An opsonic phagocytosis assay for *Plasmodium falciparum* sporozoites

Ryan W. J. Steel,[a] Brandon K. Sack,[a] Moriya Tsuji,[b] Stefan H. I. Kappe,[a,c]#

Center for Infectious Disease Research, formerly Seattle Biomedical Research Institute, Seattle, Washington, USA[a]; HIV and Malaria Vaccine Program, Aaron Diamond AIDS Research Center, Affiliate of The Rockefeller University, New York, New York, USA[b]; Department of Global Health, University of Washington, Seattle, Washington, USA[c]

Running Head: Opsonization assay for *P. falciparum* sporozoites

Address correspondence to Stefan H.I. Kappe, Stefan.Kappe@cidresearch.org

Abstract

Plasmodium falciparum malaria remains the deadliest parasitic disease worldwide. Vaccines targeting the pre-erythrocytic sporozoite and liver stages have the potential to entirely prevent blood stage infection and disease, as well as onward transmission. Sporozoite surface and secreted proteins are leading candidates for inclusion in a pre-erythrocytic, antibody-based vaccine. Preclinical functional assays to identify humoral correlates of protection *in vitro*, and to validate novel sporozoite protein targets for inclusion in multi-subunit vaccines currently do not consider the interaction of sporozoite-targeting antibodies with other components of the immune system. Here, we describe the development of a simple flow cytometric assay to quantitatively assess the ability of antibodies directed against *P. falciparum* sporozoites to facilitate their phagocytosis. We demonstrate that this *S*porozoite *O*psonic *P*hagocytosis *A*ssay (SOPA) is compatible with both monoclonal antibodies and human immune serum, and can be performed using cryopreserved *P. falciparum* sporozoites. This simple, accessible assay will aid with assessing antibody responses to vaccination with *Plasmodium* antigens and their interaction with phagocytic cells of the immune system.

1. Introduction

The parasite *Plasmodium falciparum* (*Pf*) causes hundreds of millions of clinical malaria infections and half a million deaths annually (1). The parasite is transmitted by deposition of sporozoite stages into the skin through the bite of infected *Anopheles* mosquitoes. Motile sporozoites then move in the dermis to locate a capillary, gaining access to the circulation through which they sequester in the liver. Here sporozoites select and infect hepatocytes and the parasite then develops as a liver stage, producing tens of thousands of red blood cell-infectious merozoites that initiate blood stage infection. Stages prior to merozoite emergence from hepatocytes are collectively known as pre-erythrocytic (PE) stages. PE infection is asymptomatic, caused by a relatively small number of parasites, and their elimination prevents progression to blood stages of infection, responsible for the clinical symptoms of malaria and onward transmission of the parasite (2). These features render PE stages attractive vaccine targets.

Sporozoite surface and secreted proteins are rational antibody targets for vaccines that aim to block sporozoite infection. The most advanced PE vaccine candidate consists of the circumsporozoite protein (CSP), which is abundantly expressed on the sporozoite surface. Immunization with a CSP-based vaccine, "RTS,S/AS01", a virus-like particle expressing CSP adjuvanted with AS01, achieved modest protection in malaria endemic area phase 3 pediatric clinical trials that was in part mediated by antibodies (3, 4). An alternative approach, vaccination with live-attenuated sporozoites that are unable to progress to blood stage, also engenders sporozoite-neutralizing antibody responses that, together with cellular responses against the liver stages, confer high-level, sterilizing protection in controlled human malaria infection (CHMI) studies (5-7).

Antibodies against sporozoite proteins can block PE infection in a number of ways, including immobilizing sporozoites in the skin, in the blood, or by directly blocking their entry into the liver parenchyma and subsequent hepatocyte infection (8-10). The effects of PE stage antibodies on these different sporozoite activities can be partially assessed by *in vitro* assays modeling the transition of the sporozoite from the skin to the liver. These assays include the sporozoite gliding motility and cell traversal assays that model parasite activities in the skin and liver (7, 11), sporozoite invasion assays for hepatocyte infection (12-14), and assays measuring liver stage development within primary hepatocytes (15). Antibodies are tested for inhibitory activity in these assays and the collective results are then used

to prioritize candidate antigens for further development (16). However, none of the aforementioned assays currently analyze the downstream effects of anti-sporozoite antibodies in conjunction with cellular components of the immune system.

In addition to the blocking and neutralizing properties of antibodies, phagocytic cells in the skin and liver have the potential to engulf sporozoites that are decorated with antibodies, eliminate them, and initiate an adaptive immune response through antigen presentation to T-cells (17). Indeed, opsonization of sporozoites by antibodies has been shown to increase their phagocytosis and elimination by Kupffer cells of the liver (18), and opsonic phagocytosis of recombinant *Pf*CSP has been reported to correlate with protection using plasma from RTS,S immunized individuals (19). However, existing assays do not measure this potentially important role of antibodies and thus this aspect is not included in guiding PE vaccine development for *Pf*, despite an increasing awareness of the importance of antibody-mediated phagocytosis of blood stage parasites. Here, we have developed a novel Sporozoite Opsonic Phagocytosis Assay (SOPA) that can be included in candidate vaccine antigen screening. The SOPA uses the human Fc receptor positive monocytic cell line THP1 suitable for phagocytosis studies (20), and live or cryopreserved *Pf* sporozoites. We demonstrate that the rapid, flow cytometry based assay can be used with monoclonal antibodies (mAbs) against CSP, as well as blood sera and purified IgG from individuals immunized with a *Pf* genetically attenuated parasite (*Pf*GAP 3KO)(21). The resulting assay will be useful in preclinical analysis of antibody function against sporozoites, and analysis of humoral responses to PE vaccination in clinical trials.

2. Materials and Methods

2.1 THP1 cell culture

THP1 cells were maintained in RPMI 1640 complete media containing 10% FBS (Thermo), 200 U/mL penicillin-streptomycin (Thermo) and 2.5 µg/mL amphotericin B (Thermo) at a cell density between 0.1 – 1.0 x 10^6 cells per mL. Pretreatment of THP1 cells with Cytochalasin D (Sigma) or its vehicle (DMSO) to inhibit actin polymerization in some experiments was performed for 30 minutes at 37°C, followed by two washes in complete media to remove residual compound before the addition of cells to sporozoites.

99

100 **2.2 Mosquito rearing, sporozoite production and isolation**

101 Mosquito rearing and *Pf* sporozoite production were performed as previously described (22). Briefly, *Pf*
102 NF54 gametocyte cultures were fed to adult female *A. stephensi* mosquitoes 3 – 7 days after their
103 emergence. Blood-fed mosquitoes were then maintained according to standard protocols for 14 days,
104 after which their salivary glands were dissected and infectious sporozoites isolated by grinding and
105 centrifugation. In some experiments cryopreserved sporozoites were used. Following their dissection,
106 sporozoites to be cryopreserved were first purified using a discontinuous density gradient (23), then
107 resuspended in Schneider's insect media (Lonza) containing 300 mM trehalose and 200 µg/mL DOPC.
108 Resuspended sporozoites were incubated at room temperature for 10 minutes before addition of
109 hydroxylethylstarch to a final concentration of 1.7% v/v followed by rapid freezing by immersion in
110 liquid nitrogen. Prior to their use in the SOPA, cryopreserved sporozoites were thawed at room
111 temperature, pelleted by centrifugation, and cryopreservation media removed.

112

113 **2.3 Generation of *Pf*GAP immunized clinical samples**

114 Naïve and immune sera from human volunteers immunized with a *Pf* genetically attenuated parasite
115 (*Pf*GAP 3KO) in a phase Ia clinical trial were collected as described elsewhere (21). Briefly, volunteers
116 received a single exposure of 150 – 200 bites from *Pf*GAP infected *A. stephensi* mosquitoes. Sera was
117 collected from volunteers before infection (naive sera), and 13 days after infection (immune sera), and
118 purified IgG (pIgG) isolated from these sera using Protein G columns (GE, cat #28-9852-55).

119

120 **Generation and human chimerization of 3C1 monoclonal antibody against *P. falciparum* CSP**

121 The coding sequence of *Pf*CSP (3D7 strain) excluding C-terminal GPI anchor was cloned into the pET20b
122 vector, expressed in the BL21(DE3) *E. coli* strain and protein purified by Ni-Affinity Chromatography.
123 Subsequent immunization of mice and hybridoma fusion was performed by Green Mountain Antibodies,
124 Inc. (Burlington, VT). Briefly, mice were primed with 50 µg of *Pf*CSP emulsified with Complete Freund's
125 Adjuvant (CFA), followed by weekly immunization of 50 µg of *Pf*CSP emulsified with TiterMax® (Sigma) or
126 SAS® (Sigma). One week following the seventh immunization, B cells were isolated from lymph nodes,

fused with a mouse myeloma cell line, and positive clones secreting anti-*Pf*CSP IgG selected by ELISA. The leading clone 3C1 was selected for expression as a chimeric antibody with human constant regions. The DNA sequences encoding the heavy- and light-chain variable genes for antibody 3C1 were amplified by polymerase chain reaction and cloned into expression vectors containing the human κ light-chain constant region and human γ1 heavy-chain constant region. The expression vectors were transfected into HEK293 cells for transient production of chimeric 3C1-hIgG1. The cells were grown in chemically-defined medium and the full-length 3C1-hIgG1 was purified from the cell culture supernatant by protein A affinity chromatography (GE Healthcare Protein A resin).

2.4 Sporozoite opsonic phagocytosis assay (SOPA)

An overview of the assay workflow is shown (Figure 1). Salivary gland *Pf* sporozoites were first prepared in complete media at a concentration of 8 x 10^5 / mL, then sporozoites opsonized with 3C1 hIgG1, non-specific human IgG (hIgG), or sera or pIgG derived from patients immunized with a *Pf*GAP (21). Sporozoites were then incubated at 37°C for 15 minutes, before 1.5 x 10^4 opsonized sporozoites were plated into triplicate wells of a 96-well V-bottomed plate. THP1 cells prepared at 1 x 10^6 / mL in complete media were added to each well at a 1:1 volume with sporozoites to achieve a ratio of sporozoites:cells of 1:3. The plate was then centrifuged at 500 x g for 3 minutes, and incubated at 37°C for 15 minutes. Cells were then immediately fixed without washing in Perm/Fix buffer (BD Biosciences), blocked and permeabilized in Perm/Wash buffer (BD Biosciences), then stained with a monoclonal antibody directed against *Pf*CSP conjugated to Alexa Fluor 647 (clone 2A10). THP1 cells containing sporozoites were identified by flow cytometry using a BD-LSRII flow cytometer (BD Biosciences), and data analysis performed using FlowJo software (TreeStar). In some experiments, a F(ab')2 fragment of 3C1 generated by pepsin digestion of the Fc region (Thermo, Cat # 44688) was used in place of the full length 3C1.

2.5 Statistical Analysis

Data were analyzed using Graphpad Prism (version 6) for Windows, and considered significant when $p<0.05$.

Clinical and Vaccine Immunology

3. Results

3.1 Development and validation of the SOPA

We developed a flow cytometry-based assay to measure the capacity of antigen-specific antibodies to facilitate opsonic phagocytosis of sporozoites by the Fc-receptor positive THP-1 cell line (Figure 1). In developing the assay, we determined that a ratio of sporozoites:cells of 1:3 with a 15-minute incubation was optimal (Figure S1). Using the sporozoite surface marker CSP, the assay measures changes in the frequency of intracellular parasites when *Pf* sporozoites are opsonized with either monoclonal antibodies or clinical immune sera and then phagocytosed. Incubation of *Pf* sporozoites with a human chimeric monoclonal antibody reactive against *Pf* CSP (3C1 hIgG1) (Figure S2), but not control non-specific hIgG, increased the frequency of intracellular parasites at concentrations as low as 1 ng/mL ($p<0.05$) with an EC_{50} of 7.1 ng/mL (Figure 2A, 2B). THP1 cells not incubated with sporozoites were negative for CSP, and used as an additional negative control in all assays (data not shown).

Having shown that antigen-specific antibodies facilitated an increase of phagocytic cells positive for the sporozoite marker CSP, we set out to further substantiate that this was due to opsonic phagocytosis of sporozoites. We generated F(ab')2 fragments of 3C1 and verified digestion of the Fc region by PAGE under non-reducing conditions (data not shown). When used in the SOPA, these 3C1 F(ab')2 fragments were unable to mediate the uptake of sporozoites by phagocytic cells when used at the same concentration as full length 3C1, instead showing low uptake levels similar to control hIgG (Figure 2C). Phagocytosis is an active cell-mediated process, requiring actin polymerization in the phagocyte that can be inhibited by cytochalasin D. To verify that the assay in fact measures active phagocytosis of sporozoites in the presence of 3C1 mAb, we pretreated phagocytic cells with cytochalasin D or its vehicle for 30 minutes, followed by washing out the drug to prevent inhibition of sporozoite motility. Cells treated with cytochalasin D, but not its vehicle, showed a significant 3.1-fold reduction in the number of sporozoite-containing phagocytic cells (Fig. 2D). Finally, we performed the SOPA on permeabilized, as well as unpermeabilized cells (Fig. 2E). While hIgG opsonized sporozoites showed similar levels of CSP^+ cells regardless of permeabilization (indicating some surface-associated sporozoites are detected in the assay), an increase in CSP^+ events with 3C1 mAb was seen only in permeabilized cells. Together, these data show that the increase in CSP^+ phagocytic cells we observe in the SOPA

Clinical and Vaccine Immunology

requires an antigen-specific antibody (Fig. 2A-B) and antibody interaction with phagocytic cells via their Fc region (Fig. 2C) that is dependent on actin polymerization in the THP1 cells (Fig. 2D), resulting in an increase in intracellular sporozoites (Fig. 2E). These data are consistent with the SOPA measuring opsonic phagocytosis of *Pf* sporozoites by THP1 cells.

3.2 Measurement of SOPA response with sera of whole sporozoite-immunized volunteers

Having demonstrated proof of concept for the SOPA using a *Pf*CSP-specific mAb, we next sought to determine the compatibility of the assay with sera from humans immunized with whole *Pf* parasites (21).

Sera from two volunteers that had received a single high dose of a triple gene deletion-attenuated sporozoite (*Pf*GAP 3KO) (21) was tested in a dilution series in the SOPA to identify the dilution range yielding the greatest fold change between naive and immune sera (Figure 3). Compared to untreated sporozoites, we observed differences in the response of the volunteers' naive sera, as well as the maximum fold change between matched naive and immune sera in the SOPA. One volunteer showed a negligible increase in CSP^+ cells with naive sera at all tested dilutions (Fig. 3A), while in the other volunteer a significant increase in CSP^+ cells was observed at naive sera dilutions of less than 160-fold (Fig. 3B). In both volunteers, the greatest fold change between naive and immune sera was observed at 80 to 160-fold dilutions, and at the earliest dilution where naive sera had a negligible effect in the assay. We observed similar differences between volunteers when purified IgG, rather than whole sera was used in the SOPA (Figure S3).

To test the reproducibility of the assay, as well as determine its capacity to measure differences in the SOPA response between individuals, we screened the sera of five *Pf*GAP 3KO immunized volunteers at an 80-fold dilution in the assay (Figure 4). The maximum fold change between naive and immune sera from these five volunteers revealed significant differences between the responses of their samples (Fig. 4A-B). These differences were reproduced in a second, independently performed assay, and a significant correlation was observed between the two assays runs demonstrating reproducibility of results (Fig. 4C). Although protection data is not yet available for the *Pf*GAP 3KO, these reproducible differences

demonstrate that the SOPA could be a useful assay for identifying correlates of protection with human clinical samples. Importantly, the magnitude of response of clinical samples in the SOPA did not necessarily correlate with titers of antibodies against *Pf*CSP (Fig. 4D) (21), suggesting that sporozoite antigens other than CSP might be able to facilitate opsonic phagocytosis in this assay.

The above experiments were performed using freshly isolated *Pf* sporozoites, the production of which requires specialized insectary facilities and personnel not available in many laboratories. Methods to cryopreserve *Pf* sporozoites have been developed, making them more broadly available for research. We thus tested cryopreserved *Pf* sporozoites in the SOPA to determine their compatibility with the assay (Figure 5). Opsonization of cryopreserved *Pf* sporozoites with 3C1 or immune sera from *Pf*GAP 3KO immunized volunteers resulted in an increase in sporozoite-containing phagocytic cells similar to that seen with fresh sporozoites, indicating that the assay will not be restricted to fresh sporozoites.

4. Discussion and Conclusion

The contribution of sporozoite-targeted antibodies to protective PE immunity is increasingly recognized for both subunit- and whole attenuated sporozoite vaccination (5, 24). These antibodies can act at multiple points during the journey of sporozoites from the dermis to the liver, and a number of *in vitro* assays are available to measure inhibition of distinct processes at multiple steps in this journey. These include assays for inhibition of motility in the skin as sporozoites search for a capillary, traversal of multiple cell types as sporozoites gain access to the circulation and subsequently to the liver parenchyma, as well as ultimately the invasion of hepatocytes by sporozoites (16). While these assays are certainly important tools in the analysis of humoral responses against subunit and whole sporozoite vaccination, they measure only neutralization of sporozoite protein functions and do not consider the interaction of these parasite-bound antibodies with other immune components.

The significance of antibody effector functions, mediated by the interaction of antibody Fc regions with other immune components, has been recognized in functional assays against the blood stages of malaria infection. The growth inhibition assay (GIA) quantifies reductions in merozoite invasion of red blood cells with antibodies against merozoite proteins (25). The addition of complement or phagocytic cells to the

GIA increases inhibition by most antibodies in this assay, and increases the capacity of the assay to predict protection (26-31). Recently, complement fixation by antibodies recognizing glycan residues on sporozoites has been shown to be protective *in vivo* (32), suggesting that the role of Fc-mediated functions should be more carefully considered in PE immunity and vaccine development as well. The sporozoite opsonic phagocytosis assay (SOPA) that we report here is the first description of a functional assay using *Pf* sporozoites that considers the interaction of antibodies with other immune components via their Fc-mediated effector functions.

Opsonic phagocytosis of sporozoites could be expected to augment clearance of the parasite from the host, as well as enhance the initiation of adaptive immune responses by phagocytic cells. Sporozoite traversal of the sinusoidal barrier in the liver has been shown to facilitate their evasion of phagocytosis and clearance by Kupffer cells (33), and incubation of sporozoites with specific antibodies increases parasite clearance by Kupffer cells *in vitro* (18). These studies indicate that antibodies bound to sporozoites could enhance their clearance at the parasites' final barrier to the establishment of liver stage infection. In the skin draining lymph nodes, $CD11c^{+}$ and $CD8\alpha^{+}$ dendritic cells appear important for the initiation of protective CD8+ T-cell responses observed in whole sporozoite immunizations (34). Enhanced phagocytosis and antigen presentation has been described for a variety of pathogens with opsonizing antibodies (17), and antibodies recognizing sporozoites could be expected to enhance adaptive immunity during *Pf* infection. Finally, opsonic phagocytosis has been investigated in the context of RTS,S immunization. In that study, plasma from immunized volunteers was able to facilitate uptake of recombinant *Pf*CSP and this correlated with protection (19). It is noteworthy to highlight that this previous assay used a protein corresponding to the immunogen of RTS,S immunization, whereas the SOPA that we describe uses whole *Pf* sporozoites. The use of sporozoites in our assay constitutes a significant improvement over previous approaches, as all sporozoite antigens are present in their natural parasite context and the activity of antibodies to proteins other than CSP can be investigated.

Here, we have developed a simple, medium throughput *in vitro* assay able to reproducibly measure the antibody-mediated opsonic phagocytosis of whole *Pf* sporozoites. This sporozoite opsonic phagocytosis assay (SOPA) is efficient with both clinical sample (1 – 4 µL of sera per sample is required per sample per experiment) and sporozoites (1.5×10^{6} sporozoites is sufficient to test 14 clinical samples with matched

Clinical and Vaccine Immunology

naïve and immune serum), and can be completed in less than one day. Using the SOPA we observed significant differences in opsonic phagocytosis between five volunteers immunized with a PfGAP 3KO vaccine that did not correlate with anti-PfCSP titers. While the small samples size in this study is a limitation, these data suggest that antigens other than CSP may be able to contribute to opsonic phagocytosis of sporozoites. It is also possible that the difference in SOPA response observed between these volunteers is related to the specific IgG subclasses of the opsonizing antibodies present in the samples, an analysis not performed here. It will be important to explore these possibilities in future studies. Nevertheless, the SOPA we have developed appears sensitive enough to enable correlative analyses to be performed in the future using samples from subjects with known protection outcomes.

We have performed our analysis using the fold-change between naive and immune sera, although it is important to note that alternative methods of analysis are possible. For example, the percent CSP^+ cells in immune sera at a given dilution could be employed. Indeed, the worst performer amongst analyzed volunteers, where fold change is reported, also exhibited the highest percent of CSP^+ cells using immune sera at an 80-fold dilution (Fig. 4). As protection was not assessed in this PfGAP 3KO phase Ia study, we are unable to resolve which method best reflects the contribution of opsonic phagocytosis to protection *in vivo*, and the predictive power of the assay remains to be determined.

5. Acknowledgements

The 3C1 monoclonal antibody used in this research was provided as a chimeric 3C1 hIgG1 by the PATH Malaria Vaccine Initiative. We thank the insectary at the Center for Infectious Disease Research for infected and uninfected mosquito preparation.

6. Funding Information

Work by the authors is funded by the PATH Malaria Vaccine Initiative, and the National Institute of Allergy and Infectious Disease (NIAID) (grant number NIH 5 F32 AI114113-03).

Clinical and Vaccine Immunology

References

1. **Organization WH.** 2015. World Malaria Report 2015.
2. **Vaughan AM, Aly ASI, Kappe SHI.** 2008. Malaria parasite pre-erythrocytic stage infection: Gliding and hiding. Cell Host & Microbe **4:**209-218.
3. **White MT, Bejon P, Olotu A, Griffin JT, Riley EM, Kester KE, Ockenhouse CF, Ghani AC.** 2013. The relationship between RTS,S vaccine-induced antibodies, CD4(+) T cell responses and protection against Plasmodium falciparum infection. PLoS One **8:**e61395.
4. **RTS SCTP.** 2015. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. The Lancet **386:**31-45.
5. **Ishizuka AS, Lyke KE, DeZure A, Berry AA, Richie TL, Mendoza FH, Enama ME, Gordon IJ, Chang L-J, Sarwar UN, Zephir KL, Holman LA, James ER, Billingsley PF, Gunasekera A, Chakravarty S, Manoj A, Li M, Ruben AJ, Li T, Eappen AG, Stafford RE, K C N, Murshedkar T, DeCederfelt H, Plummer SH, Hendel CS, Novik L, Costner PJM, Saunders JG, Laurens MB, Plowe CV, Flynn B, Whalen WR, Todd JP, Noor J, Rao S, Sierra-Davidson K, Lynn GM, Epstein JE, Kemp MA, Fahle GA, Mikolajczak SA, Fishbaugher M, Sack BK, Kappe SHI, Davidson SA, Garver LS, Bjorkstrom NK, Nason MC, Graham BS, Roederer M, Sim BKL, Hoffman SL, Ledgerwood JE, Seder RA.** 2016. Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. Nat Med **22:**614-623.
6. **Epstein JE, Richie TL.** 2013. The whole parasite, pre-erythrocytic stage approach to malaria vaccine development: a review. Curr Opin Infect Dis **26:**420-428.
7. **Behet MC, Foquet L, van Gemert GJ, Bijker EM, Meuleman P, Leroux-Roels G, Hermsen CC, Scholzen A, Sauerwein RW.** 2014. Sporozoite immunization of human volunteers under chemoprophylaxis induces functional antibodies against pre-erythrocytic stages of Plasmodium falciparum. Malar J **13:**136.
8. **Vanderberg JP, Frevert U.** 2004. Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes. Int J Parasitol **34:**991-996.
9. **Kebaier C, Voza T, Vanderberg J.** 2009. Kinetics of mosquito-injected Plasmodium sporozoites in mice: fewer sporozoites are injected into sporozoite-immunized mice. PLoS Pathog **5:**e1000399.
10. **Sack BK, Miller JL, Vaughan AM, Douglass A, Kaushansky A, Mikolajczak S, Coppi A, Gonzalez-Aseguinolaza G, Tsuji M, Zavala F, Sinnis P, Kappe SH.** 2014. Model for in vivo assessment of humoral protection against malaria sporozoite challenge by passive transfer of monoclonal antibodies and immune serum. Infect Immun **82:**808-817.
11. **Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, Nussenzweig V, Rodriguez A.** 2001. Migration of Plasmodium sporozoites through cells before infection. Science **291:**141-144.
12. **Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A, Richman A, Chakravarty S, Manoj A, Velmurugan S, Li M, Ruben AJ, Li T, Eappen AG, Stafford RE, Plummer SH, Hendel CS, Novik L, Costner PJ, Mendoza FH, Saunders JG, Nason MC, Richardson JH, Murphy J, Davidson SA, Richie TL, Sedegah M, Sutamihardja A, Fahle GA, Lyke KE, Laurens MB, Roederer M, Tewari K, Epstein JE, Sim BK, Ledgerwood JE, Graham BS, Hoffman SL.** 2013. Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine. Science doi:10.1126/science.1241800.

13. **Kaushansky A, Rezakhani N, Mann H, Kappe SH.** 2012. Development of a quantitative flow cytometry-based assay to assess infection by Plasmodium falciparum sporozoites. Mol Biochem Parasitol **183:**100-103.
14. **Finney OC, Keitany GJ, Smithers H, Kaushansky A, Kappe S, Wang R.** 2014. Immunization with genetically attenuated P. falciparum parasites induces long-lived antibodies that efficiently block hepatocyte invasion by sporozoites. Vaccine **32:**2135-2138.
15. **Zou X, House BL, Zyzak MD, Richie TL, Gerbasi VR.** 2013. Towards an optimized inhibition of liver stage development assay (ILSDA) for Plasmodium falciparum. Malar J **12:**394.
16. **Steel RWJ, Kappe SH, Sack BK.** In Press. An expanding toolkit for preclinical pre-erythrocytic malaria vaccine development: bridging traditional mouse malaria models and human trials. Future Microbiology.
17. **Joller N, Weber SS, Oxenius A.** 2011. Antibody - Fc receptor interactions in protection against intracellular pathogens. European Journal of Immunology **41:**889-897.
18. **Seguin MC, Ballou WR, Nacy CA.** 1989. Interactions of Plasmodium berghei sporozoites and murine Kupffer cells in vitro. J Immunol **143:**1716-1722.
19. **Schwenk R, Asher LV, Chalom I, Lanar D, Sun PF, White K, Keil D, Kester KE, Stoute J, Heppner DG, Krzych U.** 2003. Opsonization by antigen-specific antibodies as a mechanism of protective immunity induced by Plasmodium falciparum circumsporozoite protein-based vaccine. Parasite Immunology **25:**17-25.
20. **Chanput W, Mes JJ, Wichers HJ.** 2014. THP-1 cell line: an in vitro cell model for immune modulation approach. Int Immunopharmacol **23:**37-45.
21. **Kublin JG, Mikolajczak SA, Sack BK, Fishbauger M, Seilie A, Shelton L, VonGoederdt T, Firat M, Magee S, Fritzen E, Betz W, Kain HS, A. DD, Steel RWJ, Vaughan AM, Sather DN, Murphy SC, Kappe SHI.** Complete attenuation of a genetically engineered Plasmodium falciparum parasite in humans. Science Translational Medicine ***In Press***.
22. **Vaughan AM, Mikolajczak SA, Wilson EM, Grompe M, Kaushansky A, Camargo N, Bial J, Ploss A, Kappe SH.** 2012. Complete Plasmodium falciparum liver-stage development in liver-chimeric mice. J Clin Invest **122:**3618-3628.
23. **Kennedy M, Fishbaugher ME, Vaughan AM, Patrapuvich R, Boonhok R, Yimamnuaychok N, Rezakhani N, Metzger P, Ponpuak M, Sattabongkot J, Kappe SH, Hume JC, Lindner SE.** 2012. A rapid and scalable density gradient purification method for Plasmodium sporozoites. Malar J **11:**421.
24. **Olotu A, Lusingu J, Leach A, Lievens M, Vekemans J, Msham S, Lang T, Gould J, Dubois MC, Jongert E, Vansadia P, Carter T, Njuguna P, Awuondo KO, Malabeja A, Abdul O, Gesase S, Mturi N, Drakeley CJ, Savarese B, Villafana T, Lapierre D, Ballou WR, Cohen J, Lemnge MM, Peshu N, Marsh K, Riley EM, von Seidlein L, Bejon P.** 2011. Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. Lancet Infect Dis **11:**102-109.
25. **Persson KE, Lee CT, Marsh K, Beeson JG.** 2006. Development and optimization of high-throughput methods to measure Plasmodium falciparum-specific growth inhibitory antibodies. J Clin Microbiol **44:**1665-1673.
26. **Boyle MJ, Reiling L, Feng G, Langer C, Osier FH, Aspeling-Jones H, Cheng YS, Stubbs J, Tetteh KK, Conway DJ, McCarthy JS, Muller I, Marsh K, Anders RF, Beeson JG.** 2015. Human antibodies fix complement to inhibit Plasmodium falciparum invasion of erythrocytes and are associated with protection against malaria. Immunity **42:**580-590.
27. **Kumaratilake LM, Ferrante A.** 2000. Opsonization and phagocytosis of Plasmodium falciparum merozoites measured by flow cytometry. Clin Diagn Lab Immunol **7:**9-13.

28. **Tiendrebeogo RW, Adu B, Singh SK, Dziegiel MH, Nébié I, Sirima SB, Christiansen M, Dodoo D, Theisen M.** 2015. Antibody-Dependent Cellular Inhibition Is Associated With Reduced Risk Against Febrile Malaria in a Longitudinal Cohort Study Involving Ghanaian Children. Open Forum Infectious Diseases **2:**ofv044.
29. **Osier FHA, Feng GQ, Boyle MJ, Langer C, Zhou JL, Richards JS, McCallum FJ, Reiling L, Jaworowski A, Anders RF, Marsh K, Beeson JG.** 2014. Opsonic phagocytosis of Plasmodium falciparum merozoites: mechanism in human immunity and a correlate of protection against malaria. Bmc Medicine **12**.
30. **Hill DL, Eriksson EM, Carmagnac AB, Wilson DW, Cowman AF, Hansen DS, Schofield L.** 2012. Efficient Measurement of Opsonising Antibodies to Plasmodium falciparum Merozoites. Plos One **7**.
31. **Wilson DW, Fowkes FJI, Gilson PR, Elliott SR, Tavul L, Michon P, Dabod E, Siba PM, Mueller I, Crabb BS, Beeson JG.** 2011. Quantifying the Importance of MSP1-19 as a Target of Growth-Inhibitory and Protective Antibodies against <italic>Plasmodium falciparum</italic> in Humans. PLoS ONE **6:**e27705.
32. **Yilmaz B, Portugal S, Tran TM, Gozzelino R, Ramos S, Gomes J, Regalado A, Cowan PJ, d'Apice AJ, Chong AS, Doumbo OK, Traore B, Crompton PD, Silveira H, Soares MP.** 2014. Gut microbiota elicits a protective immune response against malaria transmission. Cell **159:**1277-1289.
33. **Tavares J, Formaglio P, Thiberge S, Mordelet E, Van Rooijen N, Medvinsky A, Ménard R, Amino R.** 2013. Role of host cell traversal by the malaria sporozoite during liver infection. The Journal of Experimental Medicine **210:**905-915.
34. **Cockburn IA, Zavala F.** 2016. Dendritic cell function and antigen presentation in malaria. Current Opinion in Immunology **40:**1-6.

Figure 1: Analysis of the *P. falciparum* sporozoite opsonic phagocytosis assay (SOPA) by flow cytometry. Sporozoites are incubated either alone, in the presence of antibodies or clinical immune sera for 15 minutes before addition of THP1 cells at a ratio of sporozoites:cells of 1:3. Sporozoites and cells are centrifuges briefly, then after a 15-minute incubation cells are fixed, permeabilized, and sporozoites detected using a fluorescently conjugated monoclonal antibody directed against *Pf*CSP.

Figure 2: Opsonized *P. falciparum* sporozoites are efficiently phagocytosed by THP1 cells. (A) Sporozoites were incubated in media alone (untreated), human IgG (hIgG), or a monoclonal hIgG antibody directed against *Pf*CSP (clone 3C1) before the addition of THP1 cells and analysis by flow cytometry. The presence of 3C1, but not hIgG at 10 µg/mL facilitated a significant increase in the percent of CSP^+ cells relative to untreated sporozoites. **(B)** The 3C1 and hIgG antibodies were titrated in the SOPA to determine the sensitivity of the assay. Significant increases in CSP^+ cells when sporozoites were opsonized with h3C1 was observed at concentrations of 1 ng/mL and higher, with an EC_{50} of 7.1 ng/mL. A negligible effect of hIgG was observed in the assay that performed similarly to untreated sporozoites (dashed line) at all tested concentrations. F(ab')2 fragments of mAb 3C1 lacking the hIgG Fc region did not facilitate phagocytic uptake of sporozoites **(C)** but uptake in the presence of full 3C1 was reduced by pretreatment of THP1 cells with 200 µM cytochalasin D **(D). (E)** The increase in CSP^+ cells with h3C1-opsonized sporozoites was not observed when non-permeabilized cells were used in the assay, demonstrating the increase observed is due to intracellular sporozoites. Data are the mean ± SD from representative experiments, analyzed either by One-way ANOVA with Sidak's planned comparison testing (A, C-D) or Two-way ANOVA with Bonferroni's multiple comparison testing; *$p<0.05$, ****$p<0.0001$.

Figure 3: Determining parameters for use of human sera in the SOPA. Naive (blue line) and immune (red line) sera from two volunteers (A and B) immunized with *Pf*GAP 3KO were tested in the SOPA across a dilution series. For one volunteer naive sera performed comparably to untreated sporozoites (dashed line) at all tested dilutions (A), while in another a background effect of naive sera was observed at dilutions of less than 160-fold (B). In both volunteers the percentage of CSP^+ cells with immune sera peaked at 80-fold dilution, with the greatest fold change between naive and immune sera (yellow line) seen at dilutions of 80-fold or above. Data are the mean ± SD from a representative experiment.

458

459 **Figure 4: Reproducible differences between clinical sera samples are measured by the SOPA. (A)** Naive
460 (Day 0) and matched immune (Day 13 post immunization) sera from five volunteers immunized with
461 *Pf*GAP 3KO were tested in the SOPA (dashed line indicates untreated sporozoites). The fold change
462 between matched sera for each volunteer was calculated, and statistically significant differences
463 between the responses of the volunteers was observed **(B)**. The experiment was performed a second
464 time, and the mean fold change between the naive and immune sera for each of the two independent
465 experiments was significantly correlated using Pearson's Correlation **(C)**. No correlation was seen
466 between CSP antibody titers and fold change in the SOPA for either of these two experiments **(D)**. Data
467 in (A) and (B) represent the mean ± SD of one representative experiment; analyzed by One-way ANOVA
468 with Bonferroni's post-hoc test; *$p<0.05$, **$p<0.01$, ***$p<0.001$. Data in (C) and (D) are analyzed by
469 Pearson correlation.

470

471 **Figure 5: Use of cryopreserved sporozoites in the SOPA.** Cryopreserved *Pf* sporozoites were tested in
472 the SOPA with the monoclonal antibody 3C1 and hIgG (A), as well as naive and immune sera from a
473 volunteer immunized with *Pf*GAP 3KO (B). A significant increase in CSP^+ cells was seen using both 3C1
474 and immune sera, demonstrating the compatibility of frozen sporozoites with the SOPA. Data are the
475 mean ± SD from a representative experiment, analyzed by One-way ANOVA (A) and student's t-test (B);
476 ****$p<0.0001$.