

Short Communication

A simple, ex vivo phagocytosis assay of *Plasmodium vivax* merozoites by flow cytometry

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ABSTRACT

As phagocytosis is the first line of defense against malaria, we developed a phagocytosis assay with *Plasmodium vivax* (*P. vivax*) merozoites that can be applied to evaluate vaccine candidates. Briefly, after leukocyte removal with loosely packed cellulose powder in a syringe, *P. vivax* trophozoites matured to the merozoite-rich schizont stages in the presence of the E64 protease inhibitor. The Percoll gradient-enriched schizonts were chemically disrupted to release merozoites that were submitted to merozoite opsonin-dependent phagocytosis in two phagocytic lines with human and

mouse antibodies against the N- and C-terminal domains of *P. vivax* Merozoite Surface Protein-1. The resulting assay is simple and efficient for use as a routine phagocytic assay for the evaluation of merozoite stage vaccine candidates.

KEYWORDS: *Plasmodium vivax*; merozoites; MSP1; opsonizing antibodies.

Based on *Plasmodium falciparum* studies, merozoite opsonization appears to be correlated to immunity against malaria, and such merozoite phagocytosis assays could be useful to aid preclinical vaccine development and evaluate vaccine clinical trials. (Boyle et al., 2010; Chan et al., 2012; Chiu et al., 2015). Merozoite phagocytosis has never been evaluated in *P. vivax*, hence, we adapted standardized protocols to develop a merozoite phagocytosis assay with saponin-treated *P. vivax* schizonts concentrated from clinical isolates, and flow cytometry is a useful tool for studying phagocytic uptake in the blood stage (Russell et al. 2012, 2015, Lopes et al. 2014). The resulting assay is a simple to evaluate opsonizing antibodies from malaria vaccine candidates.

Two PvMSP1 recombinant proteins (N- and C-terminus of *P. vivax* MSP1) were purified on glutathione-Sepharose 4B columns (Amersham-GE Healthcare Life Sciences, Sinapse Biotecnologia, São Paulo, Brazil) as previously described as GST tagged proteins (Soares et al. 1997, Nogueira et al. 2006, Versiani et al. 2013). Each protein was used to obtain mouse-immunized sera and specific, purified human IgG antibodies from malaria-infected individuals by immunoprecipitation.

For murine-immunized sera, eight-week-old female Balb/c mice (n=3) were intraperitoneally immunized with 50 µg protein of Nterm-PvMSP1, MSP1₁₉ or GST alone dissolved in 0.1 mL PBS emulsified in 1:1 ratio with complete Freund's adjuvant, and subsequent immunizations were administered in incomplete Freund's adjuvant at intervals of 20 days. Control mice received Phosphate-buffered saline (PBS) plus adjuvant. Mouse immunized sera were quantified by enzyme-linked immunosorbent assay. All immunization procedures minimized discomfort to the nonhuman animals. All procedures were approved by CEUA-INPA 03/2015. Sera from mice immunized with GST were used as controls. For human antibodies, each proteins was cross-linked to carbon nanotubes (CNTs). Briefly, activated CNTs with five µM EDAC followed by 10 mM NHS in pH 7.0 phosphate buffer were linked in ration of 100 µg of protein to 500 mg of CNTs for 30 minutes, blocked with bovine serum albumin (BSA) and stored at 4 °C in the presence of sodium azide until use. We used

human plasma from malaria-exposed individuals whose anti-Nterm-PvMSP1 and anti-MSP1₁₉ antibodies were previously identified (Versiani et al. 2013). Pooled human plasma samples were added, incubated for 30 minutes under agitation at room temperature, and washed with six cycles of washing and centrifugation. For the elution of the specific anti-Nterm-PvMSP1 and anti-MSP1₁₉ antibodies, 0.1 M glycine-HCl (pH 3.0) was added and coupled CNTs were centrifuged for 12,000 × g for 5 minutes at room temperature. To minimize acid degradation, the eluate was recovered and transferred to tubes containing 1/10th volume of 1 M Tris-HCl, pH 9.0. The reactivity of the eluted human antibodies and mouse immunized sera against anti-N-term PvMSP1 and anti-MSP1₁₉ were tested using enzymatic immune assays with the respective proteins (see IgG ELISA). The best opsonizing concentration of antibodies was verified in cytometry with ethidium bromide labelled merozoites, the 0.5 µg/mL for purified human antibodies and 1:50 dilution of mouse immunized.

As sources of merozoites for the phagocytosis assays, five human malaria blood samples were collected from adult malaria patients at “*Fundação de Medicina Tropical-Dr. Heitor Vieira Dourado*”, FMT-HVD, for which consent forms were approved and five milliliters of peripheral blood was collected from infected patients (approval number CAAE 42021515.0.3001.0005-FMT-HVD). White blood cells were removed from the blood using a CF11 cellulose (Sigma) column (Sripawat et al. 2009). Briefly, 50 % hematocrit red blood cells were then passed through a 10 mL syringe containing five cm³ of loosely packed CF11-cellulose powder (Sigma, Brazil) at the bottom that was pre-sterilized by ultraviolet light. Then, *P. vivax* trophozoites were matured in 20 % hematocrit in 7.5 % glucose McCoy medium supplemented with 10 % AB+ serum at 5 % O₂, 5 % CO₂ and 90 % N₂ until the beginning of schizogony, according to previous study (Carvalho et al. 2010). After 24-30 hours of culture, parasite-infected erythrocytes were treated with trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E64), a cysteine protease inhibitor, to ensure a maximum output of merozoite-rich schizonts, with some modifications (Hill et al. 2014). E64 ensured that the schizonts were fully mature after 46 hours of culture and osmotically ruptured schizonts to release fully formed merozoites. The Percoll gradient confirmed the full schizogony of schizonts containing uninucleated, membrane-enclosed merozoites (Figure 1A). The inset in this picture shows fully formed merozoites obtained after osmotic rupture. The integrity and full morphology of merozoites were verified with an immunofluorescence assay (IFA). Free merozoites and ruptured schizonts were incubated with mouse anti-N-term PvMSP1 and anti-MSP1₁₉ antibodies in BSA-phosphate buffer in 1.5 mL micro tubes for 30 minutes at room temperature and revealed with Alexa Fluor-488 conjugated anti-mouse antibodies and DAPI were incubated for 30 minutes at room

temperature. The images were obtained with a 100x magnification lens using an Imaging System (EVOS-FL Color Imaging System, Thermo Fisher, Brazil). Despite the fragility of the parasites, anti-Nterm-PvMSP1 antibodies confirmed the expression of MSP1 in DAPI-labeled scattered schizonts (Figure 1A). Free merozoites did not have damage to their surface coating after osmotic shock and repeated washings with saponin, as revealed by anti-MSP1₁₉ opsonizing antibodies (Figure 1B). As the 19-kDa fragment (MSP1₁₉) remains attached to the merozoite surface through its glycosylphosphatidylinositol anchor, immunofluorescence with the human IgG anti-MSP1₁₉ antibodies confirmed the integrity of merozoites, as observed in classical studies (Boyle et al. 2010, Hill et al. 2012, Osier et al. 2014).

For phagocytosis assays, merozoites were labeled with 1/1,000 DNA-specific dye SYBR Green solution (ThermoFisher) and pre-opsonized with antibodies. The merozoite-containing pellets were washed once due to the fragility of the parasites and suspended in DMEM-HAM-F12 to prepare a suspension of 10⁶ merozoites/mL. Fifty microliters of free merozoites (~5x10⁴ free merozoites) and fifty microliters of murine macrophage cell line J774 (J774 cells) and human macrophage cell line THP1 were incubated at a 1:1 ratio in round bottom 96-well polystyrene microwell plates for 1-hour incubation at 37 °C in a 5 % CO₂ atmosphere (Hill et al. 2012). Each condition was performed in triplicate. After incubation, the cell suspensions were washed at 200 × g for eight minutes at room temperature to remove free parasites. The pellet was suspended and fixed in 2 % paraformaldehyde (PFA) and stored in the dark at 4 °C for a maximum of 24 hours prior to measurement using a FACSCanto II with red-blue lasers (BD Bioscience) (Chaves et al. 2016).

To optimize the functional phagocytosis assays, SYBR-labeled merozoites and phagocytic cell lines free of merozoites were acquired individually and plotted on the forward versus side scatter (FSC vs. SSC) axis, respectively (Figure 2A). We distinguished merozoite and merozoite-free phagocytic cells by a merge between both gates served to define a “phagocytic cell gate”. Dot plot charts defined in the FSC versus FL-1 axis compared phagocytosis-positive gates of pre-opsonized merozoites with anti-Nterm-PvMSP1, anti-MSP1₁₉ and anti-GST antibodies, or non-opsonized merozoites (Figure 2B).

The opsonization-dependent merozoite phagocytosis of anti Nterm-PvMSP1 and anti-MSP1₁₉ were assessed in the murine J774 and THP-1 phagocytic cell lines, after 1 hour at 37 °C under 5 % CO₂

(Figures 2CD). For murine J774 line, samples were tested in triplicate while with THP-1 they were performed in duplicate. The results were represented individually for each sample to show variability between them. The percentage of SYBR+ cells were determined by the number of cells in the phagocytosis-positive gate of fifty thousand events acquired (Figures 2CD). The percentage of SYBR+ cells and merozoite phagocytosis levels increased significantly after opsonization with the anti-Nterm-PvMSP1 and IgG anti-MSP1₁₉ antibodies. The opsonizing ability of mouse and human antibodies to anti-Nterm-PvMSP1 and anti-MSP1₁₉ antibodies were evaluated by median intensity fluorescence (MIF) and calculated among events acquired in the phagocytosis-positive gate (Figures 2EF). To standardize merozoite phagocytosis for each sample, we created the following formula for phagocytosis index ($PI = \% SYBR+ cells \times MIF$). Antisera from immunized mice and purified human antibodies against Nterm-PvMSP1 and MSP1₁₉ proteins were able to demonstrate the opsonizing ability of the tested antibodies (Figures 2GH).

Based on *P. falciparum* studies, merozoite opsonization appears to be correlated to immunity against malaria, and such merozoite phagocytosis assays could be useful to aid preclinical vaccine development and evaluate vaccine clinical trials (Hill et al. 2012, 2013, 2014, 2016, Osier et al. 2014, Chiu et al. 2015). Here, merozoite phagocytosis assay with saponin-treated *P. vivax* schizonts concentrated from clinical isolates is a simple and efficient method to evaluate opsonizing antibodies from malaria vaccine candidates. Because a lack of efficient and continuous *in vitro* culture systems has limited efforts to develop *P. vivax*-specific vaccines (Herrera et al. 2007), our technique could be beneficial for evaluating *P. vivax* merozoite stage vaccine candidates.

To achieve success in merozoite phagocytosis assays, the processing of maturation *P. vivax* isolates should be rapid and efficient (Lopes et al., 2014; Russell et al., 2015, 2012). After leukocyte and platelet removal, we were able to mature *P. vivax* schizonts without rupture using the E64 protease, similar to its use in *P. falciparum* cultures for merozoite isolation (Boyle et al., 2010; Hill et al., 2016, 2014, 2013, 2012; Osier et al., 2014). Maturation was enhanced when the cultures were started when the trophozoites were mostly in the 20-24 hour stage and largely dependent on leukocyte depletion, as demonstrated previously (Russell et al., 2012). Despite the fragility of the parasites, the integrity of the merozoite membrane was assessed with an IFA with anti-Nterm-PvMSP1 antibodies. Nterm-PvMSP1 is the major domain before the initial MSP1 proteolytic processing that releases 83, 30, and 38 kDa fragments (Soares et al., 1997). In addition, this is the first characterization of anti-Nterm-

PvMSP1 antibodies that confirms the expression of MSP1 in DAPI-labeled scattered schizonts. Moreover, full schizogony was also characterized with free merozoites collected from the supernatants of cultures, despite the use of E64. As the 19-kDa fragment remains attached to the merozoite surface through its glycosylphosphatidylinositol anchor, immunofluorescence with the human IgG anti-MSP1₁₉ antibodies confirmed the integrity of merozoites, as observed in classical studies (Boyle et al., 2010; Hill et al., 2012; Osier et al., 2014).

Our merozoite phagocytosis assay demonstrated reliability even in the presence of hemozoin, although some studies have reported that hemozoin could affect phagocyte function or generate confounding events (Boyle et al. 2010, Hill et al. 2014). Indeed, we observed unspecific phagocytosis without antibodies; nonetheless, the phagocytosis index for the anti-Nterm-PvMSP1 and anti-MSP1₁₉ antibodies was higher than in the control. Moreover, the presence of hemozoin, as a possible cause of confounding events, did not harm our merozoite phagocytosis assay, whereas an auspicious study had to modify the SSC detector to change polarized light to depolarized light to allow the detection of hemozoin (Frita et al., 2011). Additionally, these authors used SYBR green to distinguish Hz-containing intraerythrocytic parasites. SYBR is a cyanine dye label, and eventual confounding events could be compensated in the FL-3 channel by comparison to the normal human IgG antibodies and GST-immunized mice serum or without antibodies with sera even after compensation. Thus, due to the prominent need for physical modification, the presence of hemozoin did not harm our merozoite phagocytosis assay.

In conclusion, the impossibility of a continuous culture of *P. vivax* limited us to an *ex vivo* short-term culture with a specified endpoint: the schizont stage. We collectively standardized *P. vivax* short-term cultures and mature schizonts to be applied in investigations in endemic areas. The combination of a phagocytic assay and flow cytometry has become an efficient method for studies of malaria vaccine candidates or novel vaccine targets.

Authors' Contribution:

EF, FB, SL contributed with phagocytosis assay
DCBS, TBC contributed with blood collection and *P. vivax* short culture
MEA, LBO PPO contributed with protein expression
PL, YC contributed with mouse immunization and cytometer assay
MVGL, PAN contributed with manuscript preparation

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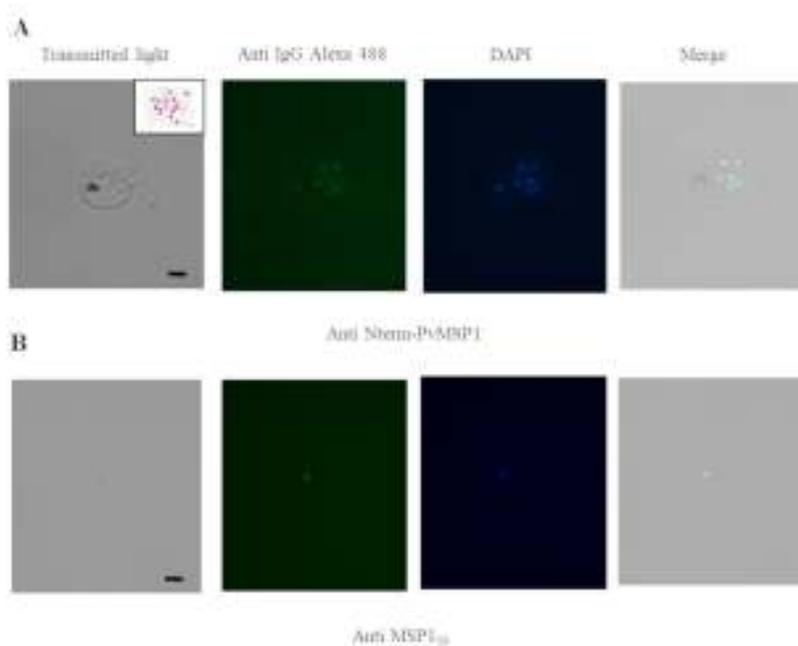


Figure 1

Figure 1. The integrity and full morphology of *Plasmodium vivax* merozoites and assessment of opsonizing antibodies were verified with an immunofluorescence assay (IFA). Blood film of parasite-infected erythrocytes treated with trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E64), a cysteine protease inhibitor, that ensured a maximum output of merozoite-rich schizonts. Schizonts were osmotically ruptured with 0.1 % saponin to release fully formed and homogeneous merozoites. The integrity of the merozoite membrane was assessed with an IFA with immunized mouse sera against the Nterm-PvMSP1 and MSP1₁₉ antibodies. A) The anti-Nterm PvMSP1 antibody revealed fully membrane enclosed merozoites. The inset in this picture shows fully formed merozoites obtained after osmotic rupture. The surface localization of the N-terminal PvMSP1

antigen is shown in mature schizonts stained with Alexa-488, mouse anti-IgG secondary antibodies, and the nucleus is stained with DAPI (panels in order: transmission light bright field, Alexa 488; DAPI, and merge). B) The opsonizing merozoite with the anti-MSP1₁₉ antibodies revealed that the surface coating did not cause damage. Bar=1 μ M.

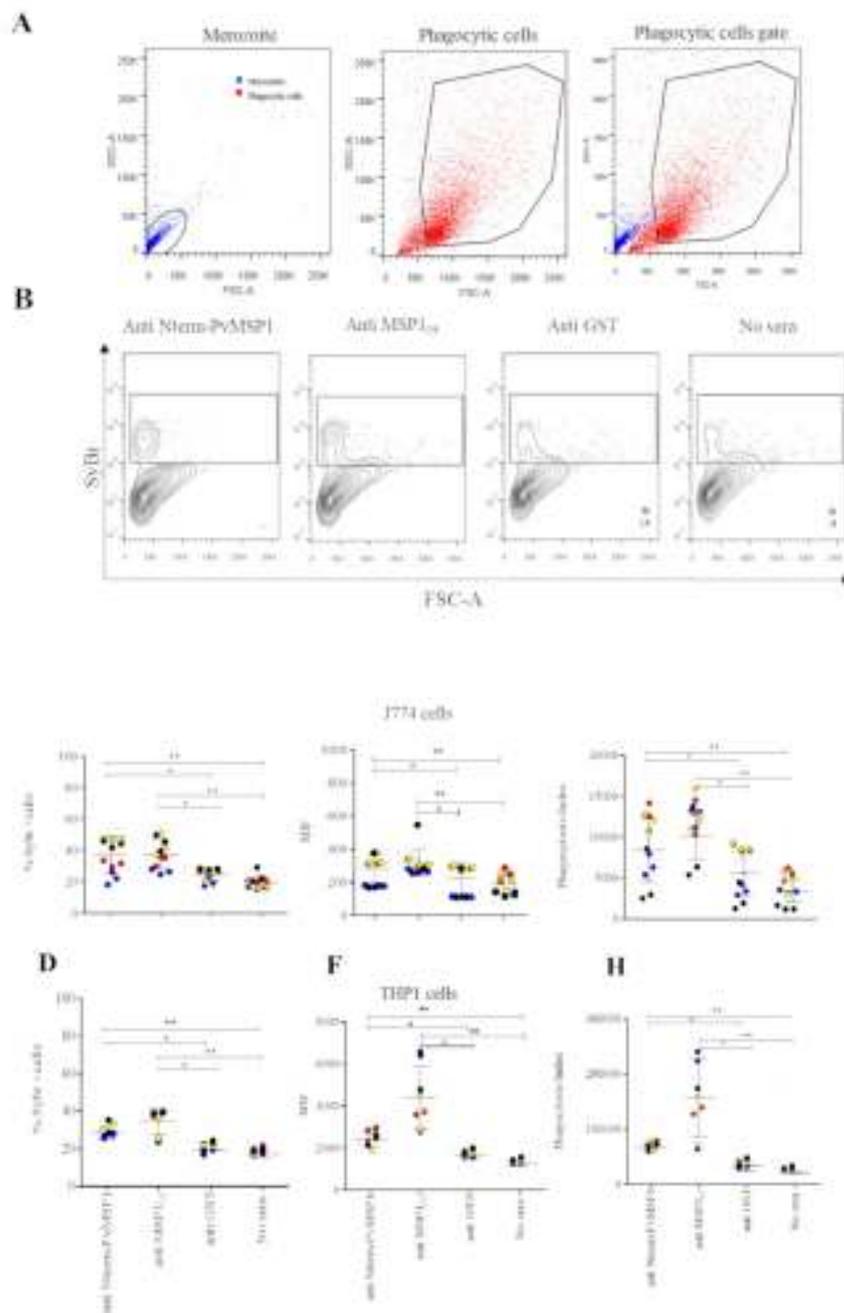


Figure 2. Optimization of the phagocytic cell line to target merozoites to evaluate the opsonizing abilities of specific antibodies. A) The suspension of merozoites was acquired and plotted on the FSC vs. SSC axis (left panel). A suspension of merozoite-free phagocytic cell lines was also plotted in the FSC vs. SSC axis (middle panel). A merge between merozoite and phagocytic cell charts served to define a “phagocytic cell gate” (right panel). B) Contour plot charts show phagocytosis-positive gates of SYBR-labeled merozoites pre-opsonized with immunized sera; respectively anti Nterm-PvMSP1, anti MSP1₁₉ anti GST mouse immunized sera, and no sera, measurement using a FACSCanto II with red-blue lasers (BD Bioscience). C-H) The opsonization-dependent merozoite phagocytosis of anti Nterm-PvMSP1 and anti-MSP1₁₉ were assessed in the murine J774 and THP-1 phagocytic cell lines. For murine J774 line, samples were tested in triplicate while with THP-1 they were performed in duplicate. For mouse antibodies, a 1:50 serum dilution in Phosphate buffered salt (PBS) of immunized sera with Nterm-PvMSP1, MSP1₁₉, and GST. The PBS was used as no sera control. For purified, human IgG antibodies, a 0.5 µg/ml of purified human IgG against Nterm-PvMSP1 and MSP1₁₉, and normal human IgG diluted in PBS. PBS was used as control. . The results were represented individually for each sample to show variability between them. Each isolate is represented by a color that is repeated in each graph. C-D) The percentage of SYBR-labelled merozoite phagocytizing cells acquired in the phagocytosis-positive gate in relation to fifty thousand events; C) murine J774; D) THP-1 phagocytic cell lines. E-F) Comparison of the median intensity fluorescence (MIF) of the SYBR-labelled merozoites of four isolates and pre-opsonized with mouse or human antibodies. E) Murine J774; F) THP-1 phagocytic cell lines. G-H) The functional opsonizing ability of these antibodies was assessed in phagocytosis assays with J774 and THP-1 cell lines in 96-well polystyrene round bottom plates. The phagocytosis index was standardized by multiplying the percentage of SYBR-labelled merozoite phagocytizing cells by the MIF. Each condition was performed in triplicate. G) Murine

J774; H) THP-1 phagocytic cell lines. All data were calculated as Repeated Measures one-way ANOVA using Holm-Sidak's multiple comparisons test. (* $p < 0.05$; ** $p < 0.005$).