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TITLE:

Measuring Naturally Acquired Phagocytosis-Inducing Antibodies to *Plasmodium falciparum* Parasites by a Flow Cytometry-Based Assay

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KEYWORDS:

phagocytosis, opsonization, antibodies, placental malaria, parasite-infected erythrocytes, IEs, VAR2CSA

SUMMARY:

The overall goal of this protocol is to provide instruction on how to measure the capacity of antibodies present in sera or plasma of individuals, naturally exposed to *Plasmodium falciparum* infection, to opsonize and induce phagocytosis of the parasite-infected erythrocytes (IEs).

ABSTRACT:

The protocol describes how to set up and run a flow cytometry-based phagocytosis assay of *Plasmodium falciparum*-infected erythrocytes (IEs) opsonized by naturally acquired IgG antibodies specific for VAR2CSA. VAR2CSA is the parasite antigen that mediates the selective sequestration of IEs in the placenta that can cause a severe form of malaria in pregnant women, called placental malaria (PM). Protection from PM is mediated by VAR2CSA-specific antibodies that are believed to function by inhibiting placental sequestration and/or by opsonizing IEs for phagocytosis. The assay employs late-stage-synchronized IEs that have been selected in vitro to express VAR2CSA, plasma/serum-antibodies from women with naturally acquired PM-specific immunity, and the phagocytic cell line THP-1. However, the protocol can easily be modified to assay the functionality of antibodies to any parasite antigen present on the IE surface, whether induced by natural exposure or by vaccination. The assay offers simple

and high-throughput evaluation, with good reproducibility, of an important functional aspect of antibody-mediated immunity in malaria. It is, therefore, useful when evaluating clinical immunity to *P. falciparum* malaria, a major cause of morbidity and mortality in the tropics, particularly in sub-Saharan Africa.

INTRODUCTION:

Malaria is a vector-borne disease caused in humans upon infection with five different species of the genus *Plasmodium*. The most prevalent species is *P. falciparum*, which is also responsible for the most morbidity and mortality[1]. Malaria clinical presentation varies from asymptomatic or benign infections to complicated/severe disease, the latter occurring mostly in children under the age of five years. Exposure to *P. falciparum* does not induce sterile immunity, but individuals living in endemic areas slowly develop immunity against the clinical disease. Protection is age/exposure dependent and immunity is normally acquired during the first 5-10 years of life[2]. Adult women are an important exception, as severe malaria can occur during pregnancy in a clinical presentation known as placental malaria (PM). PM is an important cause of abortion, stillbirth, premature delivery, low birth weight, fetal death, and maternal anemia. Resistance to PM develops over successive pregnancies[3]. Protection from PM is associated with the acquisition of antibodies against VAR2CSA-type PfEMP1[4,5], an infected erythrocyte (IE) surface antigen that binds to chondroitin sulphate A (CSA) enabling IE sequestration in the placenta. Antibodies mediate protection performing various functional activities (reviewed in[6,7]) including opsonization of IEs to induce phagocytosis. Early in vitro studies showed that antibodies can limit *P. falciparum* growth in the presence of monocytes via phagocytosis[8,9]. More recent studies have shown that higher levels of phagocytosis-inducing antibodies are associated with better pregnancy outcomes (in the context of HIV co-infection)[10,11], indicating the relevance of this effector function in the naturally acquired immune response.

Here we present a protocol to measure this function of antibodies present in human plasma/serum, using in vitro cultured IEs expressing VAR2CSA together with the monocyte line THP-1. The assay has been previously used[11–18] and is considered an improved and easier approach compared to earlier microscope-based protocols[8], since it allows testing of a larger number of antibody samples in a single run using smaller volumes of antibody and avoiding tedious and biased microscopy counting. Even though the assay has been used by multiple laboratories and its execution is simple enough, it requires careful planning and preparation, therefore, a detailed protocol would allow its application by laboratories and researchers lacking previous experience. We use, as an example, late-stage-synchronized IEs expressing VAR2CSA opsonized with antibodies present in serum collected from women with naturally acquired PM-specific immunity. However, the protocol can easily be modified to assay the functionality of antibodies to any parasite antigen present on the IE surface, whether induced by natural exposure or by vaccination.

PROTOCOL:

The human serum samples used for the results presented here were collected in a separate study[19]. Collection was approved by the Institutional Review Board of Noguchi Memorial

Institute for Medical Research, University of Ghana (study 038/10-11), and by the Regional Research Ethics Committees, Capital Region of Denmark (protocol H-4-2013-083).

0. Parasite culture

NOTE: Follow the local regulations for human pathogens handling.

1.1. Maintain *P. falciparum* parasites according to the standard protocol as described before[20] in parasite culture medium (see **Table of Materials**).

1.2. Keep the parasites tightly synchronized using sorbitol treatment as previously described[21].

1.3. To ensure VAR2CSA expression on the surface of the IE, perform repeated rounds of immune-magnetic selection using an anti-VAR2CSA antibody (e.g., PAM1.4 antibody, a cross-reactive human monoclonal VAR2CSA-specific IgG[22]) coupled to protein G-magnetic beads[23].

1.3.1. In brief, incubate late-stage trophozoite-IEs with magnetic beads coupled to an anti-VAR2CSA antibody and positively select using a magnet.

1.3.2. Expand the selected parasites in culture for a few cycles until parasitemia is at least 5%.

1.3.3. Alternatively, select parasites that bind to plastic-immobilized CSA (the receptor for VAR2CSA) as previously described[24].

1.4. To verify VAR2CSA expression on the selected parasites perform flow cytometry as previously described[25].

1.4.1. In brief, label the late-stage trophozoite-IEs with the same antibody used for the magnetic selection (step 1.3) followed by a fluorescently labeled secondary antibody.

1.4.2. Measure the IE surface reactivity by flow cytometry. Successful antibody selection normally results in a parasite population that expresses VAR2CSA in most of the parasites (≈80%).

1.5. For the phagocytosis assay, use purified mid- to late-stage trophozoite-IEs. Perform purification using magnetic separation as previously described[26].

NOTE: To accurately determine the stage of the parasites, perform Giemsa staining on blood smears followed by light microscopy observation. Follow standard procedures and morphological guidelines as described before[27]. Late-stage purification can also be performed using density gradient medium. The IE yield, however, in our experience is lower and, therefore, magnetic purification is preferable.

1.5.1. For magnetic separation, spin the parasite culture (5-10% parasitemia) at 500 *x g* for 10 min at room temperature. Remove the supernatant and re-suspend the cell pellet in 10 mL of parasite culture medium.

1.5.2. Add the parasite suspension into a CS-column coupled to a magnet (see **Table of Materials**) and let it slowly pass through the column. Trophozoite-IEs are paramagnetic (due to the presence of hemozoin) and will stay into the column mesh.

1.5.3. Wash the column with 50 mL of parasite culture medium and elute the IEs from the magnetic column using 50 mL of parasite culture medium.

1.6. Spin the eluate from 1.5.3 at 500 *x g* for 10 min at room temperature. Carefully discard the supernatant and re-suspend in 1 mL of parasite culture medium.

1.7. Using a hemocytometer, count the number of IEs (easily identified by light microscopy as erythrocytes with dark hemozoin pigment) and total cell number to calculate the final parasitemia (percentage of IEs).

NOTE: Only use purified cultures with at least 80% IEs.

1.8. Based on the number of serum/antibody samples planned for testing, estimate the total amount of IEs needed and scale up the parasite cultures accordingly. A 75 cm^2 culture flask with 25 mL of culture at 5% hematocrit and 5-10% parasitemia should yield enough IEs to run a full 96 well plate. For a full plate (42 samples plus 6 controls in duplicate), a minimum of 1.5 mL parasite suspension at 3.3 x 10^7 IEs/mL are needed.

2. THP-1 cells

NOTE: The THP-1 cell line is used in this assay. This monocyte cell line is derived from a patient with monocytic leukemia[28] and can be purchased from ATCC. Maintain the cell line according to the provider's instructions in THP-1 culture medium (see **Table of Materials**).

2.1. For regular maintenance, start THP-1 cell culture at 2-4 x 10^5 cells/mL and subculture when cell concentration reaches 8 x 10^5 cells/mL.

NOTE: Do not allow the cell concentration to exceed 1 x 10^6 cells/mL and keep track of passage number. Avoid the use of cells that are beyond passage 25. The average doubling time of the THP-1 cell line varies between 19-50 h. Determine the doubling time of the cell batch before starting the phagocytosis experiments. This will help to roughly estimate the necessary amount of culture needed for a determined number of serum samples to be tested (e.g., for a full 96 well plate, 10 mL of culture at 5 x 10^5 cells/mL are needed).

2.2. Periodically check the THP-1 cells for the surface expression of the Fcγ-receptors I (CD64), II (CD32) and III (CD16) as previously described[15].

2.2.1. In brief, stain the THP-1 cells (separately) with anti-CD64, anti-CD32 and anti-CD16 fluorescently labeled antibodies (**Table of Materials**) for 30 min at room temperature (1:100 dilution prepared in 2% FBS in PBS).

2.2.2. Wash three times with 2% FBS in PBS and measure surface staining by flow cytometry.

NOTE: The THP-1 cells are negative for CD16 and positive for CD32 and CD64 as previously reported[29,30] (**Figure 1**).

2.3. For the phagocytosis assay, set up a THP-1 cell culture flask with 2.5 x 10^5 cells/mL the day before the experiment to yield around 5 x 10^5 cells/mL on the day of assay.

NOTE: Ensure 4-6 x 10^5 cells/mL are present on the day of the assay.

3. Phagocytosis assay (Figure S1)

3.1. Before starting the assay, block (>1 h) two round-bottom 96 well plates (one for the opsonization and another for the phagocytosis) using 150 µL per well of sterile 2% FBS in PBS.

NOTE: Label plate 1 as opsonization plate and use it both for ethidium bromide (EtBr) staining and opsonization. Label plate 2 as phagocytosis plate and use both for THP-1 cell plating and for the phagocytosis step.

3.2. Parasite staining and opsonization

3.2.1. Take the IE suspension prepared in 1.6 and adjust cell count to 3.3 x 10^7 IEs/mL by adding parasite culture medium and EtBr to achieve a final concentration of 2.5 µg/mL (1:40 dilution, from a 0.1 mg/mL stock).

NOTE: EtBr stains the parasite DNA, allowing detection by flow cytometry.

CAUTION: EtBr is a mutagen and skin, eye, and respiratory irritant. Use appropriate protection and dispose waste according to local regulations.

3.2.2. Remove the blocking solution from one of the 96 well plates (opsonization plate), flicking the plate and removing liquid excess over a piece of towel paper.

3.2.3. Add 30 µL per well of the IE suspension prepared above in the upper half of the plate. Leave one well empty and add 30 µL of parasite culture medium (without IEs for the THP-1 alone control). (**Figure S2A**). Incubate for 10 min at room temperature and protected from light.

3.2.4. Add 170 µL per well of parasite culture medium. Spin the plate at 500 *x g* for 3 min and remove the supernatant by flicking the plate over an appropriate waste container.

3.2.5. Wash the EtBr-labeled IEs two more times using 200 µL per well of parasite culture medium spinning the plate at 500 *x g* for 3 min. The supernatant from the first wash can be removed by flicking the plate. Carefully remove the supernatant from the second wash using a multichannel pipette to make sure the entire volume of washing medium is removed. Do not disturb the pellet.

3.2.6. Re-suspend the EtBr-labeled IEs in 30 µL of antibody/plasma/serum solution prepared at the desired concentrations in parasite culture medium.

3.2.7. Always include the following controls (**Figure S2B**): a control without IEs/THP-1 cells control (parasite culture medium); a control without any antibody or plasma/serum (un-opsonized control); a positive control using the commercially available rabbit anti-human erythrocyte antibody at 1:100 dilution (see **Table of Materials**); two negative plasma/serum controls at 1:5 dilution (a malaria-naïve pool and a pool from malaria-exposed males); and a positive serum control at 1:5 dilution (a pool from malaria-exposed women, who have previously been pregnant (preferably multigravidae)).

NOTE: A 1:100 dilution for the positive control seems to work consistently across different batches of purchased antibody (data not shown). However, it is recommended to rule out variations between batches by testing several dilutions every time a new batch of antibody is used.

3.2.8. Incubate for 45 min in the dark at 37 °C.

3.3. THP-1 cells preparation and phagocytosis

3.3.1. While the IEs are being opsonized (3.2.9), begin preparing the THP-1 cells.

3.3.2. Remove the THP-1 cells from the culture flask, spin them down (500 *x g* for 5 min at room temperature), decant the supernatant and re-suspend the pellet in THP-1 cell culture medium.

3.3.3. Spin again (500 *x g* for 5 min at room temperature), decant the supernatant and re-suspend the cell pellet in 1 mL of THP-1 cell culture medium.

3.3.4. Determine cell count in the solution prepared above and add more medium to obtain a final concentration of 5 x 10^5 cells/mL.

3.3.5. Remove the blocking solution from the remaining 96 well plate (phagocytosis plate) by flicking the plate and removing liquid excess over a piece of towel paper.

3.3.6. Add 100 µL per well of the THP-1 cell suspension prepared in 3.3.4 and put back in the cell culture incubator (**Figure S2C**).

3.3.7. Once the antibody/plasma/serum incubation time has finished, add 170 µL per well of parasite culture medium. Spin the plate at 500 *x g* for 3 min at room temperature and remove the supernatant by flicking the plate over an appropriate waste container.

3.3.8. Wash the opsonized IEs two more times using 200 µL per well of parasite culture medium, spinning the plate at 500 *x g* for 3 min. The supernatant from the first wash can be removed by flicking the plate. Carefully remove the supernatant from the second wash, using a multichannel pipette to make sure the entire volume of washing medium is removed. Do not disturb the pellet.

3.3.9. Finally re-suspend the opsonized IEs in 100 µL of pre-warmed THP-1 cell culture medium. Transfer 50 µL of opsonized IE suspension to each well in the phagocytosis plate. Since there is a total of 100 µL of IEs, each antibody/serum dilution can be run in duplicates in the phagocytosis plate (**Figure S2D**)

NOTE: The amount of IEs and THP-1 cells used in the assay correspond to a 10:1 ratio.

3.3.10. Incubate for 40 min in the dark at 37 °C, 5% CO_2.

NOTE: Do not allow the phagocytosis to proceed for more than 40 min.

3.3.11. Stop the phagocytosis by centrifugation at 4 °C (500 *x g*, 5 min) and discard the supernatant by flicking the plate. Add 150 µL of room-temperature ammonium chloride lysing solution (**Table of Materials**) and mix by pipetting, incubate for exactly 3 min.

NOTE: This step will lyse the erythrocytes that have not been phagocytosed by the THP-1 cells.

3.3.12. Stop the lysis by adding 100 µL of ice-cold 2% FBS in PBS. Spin the plate at 4 °C (500 *x g* for 3 min) and remove the supernatant by flicking the plate over an appropriate waste container.

3.3.13. Wash three times using 200 µL per well of ice-cold 2% FBS in PBS spinning the plate at 4°C (500 *x g* for 3 min). After the final wash, re-suspend in 200 µL of ice-cold 2% FBS in PBS and immediately analyze by flow cytometry.

NOTE: Previous publications have used cell fixation in 2% paraformaldehyde prior to flow cytometry[31], but the results presented here were acquired immediately after assay completion. Postponing flow cytometry data acquisition by storing the plate at 4°C **is not** recommended, since the percentage of $EtBr^+$ THP-1 cells decays rapidly (**Figure S3**).

3.4. Flow cytometry acquisition and analysis

NOTE: Any flow cytometer supporting 96 well plate format and having the appropriate

lasers/filters to measure EtBr fluorescence can be used.

3.4.1. For acquisition, gate on THP-1 cells using a linear forward-scatter (FSC) vs. linear side-scatter (SSC) plot using the wells were no IEs were added (**Figure 2A**) and acquire 10,000 events on this gate.

3.4.2. Measure fluorescence intensity for EtBr (FL3-Log) on the THP-1 gate using a histogram plot.

3.4.3. For gating, first gate on THP-1 cells in a FSC vs. SSC density plot, using the wells were no IEs were added (**Figure 2A**). Then set up a positive gate in an FL3 (EtBr) histogram, using the THP-1 cells (no IEs added) and the un-opsonized control (**Figure 2B**).

3.4.4. Copy these gates in all the other samples tested in the same plate and determine the percentage of EtBr-positive THP-1 cells (THP-1 cells that have phagocytized at least one IE). For each of the samples tested, phagocytosis can be reported as the absolute values (percentage of $EtBr^+$ THP-1 cells) or as relative phagocytosis calculated as percentage using the positive control as maximum.

REPRESENTATIVE RESULTS:

Here we present in detail a protocol that has previously been described[31] and used[11–18] to measure the capacity of antibodies targeting the surface of *P. falciparum* IEs to induce opsonization and phagocytosis by THP-1 cells.

The assay specifically measures antibody-mediated phagocytosis and, therefore, interaction with the appropriate Fc-receptors on the surface of the THP-1 cells is required. For this reason, and as mentioned in the protocol, we recommend periodically checking the expression of Fcγ-receptors on the surface of the THP-1 cells by flow cytometry. The cells should be negative for CD16 (**Figure 1A**) and positive for CD32 and CD64 (**Figure 1B,C**).

For the assay, purified late-stage IEs were labeled with EtBr and then opsonized with antibodies present in the plasma/serum of malaria-naïve or malaria-exposed individuals. Phagocytosis was measured by flow cytometry, quantifying the percentage of $EtBr^+$ THP-1 cells after 40 min co-incubation with EtBr-labeled and antibody-opsonized IEs. Initially, THP-1 cells were gated using an FSC vs. SSC density plot (**Figure 2A**). Then, an $EtBr^+$ marker was created, using an FL3 histogram on the THP-1 cells and un-opsonized IEs (**Figure 2B**). These gates were then used to analyze all the other controls and test samples.

The negative controls (including the THP-1 cells alone, the un-opsonized IE control, and the controls with malaria-naïve and malaria-exposed males) should all generate a single negative peak in the FL3 channel (**Figure 3A**) with only few events in the $EtBr^+$ marker. Accordingly, the mean phagocytosis values both as absolute $EtBr^+$ THP-1 cells and as relative phagocytosis percentages should be very low (**Figure 3B,C**, normally less than 2% for all cases). In contrast, the positive controls (including the rabbit anti-human erythrocyte antibody and the malaria-

exposed female pool) should generate traces with two peaks (**Figure 3A**): a negative one (largely overlapping with the one generated by all the negative controls) and a clearly positive and well-separated one located inside the $EtBr^+$ marker. A positive sample, as the presented example (sample from a malaria-exposed multigravid woman/NF20) should generate a similar profile as the positive controls. The mean phagocytosis values, measured as absolute $EtBr^+$ THP-1 cells and as relative phagocytosis, were normally highest for the positive control (58%/100%), followed by the malaria-exposed female pool (29%/53%), and then the single malaria-exposed woman (23%/40%). As observed in **Figure 3B,C**, where three independent experiments are presented, there was a considerable variability between experiments and we, therefore, recommend running samples intended for comparison in the same experiment. In our hands, at least four full 96 well plates can be handled by a single experienced researcher. The variability between assays was also clearly observed when two identical experiments testing several serum samples from malaria-exposed women were performed simultaneously. The same parasite preparation (after magnetic purification of late-stage IEs) and serum dilutions were used. THP-1 cells were kept in two separate flasks but seeded from the same initial flask and the experiments were performed by two different researchers. Even though the assay seems to generate consistent results when performed separately, with tight linear correlations ($r>0.9$ for both absolute and relative phagocytosis values) between phagocytosis values measured in the two experiments, the slope coefficient of the adjusted lines deviates from one, indicating the values generated in different experiments were not identical. This deviation was more evident for the absolute values (slope coefficient confidence interval 0.55-0.72) as compared to the relative values (slope coefficient confidence interval 0.68-1) (**Figure 4**). We, therefore, recommend using relative values, especially if for some reason (e.g., not enough purified IEs, more than 4 full plates, etc.) it is not possible to run all the samples in a single experiment. We also recommend running experiments intended for comparative analysis within the shortest amount of time, to avoid introducing extra variation due to drifting in PfEMP1 expression (as well as other antigens) and due to subtle differences on the THP-1 cells upon extended time in culture.

FIGURE AND TABLE LEGENDS:

Figure 1: Fc receptors expressed on the THP-1 cell surface. (**A**) Fcγ-receptor III/CD16 (red). (**B**) Fcγ-receptor II/CD32 (green). **C.** Fcγ-receptor I/CD64 (orange). Un-labeled cells are shown in blue.

Figure 2: Flow cytometry gating strategy. (**A**) THP-1 cells gated on FSC/SSC**.** (**B**) Ethidium bromide positive ($EtBr^+$) THP-1 cells in an FL3 histogram. THP-1 cells alone/no IEs added (blue), THP-1 cells incubated with un-opsonized IEs (green), and THP-1 cells incubated with IEs opsonized with a positive control (red) are shown.

Figure 3: Phagocytosis of IT4VAR04-IEs by THP-1 cells. (**A**) Representative flow cytometry histograms of one of the experiments presented in B (identified by larger symbols). (**B**) Percentage of $EtBr^+$ THP-1 cells (means and standard deviations of three independent experiments). (**C**) Same data as in B, after normalization against the corresponding positive

control. Color coding is the same in all panels: THP-alone (black), un-opsonized/no antibody control (blue), malaria-naïve control (cyan), malaria-exposed male pool (green), malaria-exposed female pool (orange), a malaria-exposed female donor (pink), and positive control/rabbit anti-human erythrocytes (red). Mean and standard deviations are shown.

Figure 4: Phagocytosis of IT4VAR04 IEs by THP-1 cells upon opsonization with serum from 10 malaria-exposed women. The plots present linear regression analysis for two identical experiments performed on the same day, but by different researchers. (**A**) Data presented as absolute values and as (**B**) relative phagocytosis values. Analysis performed using statistical analysis software.

Figure S1: Phagocytosis assay flow chart. Flow chart depicting the main steps of the assay.

Figure S2: 96 well plate experiment layout. (**A**) Layout for IEs EtBr labeling. (**B**) Layout for opsonization; 6 wells are always reserved for controls. (**C**) Layout for THP-cells plating. (**D**) Layout for phagocytosis.

Figure S3: Color coding as in Figure 3. (**A**) Flow cytometry histogram overlay of one experiment acquired immediately and (**B**) after storage at 4 °C for 12 h. (**C**) Percentage of $EtBr^+$ THP-1 cells measured before and after storage. NF## represent different malaria-exposed female donors.

DISCUSSION:

The protocol presented here has been previously described and used[12,15,17,31] to measure the capacity of antibodies targeting the surface of *P. falciparum* IEs to induce opsonization and phagocytosis by THP-1 cells. The results presented here focus on naturally acquired VAR2CSA-specific antibodies in the plasma/serum of women living in a *P. falciparum* endemic region. VAR2CSA is a type of PfEMP1 involved in placental sequestration of IEs, and a key determinant in the pathogenesis of PM.

The assay can be used for antibodies induced by immunization and/or targeting other PfEMP1 variants or any other parasite antigen present on the IE surface, provided the antibody tested interacts with the human Fcγ-receptors expressed by the THP-1 cells (CD32 and CD64). The assay is simple, high-throughput (allowing the analysis of large sample sets) and can be performed in one day. Phagocytosis is measured by flow cytometry, quantifying the percentage of $EtBr^+$ THP-1 cells after 40 min co-incubation with EtBr-labeled and antibody-opsonized IEs.

Even though the assay gives consistent results over experimental replicates, there is variability between the absolute values measured and, therefore, we recommend calculating relative values using a positive control that must always be included. We also recommend running all samples to be tested in a single experiment, to avoid inter-assay variation as discussed above. When testing serum samples collected from individuals exposed to *P. falciparum* infection, we recommend always including a set of samples from naïve individuals to be used as a control group. This control group can be used to set up a threshold to determine which of your test samples are to be considered positive.

We have previously used this approach to compare the phagocytosis-inducing capacity of sera collected from children with different malaria clinical presentations (severe vs. mild)[17].

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DISCLOSURES:
The authors have nothing to disclose.

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538

Figure 1

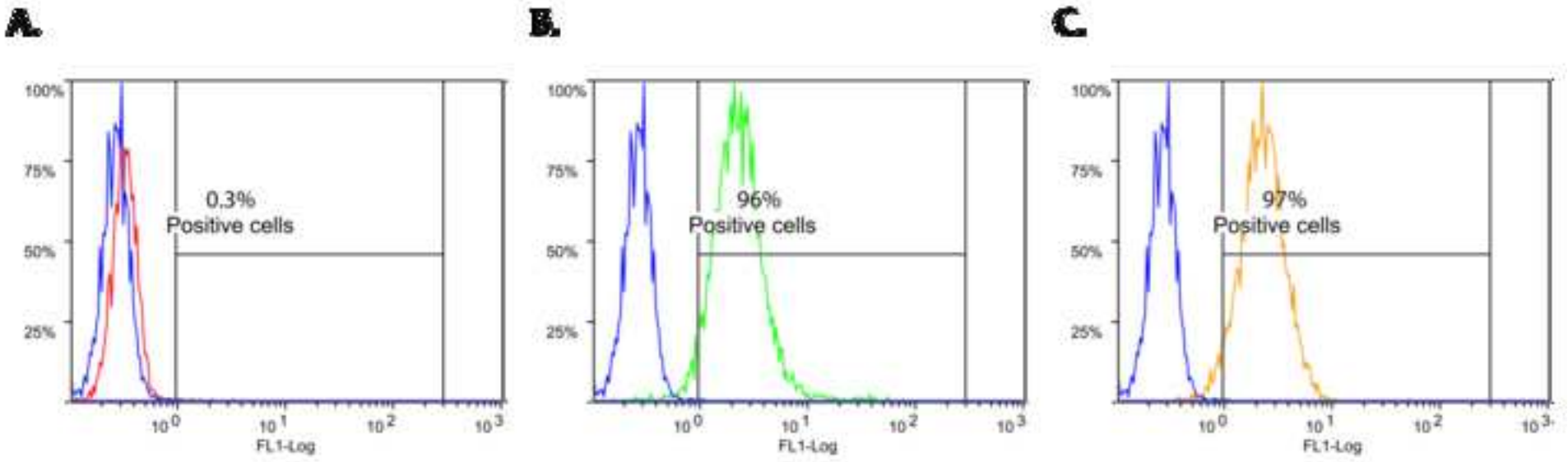

Figure 2

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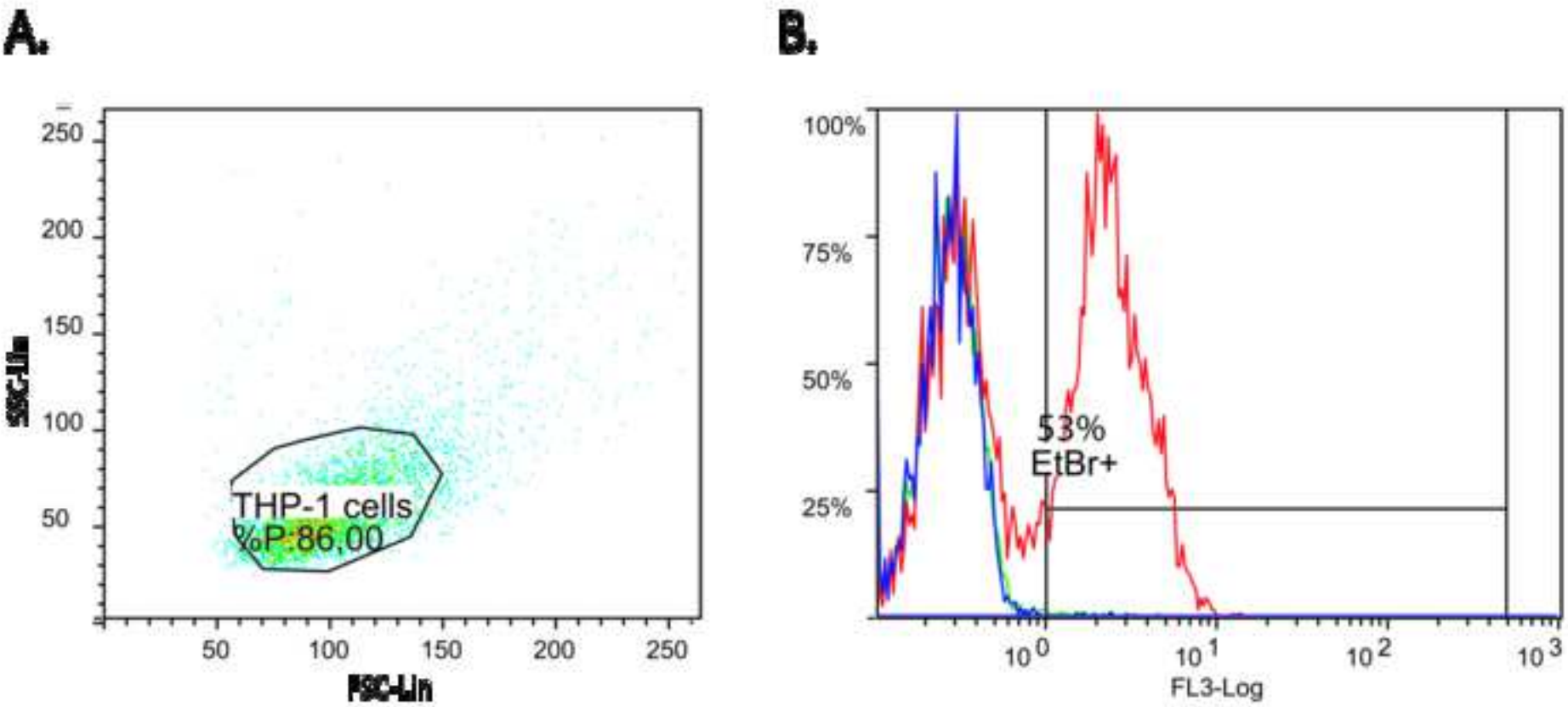

Figure 3

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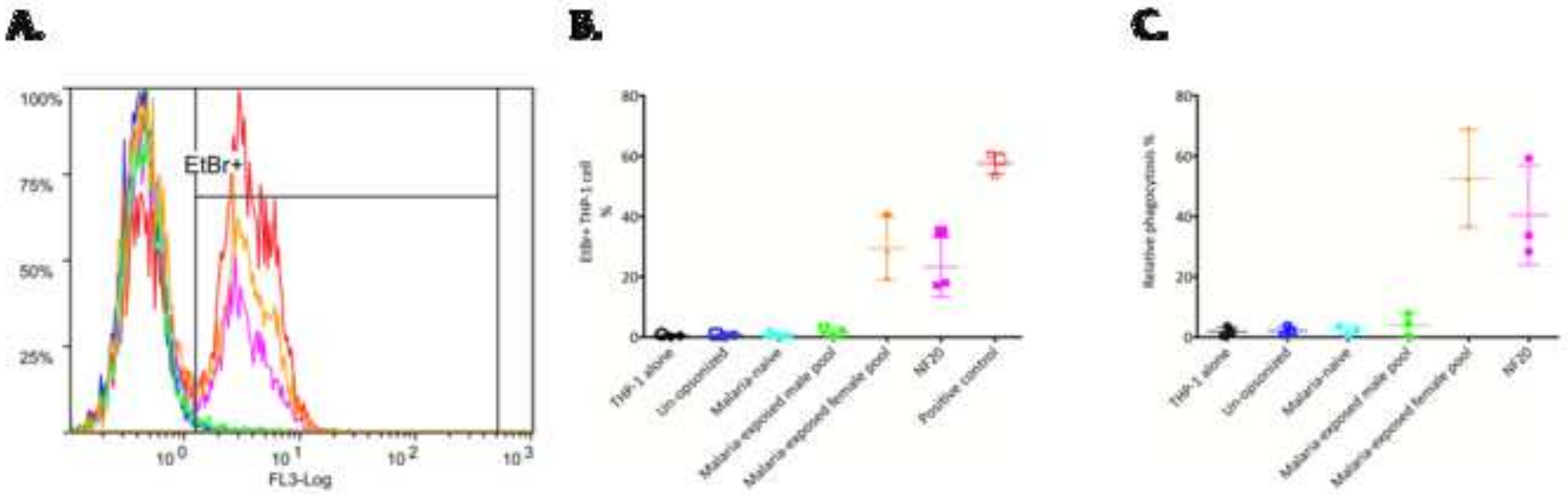

Figure 4

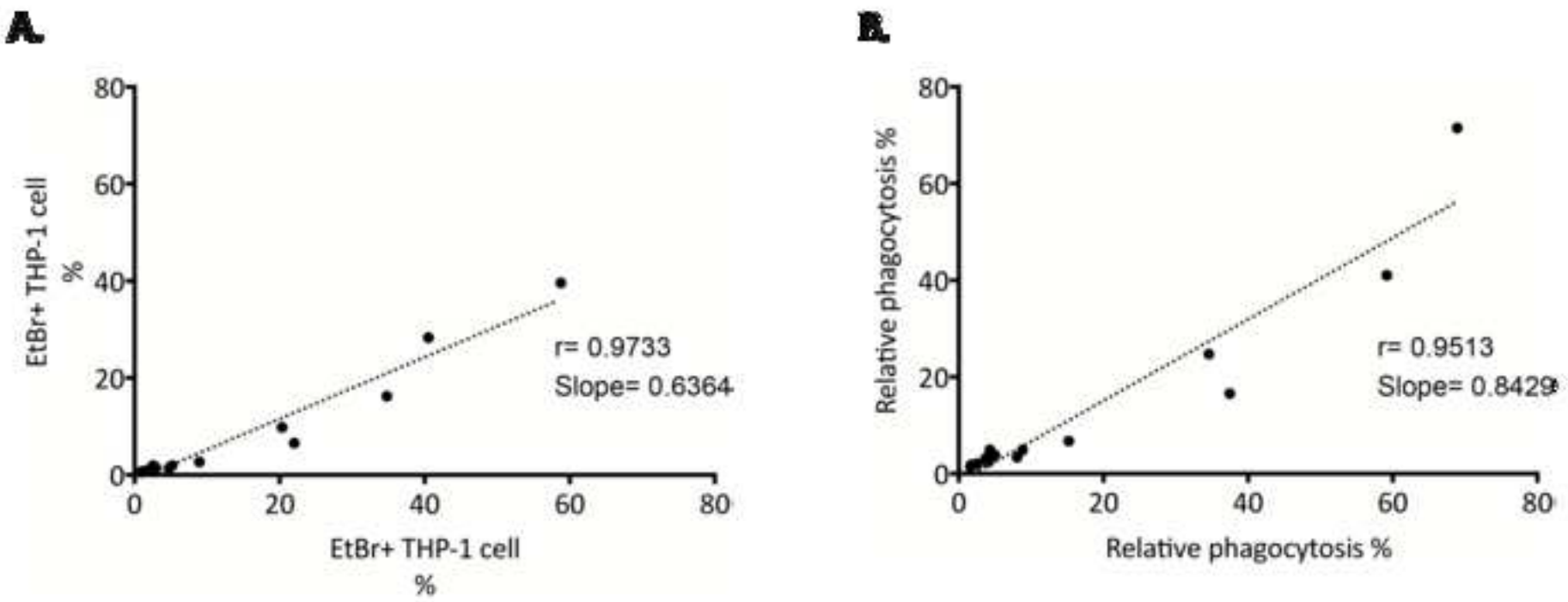

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
96 well cell culture plates, round bottom with lid	Corning	3799
AlbuMAX-II	Gibco	11021-037
AlbuMAX-II (5%)	-	-
Anti-Red Blood Cells antibody	Abcam	ab34858
DPBS	Sigma	P8622
Dynabeads Protein G	Invitrogen	10003D
Ethidium bromide solution	Sigma	E1510
FC500 flow cytometer	Beckman Coulter	
Fetal Bovine Serum (FBS)	Gibco	10099-141
FITC mouse anti-human CD16	BD Biosciences	555406 or 556618
FITC mouse anti-human CD32	BD Biosciences	552883
FITC mouse anti-human CD64	BD Biosciences	555527
FlowLogic software	technologies	
Gentamicin (10mg/mL)	Sigma	G1272
Hypoxanthine	Sigma	H9377
L-glutamine (200mM)	Sigma	G7513
Lysing solution	-	-
MACS CS-column and accesories	Miltenyi Biotec	130-041-305
Parasite culture medium	-	-
Penicillin/Streptomycin (10000U and 10mg/mL)	Sigma	P0781
RPMI-1640 medium	Sigma	R5886
THP-1 culture medium	-	-
Vario MACS magnet	Miltenyi Biotec	

Comments/Description

Any similar plate can be used, make sure it is compatible with the flow cytometer instrument you intend to use

5% AlbuMAX-II (Gibco, 11021-037), 0.2g/L hypoxanthine (Sigma, H9377) in RPMI1640 (Sigma, R5886)
Prepare **2 l al**iquots and freeze a -20°C. Use one aliquot per experiment.

Prepare a stock solution at 0.1mg/mL in RPMI1640 (R5886). Store protected from light
Any flow cytometer supporting 96 well plate format and having the appropriate lasers/filters to measure EtBr fluorescence can be
Heat inactivate before use.

Any flow cytometry analysis can be used, for example FlowJo or Winlist

15mM NH_4Cl, 10mM $NaHCO_3$, 1mM EDTA

2mM L-glutamine (Sigma, G7513), 50µg/mL Gentamicin (Sigma, G1272), 0.5% AlbuMAX-II (AlbuMAX-II 5%) in RPMI1640 (Sigm

10%FBS (Gibco, 10099-141), 2mM L-glutamine (Sigma, G7513), 100U/mL Penicillin, 0.1mg/mL Streptomycin (Sigma, P0781) in

e used.

na, R5886)

RPMI1640 (Sigma, R5886)

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.
2. Please address all the specific comments marked in the manuscript.
3. We cannot have dashes in the protocol. Please use complete sentences instead.
4 Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.
5. Please proofread the manuscript well.
We have addressed all these comments in the manuscript file.

6. In the table of materials please include all the equipment, reagents, buffers, materials used in the experiment. (e,g. Flow sorter, Flo Jo, Dynabeads not included presently).
We have added the flow cytometer used and the software used for flow cytometry analysis. Also the Dynabeads.

Figure S1

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Figure S2

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A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs
B	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs
C	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs
D	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs	IEs
E												
F												
G												
H												

B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Unopsoniz	Positive	Malaria nai	Malaria ♂	Malaria ♀	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
B	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18
C	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30
D	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42
E												
F												
G												
H												

C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1
B	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1
C	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1
D	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1
E	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1
F	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1
G	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1
H	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1	THP-1

D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Medium	Unopsoniz	Positive	Malaria nai	Malaria ♂	Malaria ♀	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
B	Medium	Unopsoniz	Positive	Malaria nai	Malaria ♂	Malaria ♀	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
C	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18
D	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18
E	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30
F	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30
G	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42
H	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42

Figure S3

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