Biochemistry

Communication

Cite This: Biochemistry 2018, 57, 516−519

pubs.acs.org/biochemistry

Malaria Derived Glycosylphosphatidylinositol Anchor Enhances Anti-Pfs25 Functional Antibodies That Block Malaria Transmission

Neeraj Kapoor,[#] Ivana Vanjak,[#] James Rozzelle,[#] Aym Berges,[#] Wei Chan,[#] Gang Yin,[§] Cuong Tran,[§] Aaron K. Sato,[§] Alexander R. Steiner,[§] Thao P. Pham,[⊥] Ashley J. Birkett,[∥] Carole A. Long,[⊥] Jeff Fairman,[#] and Kazutoyo Miura*[,⊥]

[#]SutroVax, Inc., 353 Hatch Drive, Foster City, California 94404, United States

[§]Sutro Biopharma, 310 Utah, South San Francisco, California 94080, United States

[⊥]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852, United States

[∥]PATH's Malaria Vaccine Initiative (MVI), Washington, D.C. 20001 United States

Supporting Information

ABSTRACT: Malaria, one of the most common vector borne human diseases, is a major world health issue. In 2015 alone, more than 200 million people were infected with malaria, out of which, 429 000 died. Even though artemisinin-based combination therapies (ACT) are highly effective at treating malaria infections, novel efforts toward development of vaccines to prevent transmission are still needed. Pfs25, a postfertilization stage parasite surface antigen, is a leading transmission-blocking vaccine (TBV) candidate. It is postulated that Pfs25 anchors to the cell membrane using a glycosylphosphatidylinositol (GPI) linker, which itself possesses pro-inflammatory properties. In this study, *Escherichia coli* derived extract (XtractCF^{+TM}) was used in cell free protein synthesis [CFPS] to successfully express >200 mg/L of recombinant Pfs25 with a C-terminal non-natural amino acid (nnAA), namely, *p*-azidomethyl phenylalanine (pAMF), which possesses a reactive azide group. Thereafter, a unique conjugate vaccine (CV), namely, Pfs25-GPI was generated with dibenzocyclooctyne (DBCO) derivatized glycan core of malaria GPI using a simple but highly efficient copper free click chemistry reaction. In mice immunized with Pfs25 or Pfs25-GPI, the Pfs25-GPI group showed significantly higher titers compared to the Pfs25 group. Moreover, only purified IgGs from Pfs25-GPI group were able to significantly block transmission of parasites to mosquitoes, as judged by a standard membrane feeding assay [SMFA]. To our knowledge, this is the first report of the generation of a CV using Pfs25 and malaria specific GPI where the GPI is shown to enhance the ability of Pfs25 to elicit transmission blocking antibodies.

Malaria constitutes an important global health problem, and the World Health Organization estimated that 212 million cases and 429 000 deaths from malaria occurred in 2015.[1] While those numbers are disappointingly high, the expanded application of antimalarial control measures was proven to reduce the incidence and mortality of malaria by 41% and 62%, respectively, between 2000 and 2015.[1] In addition to artemisinin-based combination therapies (ACT) which are crucial in treating malarial infections, efforts toward development of a transmission blocking vaccine (TBV) have gained much attention recently in view of their potential to accelerate malaria parasite elimination.[2] TBV is designed to elicit anti-parasite or anti-mosquito antibodies in humans with the expectation that the induced antibodies will block parasite development in the mosquito host when ingested with the malaria parasites.

Pfs25, a postfertilization mosquito-stage (zygotes and ookinetes) surface expressed malaria antigen, is one of the leading TBV candidates based on promising preclinical data; however, this promise has not been replicated in clinical studies. Multiple human phase 1 trials with Pfs25 containing TBVs have been conducted or are being conducted,[3] and have shown to induce antibodies with functional efficacy in vaccinees, as judged by the standard membrane-feeding assay (SMFA).[4−6] However, a more potent TBV is likely to be required to show the efficacy in the field. Several heterologous expression systems ranging from *Escherichia coli*, yeast, baculovirus to plants have been examined for expression of Pfs25, but most of them either suffer from poor expression yields of soluble protein or are limited by difficulty in scaling up the protein production process.[7−11] In addition to protein antigens, malarial glycosylphosphatidylinositol (GPI) linkers, which are post-translational modifications that help anchor proteins to the plasma membrane, have also been shown to be important pathogenesis factors that can induce inflammation and cause symptoms similar to acute onset of malarial infection in animal models.[12−14] However, studies with malaria vaccines using parasite GPI have resulted in inconclusive results; depending on the target antigens and vaccine platforms, some studies have shown better immunogenicity in vaccines with GPI, but the opposite in other studies.[15−19] Thus, even though significant progress has been made in the quest to develop safe and effective TBV(s), several limitations persist, which need to

Received: October 30, 2017
Revised: January 9, 2018
Published: January 11, 2018

DOI: 10.1021/acs.biochem.7b01099
Biochemistry 2018, 57, 516−519

be overcome before their potential can be realized in a field setting.[2,6]

In this study, in order to overcome limitations of protein expression and to investigate the role of GPI in a Pfs25-based vaccine, we used a proprietary *in vitro* XpressCF^{+TM} cell free protein synthesis (CFPS) expression system[20,21] to robustly and efficiently express Pfs25 (V^{24}-T^{193}), which harbors a C-terminal amber stop codon site for incorporation of a non-natural amino acid (nnAA), namely, *p*-azidomethyl phenylalanine (pAMF) followed by a 6x-histidine tag to facilitate purification (Figure 1a). Expression of Pfs25 (>200 mg/L) was

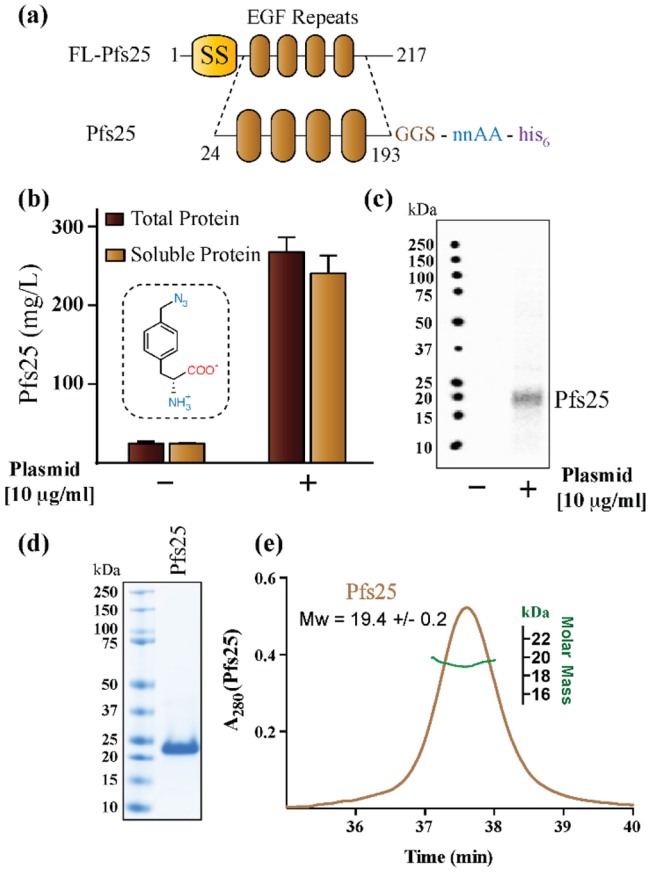

Figure 1. Pfs25 expression, purification, and biophysical characterization. (a) Schematic showing the modular architecture of full length (FL) FL-Pfs25 (aa 1−217) with a N-terminal signal sequence (SS) that was used to generate recombinant Pfs25 protein (aa 24−193) with a C-terminal extension comprised of a linker (GGS) followed by a non-natural amino acid (nnAA) incorporation site before a 6x-histidine tag for purification. (b) The yield of Pfs25 in CFPS as estimated by incorporation of ^{14}C-leucine into the translating polypeptide (inset shows the structure of the nnAA incorporated into recombinant Pfs25). (c) SDS-PAGE autoradiogram analysis of expression shows Pfs25 translation with ^{14}C-leucine incorporation. (d) Safe Blue stained SDS-PAGE analysis of purified Pfs25. (e) SEC-MALS analysis of the Pfs25.

performed as shown elsewhere[22,23] and quantitatively estimated using incorporation of ^{14}C-leucine into the translating polypeptide, which results in the generation of a single band on an autoradiogram (Figure 1b−c). Subsequently, Pfs25 was expressed, purified, and analyzed using SDS-PAGE gel, which showed >95% purity (Figure 1d). Final yield of purified Pfs25 was 120−140 mg/L. Finally, SEC MALS analysis of the purified protein showed a monodisperse species with an estimated molecular mass of 19.4 ± 0.2 kDa (Figure 1e), which is in close agreement with its theoretical molecular mass of 19.7 kDa.

Upon purification, the incorporation of the pAMF into Pfs25 was confirmed using an *in vitro* fluorescence labeling assay. As shown in Figure 2a, 50 µM purified Pfs25 was incubated with

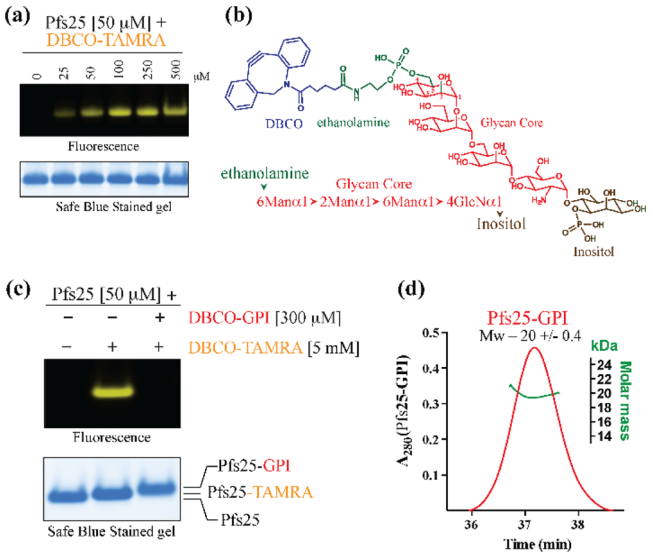

Figure 2. Pfs25 conjugation to DBCO-TAMRA and DBCO-GPI. (a) Pfs25 protein [50 µM] was incubated with increasing concentrations [0−500 µM] of DBCO-TAMRA dye at 25 °C for 2 h. Thereafter, 5 µL aliquots were analyzed using SDS-PAGE gel analysis. (b) Structure of malaria derived GPI linked to DBCO. (c) Pfs25-GPI or Pfs25 alone were incubated with a molar excess of DBCO-TAMRA to confirm stoichiometric conjugation of DBCO-GPI to pAMF sites in Pfs25. (d) SEC MALS analysis of the conjugated Pfs25-GPI (theoretical molecular mass of 20.7 kDa).

increasing concentrations of rhodamine based fluorescent dye, namely, DBCO-TAMRA, at RT for 1 h to facilitate a copper free click chemistry reaction. SDS-PAGE analysis followed by fluorescence readout was used to confirm concentration dependent enhancement in conjugate efficiency (with 250 µM or higher amounts of DBCO-TAMRA leading to saturation of conjugation). Subsequent Safe Blue staining of the gel confirmed equal protein amounts were used in all reactions. Using this information, Pfs25 [50 µM] was incubated with 300 µM of DBCO derivatized core glycan of malarial GPI (DBCO-GPI, prepared by Corden Pharma) (Figure 2b) overnight at 4 °C to facilitate stoichiometric conjugation. To confirm this, excess [5 mM] DBCO-TAMRA dye was added to the reaction mixture, and the reactions were analyzed as in Figure 2a. The lack of fluorescence postincubation with DBCO-TAMRA confirms that the pAMF site in Pfs25 was completely conjugated to DBCO-GPI (Figure 2c). Finally, SEC-MALS analysis of Pfs25-GPI conjugate showed a homogeneously eluting monodisperse species with a molecular mass of 20 ± 0.4 kDa (Figure 2d). The increase in mass in comparison to Pfs25 alone (19.4 kDa) confirms the presence of GPI attached to the C-terminal pAMF site (theoretical molecular mass of GPI is ~1.3 kDa).

To test for functional potency, mice were immunized with Pfs25 or Pfs25-GPI vaccine candidates (2 or 10 µg doses of Pfs25), and the immunogenicity was first evaluated by ELISA using an unconjugated Pfs25 protein. As shown in Figure 3A, formulations with Pfs25-GPI induced significantly higher

DOI: 10.1021/acs.biochem.7b01099
Biochemistry 2018, 57, 516−519

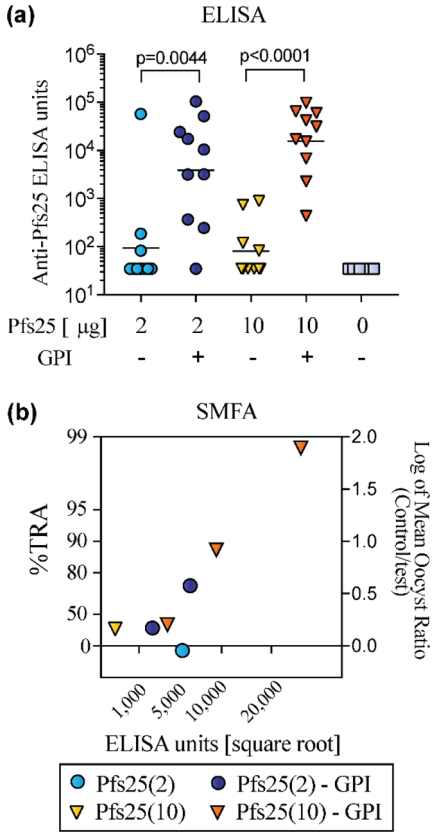

Figure 3. GPI induces higher level of functional anti-Pfs25 antibodies. (a) Anti-Pfs25 antibody levels (ELISA units) were determined on day 42. ELISA units of individual serum and geometric mean of group are shown. The levels of anti-Pfs25 antibodies between two groups (with or without GPI) were compared by a Mann−Whitney test, and the *p*-value is shown at each dose of Pfs25. (b) Purified IgGs were tested at indicated concentrations in two independent assays. Anti-Pfs25 ELISA units of each test condition are shown on a square root scale (*x*-axis), and the ratio of mean oocyst (mean oocyst density in control divided by mean in test) is plotted on a log-scale (right side of *y*-axis). The associated % inhibition (%TRA) value is shown on the left *y*-axis. The 100% TRA data point (Pfs25(2 μg)-GPI IgG tested at 750 μg/mL of total IgG, which was equivalent to 8253 ELISA units) is not shown in the figure, as the log of mean oocyst ratio is infinity.

antibody levels, both at 2 and 10 μg/dose. We then assessed functionality of the induced anti-Pfs25 antibodies by SMFA. Total IgG was purified from a pooled serum for each group, and tested at 750 μg/mL (Table S1, Assay #1). While Pfs25(2 μg)-GPI and Pfs25(10 μg)-GPI IgGs showed significant inhibitions (100 and 98.7% inhibitions in oocyst density, % TRA, respectively; $P < 0.001$ for both), Pfs25(2 μg) and Pfs25(10 μg) IgGs showed insignificant inhibitions (−10.3 and 31.2% TRA, respectively; $P \geq 0.36$). The CV-induced IgGs showed almost complete transmission reduction in the initial assay; therefore, further testing of the two IgGs was conducted at 250 and 83.3 μg/mL (Table S1, Assay #2). When the ratio of mean oocyst count (right *y*-axis, on a log-scale) was plotted against square root of antibody level (*x*-axis), there was a linear dose response, indicating that the quality of antibodies was similar in both GPI containing groups (Figure 3b).

Pfs25 is one of the most promising TBV candidates from *Plasmodium falciparum*. In this study, we have outlined a novel, highly efficient, and unique methodology for generating (>200 mg/L) and purifying recombinant Pfs25 protein that harbors a C-terminal nnAA, namely, pAMF, which can readily react with strained alkynes [Figure 1]. Additionally, we have also evaluated the adjuvant effect of malaria GPI to enhance immune response for generating functional anti-Pfs25 antibodies. As shown in Figure 2, copper free click chemistry was performed to site specifically conjugate DBCO derivatized malaria GPI to Pfs25 for generating a novel CV candidate, namely, Pfs25-GPI. Quite strikingly, not only did the immunization with Pfs25-GPI lead to higher anti-Pfs25 antibody titers, but also more importantly, only IgGs purified from Pfs25-GPI groups (and not Pfs25 group), significantly blocked oocyst development [Figure 3]. Thus, the current study for the first time shows that malaria GPI anchor can act as an adjuvant to significantly augment the transmission reducing immunity conferred by Pfs25 alone.

In 2016, WHO outlined aggressive goals to reduce the incidence of global malaria related cases and mortality rates by 40% by 2020 and to at least 90% by 2030.[1] RTS,S, the most advanced pre-erythrocytic stage vaccine, has shown encouraging results in phase 3 trials, but the vaccine efficacy against clinical malaria in 5−17 months children was 39% over four years of follow-up, for those participants receiving four doses of vaccine.[24] Therefore, controlling the spread of malaria-causing parasites using novel tools such as effective TBVs, offering higher and more durable efficacy, is still needed.[2] Apart from Pfs25, which is a postfertilization stage antigen, several prefertilization stage antigens, including Pfs230 and Pfs48/45, have also become important targets. However, expression and purification of soluble FL- or truncated fragments of Pfs230 or Pfs48/45 with correct conformation have been incredibly challenging.[25−29] Future efforts directed toward making Pfs230- and/or Pfs48/45-based TBVs, using CFPS technology, could be immensely useful. Furthermore, CFPS for malaria specific antigens with nnAA incorporation could provide an attractive opportunity for making CV candidates with enhanced potency, as shown in this study.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b01099.

A detailed description of the experimental procedures along with a table (Table S1) with details of SMFA results (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail:kmiura@niaid.nih.gov.

ORCID

Kazutoyo Miura: 0000-0003-4455-2432

Author Contributions

N.K., J.F., A.J.B., C.A.L., and K.M. designed the research, and analyzed the data. N.K., I.V., J.R., A.B., W.C., G.Y., C.T., A.K.S., A.R.S., T.P.P., and K.M. performed the research. N.K., J.F., A.J.B., C.A.L., and KM analyzed the data and wrote the manuscript with input from all authors.

Funding

The SMFA and ELISA works were supported in part by the intramural program of the National Institute of Allergy and

DOI: 10.1021/acs.biochem.7b01099
Biochemistry 2018, 57, 516−519

Infectious Diseases/NIH, and by PATH's Malaria Vaccine Initiative.

Notes

The authors declare the following competing financial interest(s): JF declares competing financial interest with Sutrovax, Inc. and JR with Sutro Biopharma.

■ ACKNOWLEDGMENTS

We thank Fraunhofer Center for Molecular Biotechnology (Newark, DE, USA) who supplied the plant expressed Pfs25MF1E protein for ELISA.

■ ABBREVIATIONS

ACT, artemisinin-based combination therapy; CFPS, cell free protein synthesis; pAMF, *p*-azidomethyl phenylalanine; nnAA, non-natural amino acid; DBCO, dibenzo cyclooctyne; TAMRA, tetramethyl rhodamine; GPI, glycosylphosphatidylinositol; TBV, transmission-blocking vaccine; CV, conjugate vaccine; SMFA, standard membrane feeding assay; TRA, transmission reducing activity

■ REFERENCES

(1) World Health Organization; *World Malaria Report*, 2016.
(2) Wu, Y., Sinden, R. E., Churcher, T. S., Tsuboi, T., and Yusibov, V. (2015) *Adv. Parasitol. 89*, 109−152.
(3) Clinicaltrials.gov; http://clinicaltrials.gov/ct2/home.
(4) Talaat, K. R., Ellis, R. D., Hurd, J., Hentrich, A., Gabriel, E., Hynes, N. A., Rausch, K. M., Zhu, D., Muratova, O., Herrera, R., Anderson, C., Jones, D., Aebig, J., Brockley, S., MacDonald, N. J., Wang, X., Fay, M. P., Healy, S. A., Durbin, A. P., Narum, D. L., Wu, Y., and Duffy, P. E. (2016) *PLoS One 11*, e0163144.
(5) Wu, Y., Ellis, R. D., Shaffer, D., Fontes, E., Malkin, E. M., Mahanty, S., Fay, M. P., Narum, D., Rausch, K., Miles, A. P., Aebig, J., Orcutt, A., Muratova, O., Song, G., Lambert, L., Zhu, D., Miura, K., Long, C., Saul, A., Miller, L. H., and Durbin, A. P. (2008) *PLoS One 3*, e2636.
(6) Nikolaeva, D., Draper, S. J., and Biswas, S. (2015) *Expert Rev. Vaccines 14*, 653−680.
(7) Kumar, R., Angov, E., and Kumar, N. (2014) *Infect. Immun. 82*, 1453−1459.
(8) Zou, L., Miles, A. P., Wang, J., and Stowers, A. W. (2003) *Vaccine 21*, 1650−1657.
(9) Lee, S. M., Wu, C. K., Plieskatt, J., McAdams, D. H., Miura, K., Ockenhouse, C., and King, C. R. (2016) *Malar. J. 15*, 405−417.
(10) Farrance, C. E., Chichester, J. A., Musiychuk, K., Shamloul, M., Rhee, A., Manceva, S. D., Jones, R. M., Mamedov, T., Sharma, S., Mett, V., Streatfield, S. J., Roeffen, W., van de Vegte-Bolmer, M., Sauerwein, R. W., Wu, Y., Muratova, O., Miller, L., Duffy, P., Sinden, R., and Yusibov, V. (2011) *Hum. Vaccines 7*, 191−198.
(11) Gregory, J. A., Li, F., Tomosada, L. M., Cox, C. J., Topol, A. B., Vinetz, J. M., and Mayfield, S. (2012) *PLoS One 7*, e37179.
(12) Naik, R. S., Branch, O. H., Woods, A. S., Vijaykumar, M., Perkins, D. J., Nahlen, B. L., Lal, A. A., Cotter, R. J., Costello, C. E., Ockenhouse, C. F., Davidson, E. A., and Gowda, D. C. (2000) *J. Exp. Med. 192*, 1563−1576.
(13) Schofield, L., and Hackett, F. (1993) *J. Exp. Med. 177*, 145−153.
(14) Schofield, L., Vivas, L., Hackett, F., Gerold, P., Schwarz, R. T., and Tachado, S. (1993) *Ann. Trop. Med. Parasitol. 87*, 617−626.
(15) Martinez, A. P., Margos, G., Barker, G., and Sinden, R. E. (2000) *Parasite Immunol. 22*, 493−500.
(16) Fanning, S. L., Czesny, B., Sedegah, M., Carucci, D. J., van Gemert, G. J., Eling, W., and Williamson, K. C. (2003) *Vaccine 21*, 3228−3235.
(17) Bruna-Romero, O., Rocha, C. D., Tsuji, M., and Gazzinelli, R. T. (2004) *Vaccine 22*, 3575−3584.
(18) Ophorst, O. J., Radosevic, K., Ouwehand, K., van Beem, W., Mintardjo, R., Sijtsma, J., Kaspers, J., Companjen, A., Holterman, L., Goudsmit, J., and Havenga, M. J. (2007) *Vaccine 25*, 1426−1436.
(19) Li, G., Basagoudanavar, S. H., and Gowda, D. C. (2008) *Parasite Immunol. 30*, 315−322.
(20) Voloshin, A. M., and Swartz, J. R. (2005) *Biotechnol. Bioeng. 91*, 516−521.
(21) Zawada, J. F., Yin, G., Steiner, A. R., Yang, J., Naresh, A., Roy, S. M., Gold, D. S., Heinsohn, H. G., and Murray, C. J. (2011) *Biotechnol. Bioeng. 108*, 1570−1578.
(22) Yin, G., Stephenson, H. T., Yang, J., Li, X., Armstrong, S. M., Heibeck, T. H., Tran, C., Masikat, M. R., Zhou, S., Stafford, R. L., Yam, A. Y., Lee, J., Steiner, A. R., Gill, A., Penta, K., Pollitt, S., Baliga, R., Murray, C. J., Thanos, C. D., McEvoy, L. M., Sato, A. K., and Hallam, T. J. (2017) *Sci. Rep. 7*, 3026.
(23) Zimmerman, E. S., Heibeck, T. H., Gill, A., Li, X., Murray, C. J., Madlansacay, M. R., Tran, C., Uter, N. T., Yin, G., Rivers, P. J., Yam, A. Y., Wang, W. D., Steiner, A. R., Bajad, S. U., Penta, K., Yang, W., Hallam, T. J., Thanos, C. D., and Sato, A. K. (2014) *Bioconjugate Chem. 25*, 351−361.
(24) The RTS,S Clinical Trials Partnership (2015) *Lancet 386*, 31−45.
(25) Chowdhury, D. R., Angov, E., Kariuki, T., and Kumar, N. (2009) *PLoS One 4*, e6352.
(26) Outchkourov, N. S., Roeffen, W., Kaan, A., Jansen, J., Luty, A., Schuiffel, D., van Gemert, G. J., van de Vegte-Bolmer, M., Sauerwein, R. W., and Stunnenberg, H. G. (2008) *Proc. Natl. Acad. Sci. U. S. A. 105*, 4301−4305.
(27) MacDonald, N. J., Nguyen, V., Shimp, R., Reiter, K., Herrera, R., Burkhardt, M., Muratova, O., Kumar, K., Aebig, J., Rausch, K., Lambert, L., Dawson, N., Sattabongkot, J., Ambroggio, X., Duffy, P. E., Wu, Y., and Narum, D. L. (2016) *J. Biol. Chem. 291*, 19913−19922.
(28) Farrance, C. E., Rhee, A., Jones, R. M., Musiychuk, K., Shamloul, M., Sharma, S., Mett, V., Chichester, J. A., Streatfield, S. J., Roeffen, W., van de Vegte-Bolmer, M., Sauerwein, R. W., Tsuboi, T., Muratova, O. V., Wu, Y., and Yusibov, V. (2011) *Clin. Vaccine Immunol. 18*, 1351−1357.
(29) Lee, S. M., Wu, C. K., Plieskatt, J. L., Miura, K., Hickey, J. M., and King, C. R. (2017) *Clin. Vaccine Immunol. 24*, e00140-17.

DOI: 10.1021/acs.biochem.7b01099
Biochemistry 2018, 57, 516−519