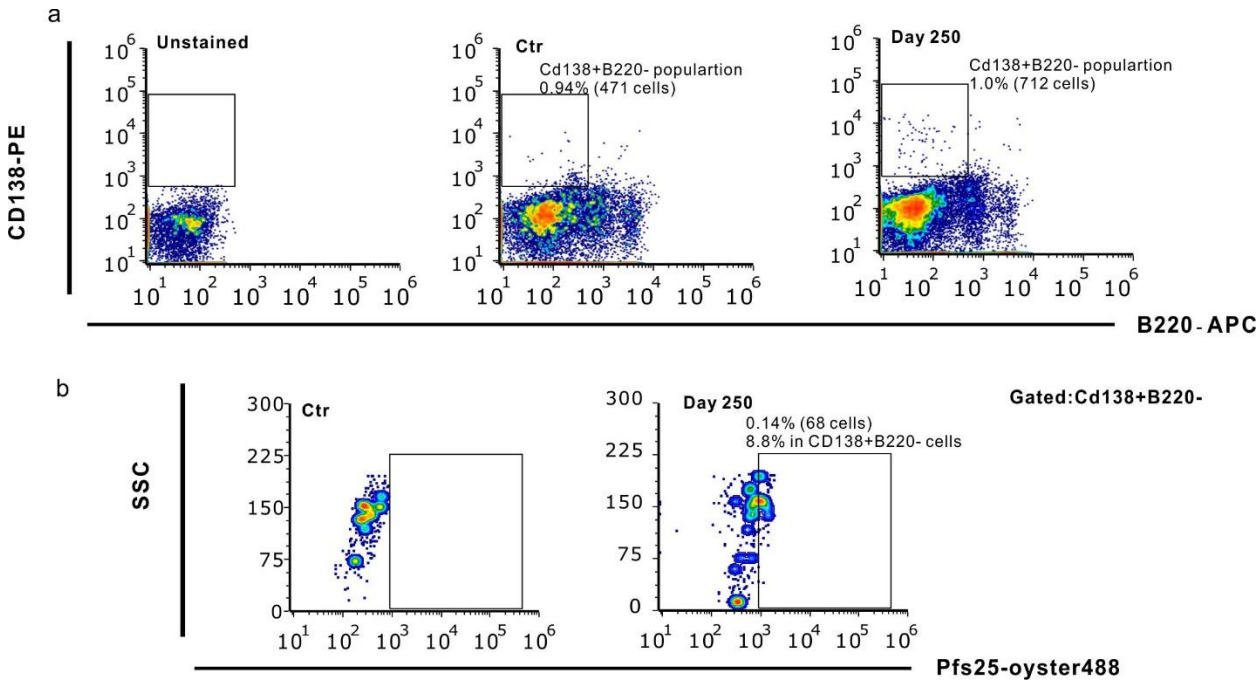

Fig S15 Gating strategy for antigen-specific long-lived plasma cells. a, The dot plot shows the CD138_PE - B220_APC (y-x axis), the cells were gated for $B220^-CD138^+$ for long-lived plasma cells. **b,** Those cells were further gated for $Pfs25^+$ based on oyster-488 labeled Pfs25 with intracellular staining. Representative plots are shown from experiments with n=6 mice.

Supplementary information

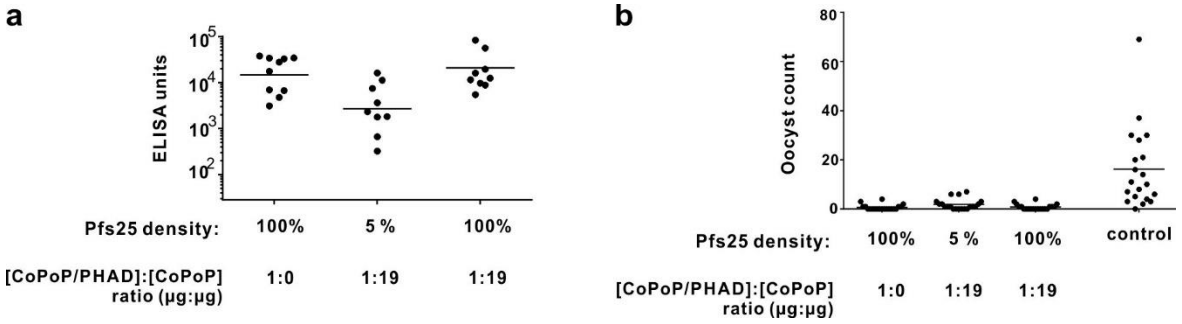

Fig S16. SNAP immunization with varying Pfs25 density. 100% density is defined as the density obtained with the Pfs25 to liposome ratio used throughout this study (i.e. a 4:1 mass ratio of PHAD to Pfs25). **a,** ELISA units and **b,** SMFA were measured following immunization with 10 ng Pfs25. Reduced density (5 %) was achieved by including CoPoP liposomes lacking PHAD, so that total PHAD dose would remain constant. Another sample was used in which 100% Pfs25 density was achieved, but additional CoPoP was added following particleization just prior to injection. The 100% density samples had significantly higher IgG production relative to the 5% density sample (One-way ANOVA, $P<0.05$). ELISA results are for n=10 mice per group and SMFA results are for n=20 mosquitos per group. Lines in the ELISA data represent geometric mean and lines in the SMFA data show arithmetic mean.

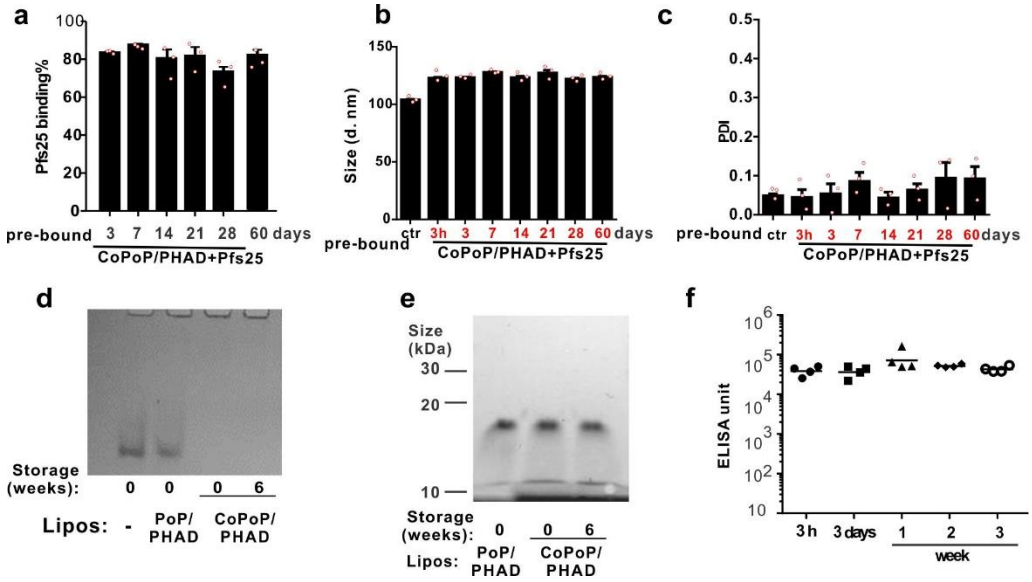

Fig S17: Stability of CoPoP/PHAD liposomes with pre-bound polypeptides. a, Stability of Pfs25 binding to CoPoP/PHAD liposomes during storage. Binding stability was assessed with the microcentrifugal filtration assay. **b,** Size and **c,** polydispersity during storage of CoPoP/PHAD liposomes with Pfs25, measured by dynamic light scattering. **d,** Native PAGE and **e,** SDS PAGE were used to check the stability of pre-bound samples after 6-weeks of storage at 4 °C. **f,** Mice were immunized with CoPoP/PHAD liposomes plus Pfs25, after different storage periods. Lines represent geometric means for n=4 mice per group (each storage time point was used to immunize a different group of mice). Bar graph for **a, b** and **c** show mean +/- std. dev with n=3 independent samples. **d** and **e** show representative gel images from three independent experiments.

Supplementary information

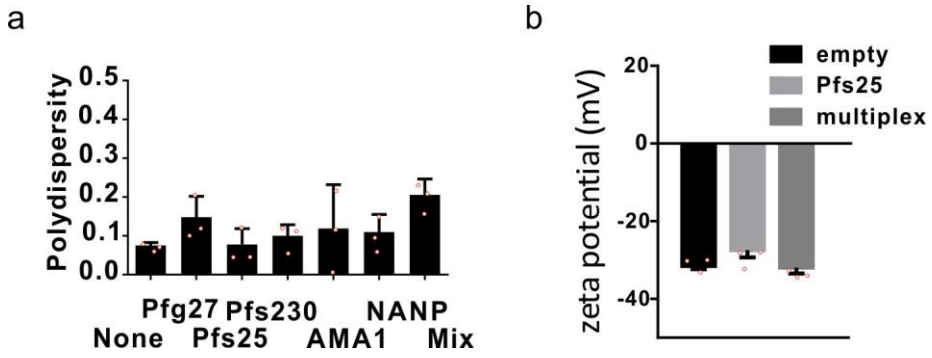

Fig S18. (a) Polydispersity and (b) Zeta potential of CoPoP/PHAD liposomes particleized with multiple his-tagged antigens, either individually or multiplexed. Values show mean +/- std. dev. for n=3 independent particleization experiments.

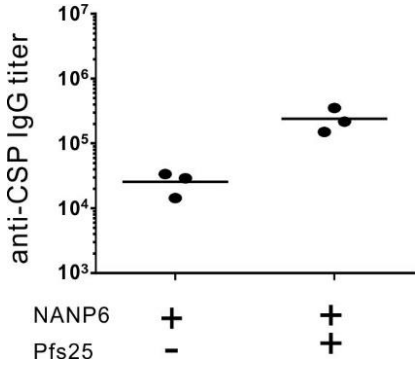

Fig S19. ICR mice immunized with 0.5 µg of NANP peptide with or without Pfs25 adjuvanted with CoPoP/3D6A liposomes. Mice were primed on day 0, boosted on day 21, and serum was collected on day 42. Lines represent geometric mean for n=3 mice per group

Supplementary information

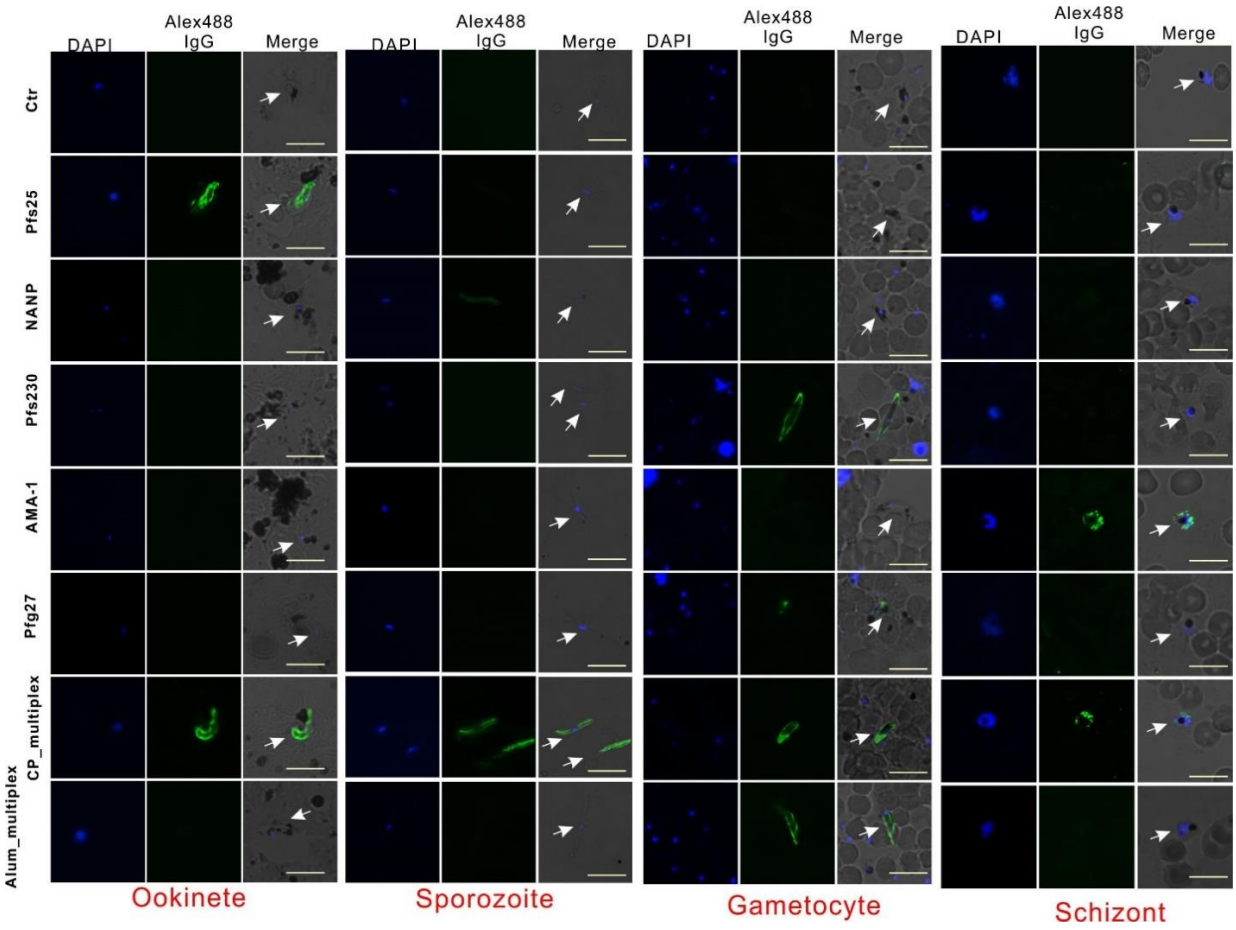

Fig S20. Immunofluorescence assay (IFA) of NF54 parasites at different life-stages (ookinetes, sporozoites, gametocytes and schizonts**).** For IFA, parasites were fixed with ice cold methanol/acetone (1:1 v/v) on the surface of a slide. Post-immune sera (1:100 dilution) was incubated with the slides, followed by staining with Alex Fluor 488 anti-mouse IgG (green) and DAPI (blue), to mark nuclei. Bar: 10 µm. The images are representative of 3 independent experiments.

Supporting Tables of SMFA results

Each SMFA test group (i.e. each table entry) was assessed with n=20 mosquitos per group using pooled serum from n=10 mice. Table entries indicate the sample dose used for immunization. Statistical testing is based on a zero-inflated negative binomial random effects model that has been described previously (Miura et al. Vaccine. 2016;34:4145-51).

Table S1: First SMFA study

Sample	IgG conc [µg/ml]	Oocyte Av.	% inhibition estimate	p-value
100 ng Pfs25, CoPoP/3D6A (0.4 µg 3D6A)	750	1.2	92.3	0.001
100 ng Pfs25, PoP/3D6A (0.4 3D6A)	750	20.1	-34.6	0.478

Supplementary information

100 ng Pfs25, CoPoP alone	750	0.6	96.3	0.001
100 ng Pfs25, Alum	750	21.4	-43.6	0.383
100 ng Pfs25 ISA720	750	27.9	-87.2	0.126
CoPoP/3D6A (0.4 µg 3D6A)	750	14.9	-	-

Table S2: Second SMFA study

Sample	Final IgG conc [µg/ml]	Oocyte Av.	% inhibition estimate	p-value
100 ng Pfs25, CoPoP/PHAD (0.4 µg PHAD)	750	0.5	97.9	0.001
30 ng Pfs25, CoPoP/PHAD (0.12 µg PHAD)	750	0.1	99.8	0.001
10 ng Pfs25, CoPoP/PHAD (0.04 µg PHAD)	750	0.5	98.1	0.001
100 ng Pfs25, CoPoP alone	750	0.3	98.8	0.001
30 ng Pfs25, CoPoP alone	750	0.1	99.8	0.001
10 ng Pfs25, CoPoP alone	750	7.3	70.0	0.004
100 ng Pfs25, CoPoP/3D6A (0.4 µg 3D6A)	750	0.3	99.0	0.001
CoPoP/PHAD (0.4 PHAD)	750	24.2	-	-

Table S3: Third SMFA study

Sample	Final IgG conc [µg/ml]	Oocyte Av.	% inhibition estimate	p-value
10 ng Pfs25, CoPoP/PHAD (100 % density)	750	0.7	96.8	0.001
10 ng Pfs25, CoPoP/PHAD + CoPoP (5% density)	750	1.9	91.0	0.001
10 ng Pfs25 CoPoP/PHAD + CoPoP (100 % density)	750	0.8	96.1	0.001
20 ng Pfs25, CoPoP/PHAD (80 ng PHAD)	750	2.5	87.8	0.001
4 ng Pfs25, CoPoP/PHAD (16 ng PHAD)	750	3.3	84.2	0.001
0.8 ng Pfs25, CoPoP/PHAD, (3.2 ng PHAD)	750	21.1	-2.4	0.942
20 ng Pfs25, CoPoP/PHAD/QS21* (80 ng PHAD)	750	1.3	93.9	0.001
4 ng Pfs25, CoPoP/PHAD/QS21* (15 ng PHAD)	750	9.7	52.8	0.088
0.8 ng CoPoP/PHAD/QS21* (3.2 ng PHAD)	750	24.6	-19.7	0.684
CoPoP/PHAD/QS21*	750	20.6	-	-

* All QS21 samples aggregated with this particular formulation

Supplementary information

Table S4: Fourth SMFA study

Sample	Final IgG conc [µg/ml]	Oocyte Av.	% inhibition estimate	p-value
100 ng Pfs25, CoPoP/PHAD (0.4 µg PHAD) Day 250	750	4.3	82.4	0.001

Table S5: Rabbit SMFA study*

Sample	Final IgG conc [mg/ml]	Oocyte Av.	% inhibition estimate	p-value
1 µg Pfs25, CoPoP/PHAD (4 µg PHAD), rabbit 1	7.5	14.1	78.8	0.002
1 µg Pfs25, CoPoP/PHAD (4 µg PHAD), rabbit 2	7.5	53.8	18.9	0.623
1 µg Pfs25, CoPoP/PHAD (4 µg PHAD), rabbit 3	7.5	3.5	94.8	0.001
10 µg Pfs25, CoPoP/PHAD (40 µg PHAD), rabbit 1	7.5	1.8	96.2	0.001
10 µg Pfs25, CoPoP/PHAD (40 µg PHAD), rabbit 2	7.5	6.8	85.3	0.001
10 µg Pfs25, CoPoP/PHAD (40 µg PHAD), rabbit 3	7.5	1.3	97.2	0.001
10 µg Pfs25, Alum, rabbit 1	7.5	43.6	6.0	0.906
10 µg Pfs25, Alum, rabbit 2	7.5	64.7	-39.3	0.417
10 µg Pfs25, Alum, rabbit 3	7.5	52.9	-13.9	0.784
10 µg Pfs25, Alum, rabbit 4	7.5	65.8	-41.7	0.392

*Rabbits vaccinated with 1ug and with 10 µg Pfs25 were processed in separate SMFA studies