Antibody response of a particle-inducing, liposome vaccine adjuvant admixed with a Pfs230 fragment

Wei-Chiao Huang1, Bingbing Deng2, Amal Seffouh3, Joaquin Ortega3, Carole A. Long3, Ragavan V. Suresh2, Xuedan He1, Kazutoyo Miura2, Shwu-Maan Lee3, Yimin Wu4 and Jonathan F. Lovell1

Pfs230 is a malaria transmission-blocking antigen candidate, expressed on the surface of *Plasmodium falciparum* gametocytes. A recombinant, his-tagged Pfs230 fragment (Pfs230C1; amino acids 443–731) formed serum-stable particles upon incubation with liposomes containing cobalt-porphyrin-phospholipid (CoPoP). In mice, immunization with Pfs230C1, admixed with the adjuvants Alum, Montanide ISA720 or CoPoP liposomes (also containing synthetic monophosphoryl lipid A; PHAD), resulted in elicitation of IgG antibodies, but only those induced with CoPoP/PHAD or ISA720 strongly reduced parasite transmission. Immunization with micrograms of Pfs230C1 admixed with identical liposomes lacking cobalt (that did not induce particle formation) or Alum was less effective than immunization with nanograms of Pfs230C1 with CoPoP/PHAD. CoPoP/PHAD and ISA720 adjuvants induced antibodies with similar Pfs230C1 avidity but higher IgG2-to-IgG1 ratios than Alum, which likely contributed to enhanced functional activity. Unlike prior work with another transmission-blocking antigen (Pfs25), Pfs230C1 was found to be effectively taken up by antigen-presenting cells without particle formation. The anti-Pfs230C1 IgG response was durable in mice for 250 days following immunization with CoPoP/PHAD, as were antibody avidity and elevated IgG2-to-IgG1 ratios. Immunization of rabbits with 20 µg Pfs230C1 admixed with CoPoP/PHAD elicited antibodies that inhibited parasite transmission. Taken together, these results show that liposomes containing CoPoP and PHAD are an effective vaccine adjuvant platform for recombinant malaria transmission blocking antigens.

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**INTRODUCTION**

Malaria is caused by *Plasmodium* parasites and is transmitted to and from humans by *Anopheles* mosquitoes. Transmission-blocking vaccines (TBVs), which block the transmission of parasites to human hosts, have been proposed as a strategy to mitigate disease spread1–3. TBVs aim to prevent transmission of parasites to other humans following a mosquito blood meal from an infected but TBV-immunized human. The host should generate circulating antibodies that, upon entering the mosquito midgut, block gametocyte fertilization4 and/or prevent oocyte development5. An effective TBV should induce long-lasting, elevated levels of functional antibodies in the host, and antibody transfer to mosquitoes during a blood meal is generally considered the most viable functional mechanism.

Pfs230 is a transmission-blocking antigen expressed on the surface of gametocytes within human red blood cells, so the host immune responses have potential to be re-boosted by natural infection, unlike some other TBV antigens like Pfs25, which is expressed only in mosquito hosts6. On the other hand, presence in the host could drive development of parasite immune escape mechanisms. However, gametocytes are located in red blood cells, so can hide within host cells7. Since red blood cells do not express major histocompatibility complexes on their surface and are not recognized by T cells, the parasite is likely to avoid clearance by the host immune response8. Pfs230 contains 3135 amino acids, and due to this large size, the full-length protein is challenging to produce in recombinant systems9. An exhaustive study of Pfs230 fragments spanning the entire protein suggested that only constructs containing the first cysteine motif domain (amino acids 589–730) induce transmission-reducing activity9. Insect cell-based production of a recombinant his-tagged N-terminal fragment of Pfs230 (amino acids 443–731), termed Pfs230C1, which induces transmission-reducing antibodies in mice, has been described previously, and was the antigen used in this study10.

IgG subclass influences transmission-blocking activity of anti-Pfs230 antibodies11. Anti-Pfs230 mouse monoclonal antibodies with transmission blocking activity have been identified for the IgG2 subclass but not the IgG1 subclass12,13. Thus, a higher IgG2-to-IgG1 ratio may be important for maximal transmission-blocking activity of anti-Pfs230 antibodies. A Pfs230 fragment with exoprotein A carrier protein (EPA) formulated with the liposomal adjuvant AS01 has been used in Phase I clinical trials (NCT02942277). AS01 contains monophosphoryl lipid A (MPLA) and QS-21 in a liposome vehicle. MPLA is a toll-like receptor-4 (TLR-4) agonist that is non-pyrogenic form of lipopolysaccharide13. MPLA can activate antigen-presenting cells for enhanced immune responses14. A synthetic version of MPLA, PHAD, which is a pure compound (as opposed to a mixture of lipids), was incorporated into the liposomes used in this study.

Vaccine adjuvants are often combined with recombinant antigens to enhance immune responses. Alum is the most common adjuvant for recombinant antigens and has a successful track record in human vaccination15. Other more potent adjuvants have been developed including Montanide ISA51 and ISA720; water in oil emulsions that have been tested in humans16. A prior trial with the TBV antigen Pvs25 with ISA51 found frequent local reactogenicity and some systemic adverse events including two cases of erythema nodosum17. ISA720 is considered more potent...
than ISA51, and therefore it is not clear whether ISA720 would be suitable for wide-scale human use in a prophylactic vaccine. Liposomes containing cobalt-porphyrin-phospholipid (CoPoP) and PHAD have not yet been tested in humans, but have been proposed as a next-generation vaccine adjuvant, and were shown to be effective with his-tagged Pf25, in mice and rabbits. That study showed that Pf25 could be admixed with CoPoP/PHAD liposomes for so-called spontaneous nanoliposome-antigen particleization (SNAP), a unique mechanism of action for a vaccine adjuvant, which induced strong transmission-reducing activity following immunization. Recombinant his-tagged antigens without further modification could readily bind CoPoP/PHAD liposomes for enhanced immune responses. Other related approaches for conversion of soluble TBV antigens to particle-based vaccines also showed promise. Pf230 fragments (amino acids 542–736) have also been shown to be effective when bioconjugated to protein toxins and outer membrane protein complexes. In this study, immunization with Pf230C1 in mice and rabbits is assessed with CoPoP/PHAD as a particle-inducing vaccine adjuvant.

RESULTS

Pfs230C1 spontaneously binds to CoPoP liposomes

CoPoP liposomes particleize his-tagged antigens with simple mixing. To assess the binding of his-tagged Pf230C1 to CoPoP liposomes, native polyacrylamide gel electrophoresis (PAGE) was used (Fig. 1a; the full blot is shown in Supplementary Fig. 1). 1.5 µg of Pf230C1 was loaded into each lane of the gel and then subjected to electrophoresis. The native gel system does not contain detergents, therefore, if the antigen is bound to liposomes, it becomes too large to enter the gel. The absence or presence of protein migration into the gel following particle formation indicates that Pf230C1 bound to liposomes containing CoPoP, but not to otherwise identical liposomes containing cobalt-free porphyrin-phospholipid (PoP). Increasing mass ratios of CoPoP:Pf230C1 from 1:1 to 4:1 resulted in increased binding of the antigen. This was consistent with an independent microcentrifugal filtration binding assay which assesses the fraction of non-bound protein that can pass through a membrane filter, as the liposomes are too large to do so. A 4:1 mass ratio of CoPoP to Pf230C1 achieved 80% binding of the antigen (Fig. 1b). Minimal Pf230C1 binding was observed when mixed with PoP/PHAD liposomes lacking cobalt. Binding kinetics showed rapid binding, with approximately 80% protein bound to CoPoP liposomes at room temperature within 1 h (Fig. 1c). Based on dynamic light scattering, the liposomes with particleized Pf230C1 were approximately 100 nm in diameter, and minimal change in size was induced by antigen binding (Fig. 1d).

Pfs230C1 immunization with CoPoP/PHAD induces functional IgG

To investigate whether immunization with the particleized antigen could generate antibodies against Pf230C1, outbred mice were vaccinated intramuscularly with 1 µg of Pf230C1, admixed prior to injection with various adjuvants. These included

![Fig. 1](image-url)
experiments were performed with \( n \) immunized with CoPoP/PHAD alone, without Pfs230C1.

From a single experiment. Scale bar, 10 \( \mu \)m. For (a, b), log10-transformed titer were analyzed by one-way ANOVA test followed by Tukey’s comparisons. *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \); ****\( p < 0.0001 \). For SMFA functional assay, the statistical differences were analyzed by a zero-inflated negative binomial model\(^{38} \).

**Fig. 2** Pfs230C1 adjuvanted with CoPoP/PHAD or ISA720, but not Alum, induces IgG with strong transmission-reducing activity. a, b Anti-Pfs230C1 IgG ELISA data (top) and standard membrane feeding assay functional assay (SMFA, bottom). a) ICR mice were immunized with 1 \( \mu \)g of Pfs230C1 with indicated adjuvants. b) ICR mice were immunized with micrograms of Pfs230C1 with Alum or nanograms of Pfs230C1 with CoPoP/PHAD or ISA720. ELISA experiments in (a, b) were performed with \( n = 10 \) independent mice; lines show geometric mean, while SMFA experiments were performed with \( n = 20 \) mosquitoes; lines show arithmetic mean. Black open circles in (a, b) represent control mice immunized with CoPoP/PHAD alone, without Pfs230C1. c) Immunofluorescence assay of NF54 parasites at gametocyte stage. Images taken from a single experiment. Scale bar, 10 \( \mu \)m. For (a, b), log10-transformed titer were analyzed by one-way ANOVA test followed by Tukey’s comparisons. *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \); ****\( p < 0.0001 \). For SMFA functional assay, the statistical differences were analyzed by a zero-inflated negative binomial model.
Fig. 3  Pfs230C1, but not Pfs25, is taken up by APCs without particleization and is effectively adjuvanted by Montanide ISA720. Anti-Pfs230C1 and anti-Pfs25 IgG ELISA (a) and SMFA (b) with indicated antigen adjuvanted with CoPoP/PHAD or ISA720. c RAW264.7 macrophage uptake of fluorophore-labeled Pfs25 or Pfs230C1 with indicated adjuvant assessed as mean fluorescence intensity (MFI) using flow cytometry. d Lymph node uptake of antigens into APCs following intramuscular injection. e Uptake ratio of antigens adjuvanted with CoPoP over ISA720 in lymph nodes. ELISA experiment in a were performed with n = 10 independent mice and lines show geometric mean. SMFA experiments in (b) were performed with n = 20 mosquitoes and lines show arithmetic mean. Bar graphs show mean±std. dev. for n = 3 independent experiments. Pfs25 asterisks in a, b, d and e indicate data re-use that was previously reported in ref. 18. For (a), the titer comparison between CoPoP/PHAD and ISA720 with indicated proteins, log-transformed data was compared with Student’s t test. For (e), one-way ANOVA followed by Tukey’s comparison was used. For e, the comparison between CoPoP/ISA720 ratio uptake in APC was compared with Student’s t test. *p < 0.05, **p < 0.01; ****p < 0.0001.

with CoPoP/PHAD and Pfs25 or Pfs230C1 induced strong SMFA activity. On the other hand, ISA720 only induced strong SMFA activity with Pfs230C1, but not with Pfs25 at the nanogram immunization dose used. To account for these differences, macrophage uptake of both Pfs25 and Pfs230C1 was compared in vitro, the cells were incubated with fluorophore-labeled Pfs25 or Pfs230C1 with CoPoP/PHAD liposomes or PoP/PHAD liposomes. Fig. 3c and Supplementary Fig. 2 show that Pfs25 was effectively taken up by macrophages when converted into particulate form with CoPoP/PHAD, but not as a soluble antigen. In contrast, Pfs230C1 was uptaken by macrophages even without being bound to liposomes. We next examined whether uptake of Pfs230C1 in APCs also occurred in vivo in draining lymph nodes. As shown in Fig. 3d, 2 days following immunization with CoPoP/PHAD or ISA720, Pfs230C1 was detected in major types of APCs, including B-cells (B220), macrophages (F4/80), dendritic cells (CD11c) and MHCII-expressing cells (I-A/I-E) with either the CoPoP/PHAD liposomes or ISA720. The gating of APC cells is shown in Supplementary Fig. 3a–d. This, again, is different compared to Pfs25, which was only taken up by APCs in draining lymph nodes when admixed with CoPoP/PHAD, and uptake was limited when combined with ISA720. Fig. 3e shows that for Pfs25, CoPoP/PHAD was more effective than ISA720 at delivering the antigen to immune cells in vivo (as determined by the differential antigen uptake ratio between the two adjuvants), suggesting that sufficient antigen delivery to APCs is an important factor for inducing transmission blocking antibodies. Uptake of Pfs230C1 into varying immune cell types was over an order of magnitude higher when CoPoP liposomes were used, relative to ISA720. However, for Pfs230C1, both adjuvants worked effectively, resulting in an inter-adjuvant antigen uptake ratio closer to unity. Taken together, these data show that enhanced antigen uptake can explain, at least in part, why only CoPoP liposomes were an effective vaccine adjuvant for Pfs25, but why both CoPoP liposomes and ISA720 were effective for Pfs230C1.

High IgG2-to-IgG1 ratios in mice immunized with Pfs230C1 and CoPoP/PHAD liposomes

We next examined the differential immune response between Alum and CoPoP/PHAD, since both adjuvant with Pfs230C1 induced high levels of IgG, yet only Pfs230C1 with CoPoP/PHAD had strong SMFA functional activity. Several independent immunization studies at varying dose levels were carried out (Supplementary Table 1). 14 groups of mice (n = 10 for each group) were immunized with Pfs230C1 and CoPoP/PHAD and nine groups of mice (n = 10 for each group) immunized with Pfs230C1 and Alum. As shown in Fig. 4a, increasing immunization doses of Pfs230C1 resulted in increasing serum IgG EU for Pfs230C1 antibodies for both CoPoP/PHAD and Alum adjuvants. To assess antibody function, SMFA activity of purified serum IgG, in terms of the transmission-reducing activity, is plotted against the amount of specific antibody present (in terms of EU) in Fig. 4b. All data points correspond to separate immunization and SMFA experiments. At higher EU, greater SMFA was only apparent with the CoPoP/PHAD post-immune antibodies. Alum samples had relatively low transmission-reducing activity regardless of the amount of antibody present. The same data is shown in Fig. 4c as the log mean oocyst ratio (LMR) as a function of the square root of EU in the feeder, which has been shown to follow a linear relationship. Although the CoPoP/PHAD data could be reasonably fit with a linear equation (indicated on the graph), Alum did not exhibit such a correlation, with consistently weak transmission-reducing activity. These results imply that CoPoP/PHAD with Pfs230C1 induced better quality of antibodies that effectively block transmission, compared to Alum, which induced less functional Pfs230C1 antibodies.

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Further studies were carried out to investigate whether the functional activity of antibodies was related to IgG subclass and avidity. As shown in Fig. 5a, a higher IgG2-to-IgG1 ratio could at least in part explain the stronger SMFA activity of CoPoP/PHAD relative to Alum. Alum Pfs230C1 antibodies had a low IgG2-to-IgG1 ratio and had weak SMFA activity. Fig. 5b shows that CoPoP/PHAD liposomes and ISA720 induced mainly IgG2 subclass antibodies in mice immunized with varying amount of Pfs230C1, the injection dose of Pfs230C1 did not correlate with the IgG2-to-IgG1 ratio. As shown in Fig. 5c, the avidity of the Pfs230C1 antibodies was similar among all the adjuvant groups. PHAD has been tested in several mouse models and reported to induce stronger IgG2 titers18,27,28, which has similar immune response as ISA72029. On the other hand, Alum has been reported to generate higher levels IgG1 antibodies30. This could be one explanation why Alum could induce Pfs230C1 specific antibodies with weak SMFA activity.

Durability of CoPoP/PHAD immunization

To assess long-lasting immune responses, mice were immunized with Pfs230C1 admixed with CoPoP/PHAD or Alum and then monitored for 250 days. As shown in Fig. 6a, following CoPoP/PHAD immunization with Pfs230C1 with injections on day 0 and day 21, the antibody levels at the end of the study (day 250) did not decrease to less than half of the day 42 levels. There was not a statistically significant decrease in the antibody levels from day 42 to day 250 for mice immunized with the CoPoP/PHAD adjuvant and a 50 ng Pfs230C1 dose (p = 0.78, based on student’s t test of log-transformed ELISA units) nor a 200 ng Pfs230C1 dose (p = 0.25). On the other hand for the groups immunized with Alum, the anti-Pfs230C1 titer significantly decreased by 64%, for the 1 μg Pfs230C1 antigen dose (p = 0.0051) and by 88% for the 15 μg antigen dose (p < 0.0001). As shown in Fig. 6b, the antibody kinetics in mice over that period showed that the Alum with 15 μg of Pfs230C1 had a steady decrease in antibody levels, whereas mice receiving CoPoP/PHAD with 50 ng Pfs230C1 maintained sustained high levels throughout.

On day 250, we observed modest transmission-reducing activity (TRA) in some groups, with 66% TRA for the 50 ng Pfs230C1 with CoPoP group and 47% TRA for the 1 μg Pfs230C1 with Alum group. All IgG samples were tested at 750 μg/mL IgG, the same total IgG concentration as the day 42 samples (Supplementary Table 1, study number MA0068-1). For the CoPoP/PHAD groups, unlike the geometric mean of the IgGs in serum, the anti-Pfs230C1 levels per total IgG following IgG pooling and purification were lower on day 250 than those in day 42 IgGs (4.1- and 2.4-time lower in 200 and 50 ng of Pfs230C1 group, respectively). The reason for this was not clear. Therefore, to compare the quality of...
DISCUSSION

In this work, we first confirmed that Pfs230C1 forms particles after simple admixing with CoPoP liposomes. Particle formation is based on the short his-tag sequence of the protein binding with the chelated cobalt of the porphyrin moieties in the bilayer. Antibodies with greater transmission-reducing function were induced with CoPoP/PHAD relative to Alum in mice. Studies have shown that Alum generally induces Th2-biased immunity responses\(^1\), while CoPoP/PHAD and ISA720 induced a higher ratio of IgG2-to-IgG1 antibodies, reflective of a Th1-biased response. A previous study has shown that the IgG2-to-IgG1 ratio is important for Pfs230C1 antibody function in the SMFA\(^1\) and this study demonstrated that IgG2 subclass was substantially greater in ISA720 and CoPoP/PHAD adjuvants. It is also possible that other factors, such as the specific antibody epitope response varied based on antigen presentation by the different adjuvants. When comparing CoPoP/PHAD and Alum adjuvants, that produced similar antibody levels, the IgG2-to-IgG1 ratio could explain the improved results of CoPoP/PHAD liposomes. However, for comparing the CoPoP/PHAD and ISA720 adjuvants, which produced similarly high IgG2-to-IgG1 ratios, the higher levels of antibodies induced by ISA720 could explain differences in SMFA activity. As mentioned above, ISA720 is a potent vaccine adjuvant that has been used in clinical studies. However, in one study, injection reactions were observed in 24% of the patients\(^2\). Although reactogenicity was not studied in this work, a prior study in mice found that CoPoP/PHAD with Pf25 had minimal local reactogenicity compared to ISA720\(^3\).

Pfs230C1 is generally an effective immunogen, while Pf25 has poor immunogenicity due to its putative hapten-like behavior\(^4\). In our previous study, we found that nanogram doses of Pf25 induced strong immunogenicity with the CoPoP/PHAD platform\(^5\), but not with other adjuvants including ISA720 or Alum. The present study sheds additional light on these observations and shows that Pfs230C1, unlike Pf25, could induce antibodies and be taken up by APC in vitro and in vivo with or without particle formation. The mechanism for the enhanced uptake of soluble Pfs230C1 in immune cells relative to Pf25 is unclear. CoPoP/PHAD with Pfs230C1 generated efficacious transmission-reducing activity with nanogram antigen doses, a durable antibody response,
and a high IgG2-to-IgG1 ratio. CoPoP/PHAD offers other features such as uniform antigen conformation and presentation on the surface of the liposomes and straightforward capability for multiplexing.

In summary, Pf230C1 could be admixed with CoPoP liposomes to induce antigen particleization. Immunization with CoPoP/PHAD generated functional antibodies in mice using nanogram antigens doses. Enhanced levels of functional antibodies were induced with CoPoP/PHAD relative to Alum, along with higher IgG2-to-IgG1 antibody ratios. Altogether, these results confirm that the CoPoP technology can effectively serve as a particle-inducing vaccine adjuvant for TBV development, resulting in strong and durable induction of functional antibodies.

**METHODS**

**Materials**

His-tagged Pf230C1 was produced in a baculovirus system as previously reported. CoPoP was produced as previously described. The following adjuvants were obtained: Montanide ISA720 (SEPPIC) and Alhydrogel 2% aluminum gel (Accurate Chemical and Scientific Corporation; Cat #A1090BS). The following lipids were used: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Corden Cat #LP-R4-057), cholesterol (PhytoChol, Wilshire Technologies), synthetic monophosphoryl lipid A Phosphorylated HexaAcyl Disaccharide (PHAD, Avanti Cat #699810) and PHAD-504 (Avanti Wilshire Technologies), synthetic monophosphoryl lipid A Phosphorylated phosphocholine (DPPC, Corden Cat #LP-R4-057), cholesterol (PhytoChol, #A1090BS). The following lipids were used: 1,2-dipalmitoyl-sn-glycero-3-

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Antibodies for flow cytometry

For antigen uptake into immune cells in draining lymph nodes, the following antibodies were obtained from Biologend: I-A/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).

**Liposome preparation**

Liposomes were prepared by ethanol injection and nitrogen-pressurized lipid extrusion in phosphate buffered saline (PBS) carried out at 60 °C followed by dialysis to remove ethanol. Final liposome concentration was adjusted to 320 μg/mL PHAD and samples were passed through a 0.2 μm sterile filter and stored at 4 °C. Liposome sizes and polydispersity index were determined by dynamic light scattering with a NanoBrook 90 plus PALS instrument after 200-fold dilution in PBS.

**Antigen-binding characterization**

Liposome binding with Pf230C1 was generally carried out by incubating protein and liposomes with a 1:4 mass ratio of protein:CoPoP. Following incubation, the sample was subjected to microcentrifugal filtration (PALL Cat #29300) and protein in the filtrate was assessed by micro BCA (Thermo Cat #23235). For gel electrophoresis, loading dye (50% glycerol, Tris-HCl (0.25 M, pH 6.8) and 0.25% bromophenol blue) was added to liposome samples and 1.5 μg of Pf230C1 with indicated liposomes were loaded into a native Tris-Glycine PAGE gel (Lonza Cat #85232) and subjected to electrophoresis and staining. CoPoP/PHAD, CoPoP and PoP/PHAD liposomes were admixed with Pf230C1. For CoPoP/PHAD and CoPoP liposomes, Pf230C1 and liposomes were mixed with 1:1, 1:2, 1:3 or 1:4 mass ratio of protein:CoPoP. Protein without liposomes was used as a control.

To determine binding saturation, 150 μL of Pf230C1 (80 μg/mL) was mixed with 150 μL of liposome containing 320 μg/mL of CoPoP or PoP in PBS for 3 h at room temperature. Samples were placed in a 100 kDa cut-off filter and stored at 4 °C. Liposome sizes and polydispersity index were determined by dynamic light scattering in a NanoBrook 90 plus PALS instrument after samples were diluted 200-fold in PBS.

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 mixed with the incubated samples and loaded into the gel. A Tris/Glycine PAGE gel (Lonza Cat #85232) and subjected to electrophoresis and staining. CoPoP/PHAD, CoPoP and PoP/PHAD liposomes were admixed with Pf230C1. For CoPoP/PHAD and CoPoP liposomes, Pf230C1 and liposomes were mixed with 1:1, 1:2, 1:3 or 1:4 mass ratio of protein:CoPoP. Protein without liposomes was used as a control.

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Labeling Pfs230C1 with fluorescent dyes

Pfs230C1 was labeled with oyster-488 tetrafluorophenylester (oyster-488, OY-488-T). Labeling was carried out with oyster-488 to Pfs230C1 molar ratio of 10:1. 150 µg of Pfs230C1 was dialysed into 100 mM sodium bicarbonate buffer (pH 9) for 4–6 h at 4 °C twice, and then labeled with oyster-488. Free dye was removed by dialysis against PBS. Pfs230C1 was also labeled with DY-405 NHS-Ester (DY-405). Labeling was carried out with DY-405 to Pfs230C1 molar ratio of 5:1. 100 µg of Pfs230C1 was dialysed in 100 mM sodium bicarbonate buffer (pH 9) for 4–6 h at 4 °C twice, and then labeled with DY-405 at room temperature for 1 h. Free dyes was removed by dialysis against PBS three times at 4 °C.
Pfs25 was labeled with DY-490-NHS-Ester (DY-490). Labeling was carried out with a DY-490 to Pfs25 molar ratio of 5:1. 100 µg of antigen was dialysed into 100 mM sodium bicarbonate buffer (pH 9) at 4 °C and then labeled with DY-490 for 1 h at room temperature, followed by dialysis against PBS three times at 4 °C to remove free dye.

Cryo-electron microscopy

Pfs230C1 (80 µg/mL) was mixed with an equal volume of CoPop/PHAD liposomes (320 µg/mL PHAD) in PBS. Reaction mixtures were incubated at room temperature for 3 h and then stored on ice until applied to the electron microscopy grids. Holey carbon grids (c-flat CF-2/2-2C-T) with a freshly applied continuous thin carbon layer were washed with chloroform. Then, grids were glow discharged at 5 mA for 10 min immediately before the application of the sample. A volume of 3.6 µL of sample was deposited in the grid and vitrification was performed in a Vitrobot (ThermoFisher) by blotting the grids once for 3 s at −2 °C 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 200 kV using a Gatan 626 single tilt cryo-holder. Images were collected in a Gatan Ultrascan 4000 4k x 4k CCD Camera System Model 895 at a nominal magnification 60,000×, which produced images with a calibrated pixel size of 1.8 Å/pixel. Images were collected with a total dose of ~50 e−/Å² using a defocus ranging from −2.7 to −3.5 µm. Images were cropped and prepared for figures using Adobe Photoshop.

Murine immunization and serum analysis

Five to six week old female CD-1 mice (Envigo) received intramuscular injections on days 0 and 21 containing the indicated antigen doses with the indicated adjuvants. Serum was collected on day 42 unless otherwise indicated and sent to the Laboratory of Malaria and Vector Research at the National Institute of Allergy and Infectious Diseases (NIAID) for analysis, which was carried out as previously described16. IgG2-to-IgG1 ratio were calculated as followed: OD value (IgG2a + IgG2b + IgG2c) / OD valueG1. Percentage of TRA were calculated negative binomial model as previously described19. IgG2-to-IgG1 ratio were calculated as followed: OD value (IgG2a + IgG2b + IgG2c) / OD valueG1. Percentage of TRA were calculated as followed: 100 x [mean number of oocysts in the test]/[mean number of oocysts in the control], and LMR is calculated based on Log10 [100/100–percentage of TRA]. Linear regression analysis for anti-Pfs230C1 IgG level in feeder (square root) to LMR were measured by signal linear regression analysis by Graphpad 6.0.

Ethics statement

All mice experiments were carried out using protocols approval of University at Buffalo Institutional Animal Care and Use Committee (IACUC). All rabbit experiments were carried out using protocols approved by the Pocono Rabbit Farm IACUC.

Reporting summary

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

All raw data are available upon request.

CODE AVAILABILITY

The RStudio code used to plot 3D figures in Fig. 5a is available in the supplementary data.

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