RESEARCH ARTICLE

Impact of *Plasmodium falciparum* small-sized extracellular vesicles on host peripheral blood mononuclear cells [version 1; peer review: 1 approved]

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Abstract

Background: Exagerated immune activation has a key role in the pathogenesis of malaria**.** During blood-stage infection, *Plasmodium falciparum* can interact directly with host immune cells through infected red blood cells (*Pf*iRBCs), or indirectly by the release of extracellular vesicles (EVs). Here, we compared the impact of *Pf*iRBCs and *P. falciparum* small-sized EVs (*Pf*sEVs, also known as exosomes) from a Kenyan clinical isolate (*Pf*KE12) adapted to short-term laboratory culture conditions on host peripheral blood mononuclear cells (PBMC).

Methods: *Pf*sEVs were isolated from cell-free culture-conditioned media by ultracentrifugation while mature trophozoite *Pf*iRBCs were purified by magnetic column separation. The *Pf*sEVs and the *Pf*iRBCs were co-cultured for 18 hours with PBMC. Cellular responses were quantified by cell surface expression of activation markers (CD25, CD69) and cytokine/chemokine levels in the supernatant.

Results: Relative to negative control conditions, *Pf*sEVs induced CD25 expression on $CD4^+$, $CD19^+$ and $CD14^+$ cells, while *Pf*iRBCs induced on $CD19^+$ and $CD14^+$ cells. Both *Pf*sEVs and *Pf*iRBCs induced CD69 on $CD4^+$, $CD8^+$ and $CD19^+$ cells. In addition, *Pf*iRBCs induced higher expression of CD69 on $CD14^+$ cells. CD69 induced by *Pf*iRBCs on $CD4^+$ and $CD19^+$ cells was significantly higher than that induced by *Pf*sEVs. Secretion of MIP1α, MIP1β, GM-CSF, IL-6, IL-8, and TNFα were significantly induced by both *Pf*sEVs and *Pf*iRBCs whereas MCP-1, IL-10, IL-17α were preferentially induced by *Pf*sEVs and IP-10 and IFN-γ by *Pf*iRBCs. Prior exposure to malaria (judged by antibodies to schizont extract) was associated with lower monocyte responses to *Pf*

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sEVs.
Conclusions: *Pf*sEVs and *Pf*iRBCs showed differential abilities to induce secretion of IL-17α and IFN-γ, suggesting that the former are better at inducing Th17, whilst the latter induce Th1 immune responses respectively. Prior exposure to malaria significantly reduces the ability of *Pf*sEVs to activate monocytes, suggesting immune tolerance to *Pf*sEVs may play a role in naturally acquired anti-disease immunity.

Keywords
Malaria, Plasmodium falciparum, small extracellular vesicles, PBMC, immune response

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Introduction

Plasmodium falciparum causes in the region of half a million deaths per year[1]. In the human host, the parasite infects both the liver and red blood cells, but it is the parasite-host interaction during the blood stage that is responsible for pathology. *P. falciparum*-infected red blood cells can interact with the host cells directly, for example through endothelial protein C receptor (EPCR) to induce inflammation[2], and also indirectly through secreted parasite factors[3–7].

One way secreted effector molecules are released from cells is through extracellular vesicles (EVs). EVs are double-layered membrane-bound nanoparticles that are released by cells. They are usually classified into two major sub-groups, small-sized and medium-sized extracellular vesicles[8] often termed as exosomes and microvesicles respectively[9,10]. Small-sized extracellular vesicles (sEVs) are vesicles with a diameter of 30–150 nm. They are generated through inward budding of the limiting membrane of late endosomes resulting in the formation of intraluminal vesicles (ILVs). Late endosomes containing many ILVs are called multivesicular bodies (MVBs) which then fuse with cell membranes and release ILVs into the extracellular space as exosomes or sEVs[8,10]. On the other hand, the diameter of the medium-sized EVs (mEVs) range between 100 and 1000 nm and are formed when part of the cell plasma membrane 'pinches-off' with part of the cytoplasm and surface receptors/proteins and are released into the extracellular milieu[10].

EVs have the ability to transfer their packaged signaling competent molecules (including proteins, lipids, nucleic acids, and metabolites) from one cell to another, consequently modifying the properties of the recipient target cell(s)[9,11–13]. Research in the pathophysiology of several diseases such as cancer[12,14,15] and infectious diseases[16–19] provide evidence for a role of EVs in mediating intercellular interactions[9,16–20]. Tumor- and pathogen-derived EVs have been shown to have the ability to abrogate the host immunological defense mechanisms as a way of evading immune responses within the host[19,21–24].

In the context of malaria, *P. falciparum* EVs (*Pf*EVs) have a role in intercellular communication as well as in inducing sexual commitment[16,17]. Extracellular vesicles reflect the molecular phenotype of the cells releasing them[12]. Analysis of the impact of host-parasite interactions mediated by *Pf*EVs may improve our understanding of the pathogenesis of severe malaria and the mechanisms through which the parasite modulates the host immune response. Furthermore, proteomic analysis revealed that *Pf*EVs are enriched in parasite proteins involved in interaction with the host cells[16,25] and have been shown to induce inflammation[16] and endothelial activation[26]. However, to date, all studies on the impact of *Plasmodium* EVs on host immune response have been performed using either rodent malaria[27,28] or using long-term laboratory-adapted *P. falciparum* isolates[16,29]. Our study is the first to use *P. falciparum* clinical isolates. The quantity and repertoire of the content of *Pf*EVs from clinical isolates appears to be different from that of long-term laboratory-adapted parasite isolates[25] and this may affect their functional impact on host immune cells. In this study, we compared the functional impact of *Pfs*EVs and the autochthonous *Pf*iRBCs of a clinical isolate adapted to short-term culture (<70 cycles) on human peripheral blood mononuclear cells (PBMC).

Methods

Plasmodium falciparum isolate

A Kenyan *P. falciparum* clinical parasite isolate (unique lab identifier, 9215) was used in this study. This isolate was obtained from a child admitted at Kilifi County Hospital with respiratory distress[30] and was adapted to *in vitro* culture (<70 cycles) and used in previous studies[31,32]. Isolate 9215 was renamed to *Pf*KE12 in unpublished genome data[33].

Harvesting and processing of parasite culture media for isolation of *Pfs*EVs

*Pf*KE12 was grown under standard culturing conditions[34]. Mycoplasma contamination was routinely monitored using PCR. The parasite cultures were tightly synchronized using D-sorbitol (Sigma) treatment and bulked up to six flasks, each containing 500 µl packed cell volume at 7% parasitemia. These cultures were grown in 40 ml of complete culture media, (RPMI 1640 + L-glutamine + Hepes + D-glucose + gentamicin + sodium-hypoxanthine) (all from Gibco) supplemented with Albumax-II (Gibco) that had previously been depleted of sEVs by ultracentrifugation at 150,000×*g* for 2 hours. The culture media added at an early ring stage was harvested after 24 hours when the parasites were in the mature trophozoite stage (herein referred to as the rings-to-trophozoite or the RT sample). In this study, the culture-conditioned media (CCM) from the RT sample was harvested as previously described (Abdi *et al.*, 2017) and is shown in Figure 1A. Briefly, the parasite culture was transferred to a 50-ml Falcon tube and centrifuged at 440×*g* for 5 minutes to pellet down RBCs and the supernatant transferred to a new 50-ml falcon tube. The supernatant (CCM) was then centrifuged once at 440×*g* for 5 minutes to remove any remaining RBCs and the supernatant transferred into a new 50-ml Falcon tube. This was followed by centrifugation twice at 2,000×*g* for 10 minutes, once at 3,600×*g* for 10 minutes and once at 15,000×*g* for 30 minutes to pellet out mEVs.The mEVs pellet was then resuspended in 1×PBS and stored at -80°C until use (this pellet will be referred to as *Pf*mEVs if from *P. falciparum* CCM). The resultant supernatant from the final centrifugation at 15,000×g above was filtered through a 0.2-µm filter (FiltoSpurS, Sarstedt) and stored at -80°C until use. Uninfected red blood cells (uRBCs) freshly obtained from a donor or stored for a month at 4°C were also both incubated at 37°C in culture media for either 24 or 48 hours, the CCM harvested and processed as described for the *P. falciparum*-infected red blood cells cultures. The uRBCs used for CCM harvesting were of the same batch as the one used in maintaining the iRBCs cultures for *P. falciparum* CCM harvesting.

Isolation of sEVs from culture-conditioned media

The 0.2-µm-filtered CCM supernatant stored at -80°C was thawed on ice and transferred under sterile conditions into quick-seal ultracentrifuge tubes (Beckman Coulter cat# 343322) that were then heat-sealed. The sealed tubes were next ultracentrifuged using Optima XE-90 ultracentrifuge in a pre-cooled (4°C)

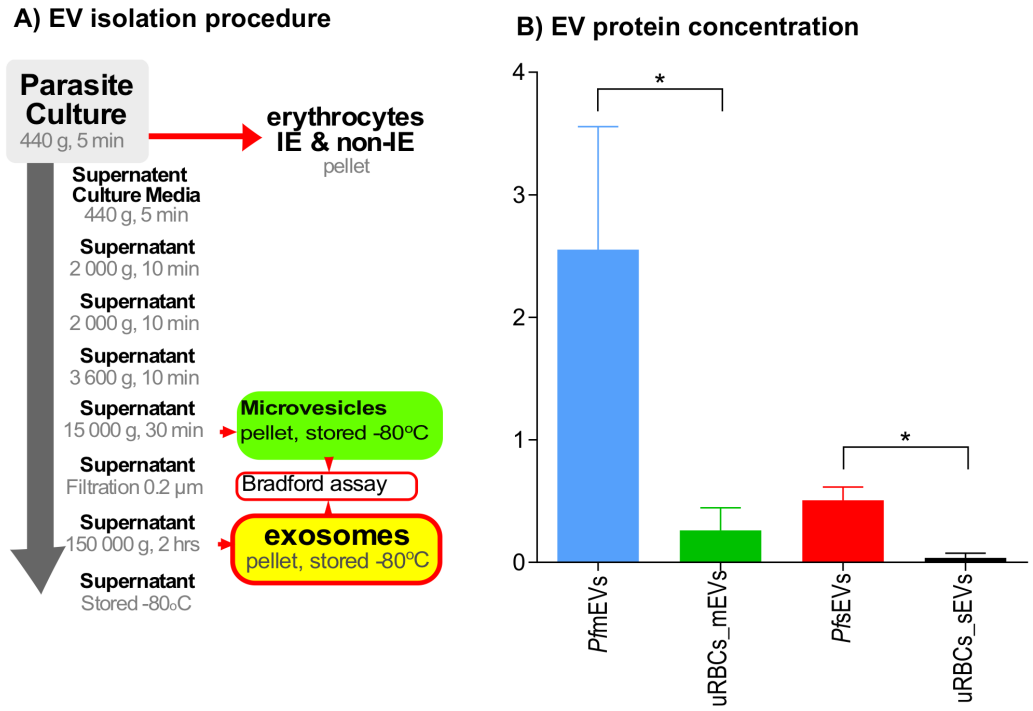

Figure 1. EV isolation procedure and the protein concentration of the isolated EVs. (**A**) Schematics showing the procedure for EV isolation from culture conditioned media. (**B**) The bar graphs show the crude protein concentration of medium EVs (mEVs) and small EVs(sEVs) isolated from *P. falciparum (Pf)* infected and uninfected RBC (uRBCs) culture conditioned media; *Pf*mEVs, uRBCs-mEVs, *Pf*sEVs and uRBC-sEVs using Bradford assay. Each bar represent data from three biological replicates. *Significant difference using Mann-Whitney U-Test.

70.1Ti fixed-angle rotor (Beckman Coulter) at 150,000×*g* for 2 hours at 4°C (Figure 1A). This approach is likely to enrich for sEVs, based on their size and the 0.2-µm filter cut-off and henceforth, referred to as *Pf*sEVs. The pellet was then washed twice by re-suspending in ice-cold 1× PBS followed by ultracentrifugation at 150,000×*g* for 2 hours at 4°C between washes. The final *Pf*sEVs pellet was re-suspended in 400µl of ice-cold 1× PBS, aliquoted and stored at -80°C until use (Figure 1A). The Bradford protein assay was used to determine the protein concentration in the *Pf*sEVs following the manufacturer's instructions (Figure 1A). The isolated *Pf*sEVs were also tested for mycoplasma contamination. The processed uRBCs' CCM was similarly subjected to the above isolation process to obtain the equivalent of *Pf*sEVs from uninfected RBCs (uRBCs-sEVs).

Isolation of infected red blood cells (iRBCs)

Plasmodium falciparum-infected RBCs (*Pf*iRBCs) were isolated from the *P. falciparum* culture using magnetic-activated cell sorting (MACS) columns (Miltenyi Biotec). Tightly synchronized culture at 7% parasitemia was taken through MACS separation columns during the late trophozoite stages. The MACS-purified iRBCs were then washed twice with incomplete culture media (RPMI 1640[Sigma] + L-glutamine[Gibco] + Hepes[Gibco] + D-glucose[Gibco] + gentamicin[Gibco] + sodium-hypoxanthine[Gibco]) and the supernatant aspirated out. Glycerolyte was added to the MACS-purified *Pf*iRBCs at 3:1vol/vol ratio, stored at -80°C in small aliquots until use. Uninfected RBCs (uRBCs) that were cultured for CCM harvesting (as explained above) were washed twice in incomplete culture media (after CCM harvesting) and stored in appropriate volumes of glycerolyte in -80°C until use.

PBMC isolation

PBMC were obtained from 20 adult Kenyan volunteers. Fresh heparinized whole blood (~20-30 mls) was obtained from each consenting adult donor for plasma and PBMC isolation. The blood was centrifuged at 440×*g* for 5 minutes to remove plasma that was then stored at -80°C. The cells were topped-up with a wash buffer, R0 (RPMI 1640 supplemented with L-glutamine and Penicillin/Streptomycin [Gibco]) equivalent to 5 volumes of the cell pellet the plasma volume aspirated out. The blood was then layered on Lymphoprep™ (Stemcell Technologies) at a Lymphoprep to blood ratio of 1:2 and centrifuged at 440×*g* for 20 minutes at room temperature. The PBMC layer between the Lymphoprep and media was aspirated out into a new 50ml Falcon tube and washed twice in R0 at 360×*g* for 7 minutes and 4°C. The washed PBMC were resuspended in ice-cold freezing medium (10% dimethyl sulfoxide (DMSO) in fetal calf serum (FCS)) and stored overnight at -80°C in Mr. Frosty (Thermo Scientific) before being transferred to liquid nitrogen until use. Prior to storage or use, PBMC number and viability

was determined by Trypan blue exclusion using the Vi-CELL XR 2.03 counter (Beckman Coulter, USA) and/or hemocytometer chamber.

PBMC stimulation assays

A 1×10^6 PBMC per stimulation condition were co-cultured with *Pf*iRBC or *Pf*sEV in 96-well U-bottomed cell culture plates (Greiner Bio-One).

The stimulation conditions included: *Pf*sEVs added at a determined concentration of 20µg/ml based on consideration from a previous experiment by Mantel *et al.*[16], and 1×10^6 MACS-purified iRBCs (*Pf*iRBCs). The positive controls were *Staphylococcal* enterotoxin B (SEB) at 2.5 µg/ml as a polyclonal activator and CpG-ODN (2.5 µg/ml). Lipopolysaccharide (LPS) at 200 ng/ml was included as an additional positive control for a subset of experiments that involved PBMC from 12 donors. Co-culture wells with (PBMC + cell growth medium only) and (PBMC + 1×10^6 uRBC) were included in each experiment as negative controls. These uRBCs were of the same batch as that used to culture the parasites. The PBMC were co-incubated with the stimulants for 18 hours in a humidified incubator at 37°C and 5% CO_2.

Cells were harvested after 18 hours stimulation, washed in fluorescence activated cell sorting (FACS) buffer (1× PBS + 5% FCS + 0.01% sodium azide) and then stained with 30 µl of fluorescently-labeled antibody cocktail containing: Phycoerythrin (PE)-Cyanine (Cy) 5-conjugated anti-human CD3 [BioLegend, Cat#: 300410, Clone: UCHT1]/Brilliant Violet (BV)-785 anti-human CD3 [BioLegend, Cat#: 317330, Clone: OKT3], PE-Cy7-conjugated anti-human CD4 [BioLegend, Cat#: 317414, Clone: OKT4], PE-CF594 Mouse anti-human CD8, [BD Biosciences, Cat#: 562282 Clone: RPA-T8]/Alexa Fluor 700-conjugated anti-human CD8a [BioLegend, Cat#: 301028 Clone: RPA-T8, 0.5mg/ml], Pacific Blue-conjugated anti-human CD19 [BioLegend, Cat#: 982404, Clone: HIB19, 200µg/ml]/PE-Cy5 anti-human CD19 [BioLegend Cat#: 302210, Clone: HIB19], BV650-conjugated anti-human CD14 [BioLegend, Cat#: 301836, Clone: M5E2], Alexa Fluor 488-conjugated anti-human CD69 [BioLegend, Cat#: 310916, Clone: FN50], BV711-conjugated anti-human CD25 [BioLegend, Cat#: 302636, Clone: BC96], and Fixable Viability Dye eFluor® 780, [eBioscience, Cat#: 65-0865-18]. All antibodies were used at a 1:200 dilution apart from BV711 anti-human CD25 that was used at a 1:100 dilution. (Those antibodies without indicated concentrations have either lot-specific concentrations or pre-diluted for use at recommended volume per test).

The cells were stained for 30 minutes at 4°C and washed twice before being re-suspended in 300 µl of FACS-flow buffer (BD Biosciences). Cells were acquired on the LSRFortessa™ cell analyzer (BD Biosciences). At least 100,000 events were acquired per stimulation condition and at least 170,000 events for the *Pf*iRBCs and uRBCs conditions since these had an extra cell lysis step prior to staining. Data were analyzed using FlowJo® software version 10.0 (Tree Star).

ELISA and LUMINEX assays

Cell free culture supernatants from the stimulation assays (above) were aspirated and stored at -20°C prior to cytokine analysis. Supernants used for this assay were all from the subset of experiments where LPS was included as an additional positive control condition. Supernants were thawed on ice and used to quantify a total of 29 analytes; IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-15, IL-17A, TNF-α, TNF-β, GM-CSF, G-CSF, IL-12 (p40), IL-12 (p70) , IL-8, EGF, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), VEGF, and Eotaxin (CCL11). Analytes were measured using the MILLIPLEX Human Cytokine/Chemokine Magnetic bead 29-Plex assay (catalogue #HCYTMAG-60K-PX29) from Merck-Millipore following the manufacturer's instructions. A total of 25 µl of the culture supernatant was diluted 1:5 times in assay medium and incubated with 25 µl of anti-cytokine antibody-coupled magnetic beads for 2 hours at room temperature while shaking at 500 rpm in the dark. The beads were then washed twice and incubated with 25 µl of biotinylated detector antibody for 1 hour at room temperature, before addition of streptavidin R-phycoerythrin and further incubation for 30 minutes (between each washing step, the beads were retained in the plate using a magnetic separator). After a final wash, beads were re-suspended in 150 µL of LUMINEX Drive Fluid and 100 beads counted for each cytokine in a MAGPIX reader running on MAGPIX xPOTENT 4.2 software (Luminex Corporation). Analyte concentrations were calculated (via Milliplex Analyst v5.1 [VigeneTech]) from the mean fluorescence intensity expressed in pg/mL using standard curves with known concentrations of each analyte. In addition to the 29 analytes measured using the Luminex platform, TGF-β levels were measured using an ELISA kit (ThermoFisher; cat# BMS249-4) following the manufacturer's protocol.

Anti-schizont antibody ELISA

Frozen plasma samples from the PBMC donors were thawed and used in an anti-schizont ELISA to determine prior exposure to malaria. ELISA plates were coated with 100 µl of 1:6000 diluted crude schizont extract/lysate and incubated overnight at 4°C. The plates were then aspirated and blocked with 1% skimmed milk and incubated for 5 hours at room temperature with washes between each step. This was followed by addition and an overnight incubation with 100µl of the 1:1000 diluted plasma, 3 hours incubation at room temperature with 100 µl of HRP-conjugated Rabbit anti-human IgG (Thermo Scientific) and final incubation with 100µl of the o-Phenylenediamine dihydrochloride (OPD) substrate for 15 minutes. The reaction was stopped with 25 µl of 2 M H_2SO_4 and the plates read at 492 nm on a Synergy 4 (Bio Tek) plate reader, recording the samples' optical densities (OD).

Data analysis

The flow cytometry data from the FlowJo® analysis and ELISA data analyses were performed using Prism 6.01 (GraphPad). Mann-Whitney U-test was used to compare continuous variables between two conditions. The chemokine/cytokine dataset

was normalized using Yeo Johnson transformation and t-test was used to compare between two conditions. The unstimulated PBMC cutlture medium (media) was used as the background negative control condition for both *Pf*sEVs and *Pf*iRBCs. For *Pf*iRBCs, relative comprison to uRBCs condition was also shown. To test the impact of prior malaria exposure on PBMC response to *Pf*sEVs and *Pf*iRBCs stimulation, the anti-schizont IgG response in the contemporaneous plasma sample of each PBMC sample was related,using Spearman's rank correlation, to the induced expression of the activation markers on $CD4^+$, $CD8^+$, $CD19^+$, $CD14^+$cells following stimulation with either *Pf*sEVs or *Pf*iRBCs. Due to small sample size (N=8), the same test was not done for the cytokine/chemokine data. For all tests, *P* values were considered significant if <0.05.

Ethical statement

Ethical approval was obtained from Kenya Medical Research Institute Scientific and Ethical Review Unit (KEMRI/SERU/CGMRC/022/3149), and written informed consent was obtained from the PBMC sample donors. The study methods were carried out in accordance with the approved guidelines.

Results

Infected RBCs release a greater quantity of small-sized extracellular vesicles (sEVs) than uninfected RBCs

mEVs and sEVs were isolated from culture conditioned media (CCM) of *Pf*iRBCs from a Kenyan clinical isolate (*Pf*KE12) and uRBCs as shown in Figure 1a. We have previously demonstrated successful isolation of sEVs from *Pf*iRBCs CCM by transmission electron microscopy[25] using the protocol described in the methods and schematically represented in Figure 1a. In this study, we used the isolated mEVs and sEVs protein concentration as a proxy for EV abundance[8]. As shown in Figure 1B, the mean protein concentration of mEVs from *Pf*iRBCs CCM (*Pf*mEVs) was ~4.9-fold higher than that of uRBCs (uRBCs-mEVs), as would be expected when parasite proteins are packaged in the mEVs.

The mean protein concentration of the sEV fraction from fresh or aged uRBCs CCM was negligible (Figure 1B), consistent with previous reports that show mature RBCs primarily release mEVs (microvesicles) but not sEVs (exosomes)[35].

Both *Pf*iRBCs and *Pf*sEVs induced expression of at least one of the activation markers on T cells, B cells and monocytes

PBMC samples from 20 healthy Kenyan adult donors were co-cultured with *Pf*iRBCs and *Pf*sEVs from the same isolate (*Pf*KE12). In addition to the test conditions we included unstimulated control (PBMC' culture medium) and uRBCs as negative controls; and CpG/SEB as positive control conditions. For 12 of the 20 PBMC samples, LPS was also included as an additional positive control condition. Our inability to detect meaningful protein content in the sEV fraction isolated from uRBCs CCM equivalent to the *Pf*sEVs used in this study excluded the possibility of using uRBC-sEVs as a negative control in the PBMC stimulation experiments.

Following co-culturing of the PBMC with the stimulants, the expression of the surface activation markers, CD25 and CD69 on T cells, B cells and monocytes was assessed by flow cytometry and analysed using FlowJo Software v.10.6.2. *Extended data*, Figure S1[36] shows the FlowJo gating strategy to enumerate different cell populations. Raw flow cytometry files are available as *Underlying data*[36].

*Pf*sEVs induced significantly higher expression of CD25 on $CD4^+$ T cells, $CD19^+$ B cells and $CD14^+$ compared to the background media condition (Figure 2A–C). In contrast, the effect of *Pf*iRBCs was apparent only on the antigen-presenting cells, $CD19^+$ B-cells and $CD14^+$monocytes (Figure 2B, C) with a significantly higher CD25 expression relative to the background condition (media or uRBCs).

Both *Pf*sEVs and *Pf*iRBCs induced a higher expression of CD69 on $CD4^+$, $CD8^+$ and $CD19^+$ relative to the background condition (Figure 2D–F). Additionally, *Pf*iRBCs induced significant CD69 expression on $CD14^+$ cells (Figure 2G). Notably, *Pf*iRBCs induced a higher CD69 expression on $CD4^+$ and $CD19^+$ cells relative to that induced by *Pf*sEVs (Figure 2E, F). Taken together, the above result shows that both *Pf*sEVs and *Pf*iRBCs can activate T and B cells and monocytes; but *Pf*sEVs tended to induce higher expression of the activation marker, CD25 while *Pf*iRBCs preferentially induced higher CD69.

To exclude the possibility of low-level Mycoplasma contamination that may be enriched during *Pf*sEVs isolation, *Pf*sEV-DNA extract was used in a PCR-based test. No contamination was detected (*Extended data*, Figure S2)[36].

Pfs EVs and *Pf*iRBCs induced common and unique cytokine/chemokine profile

For 8 out of the 20 PBMC samples , levels of 29 different cytokines/chemokines in the culture supernatant induced by each of the stimulants were measured using Luminex. Additionally, TGF-β was measured by ELISA. IL-3, IL-4, and IL-5 levels were below the limit of detection with both *Pf*sEVs and *Pf*iRBCs (*Extended data*, Figure S3)[36]. The levels of 16 cytokines/chemokines induced by *Pf*sEVs and by *Pf*iRBCs were not significantly greater than the levels seen in the negative controls using two-sided t-test, however, on one-sided t-test, IL-15 and G-CSF induced by both *Pf*sEVs and *Pf*iRBCs were significantly higher relative to the background conditions (*Extended data*, Figure S3)[36]. Output ELISA files are available as *Underlying data*[36].

Of the remaining 11 cytokines/chemokines, significant induction relative to the negative control background was observed with; a) both *Pf*sEVs and *Pf*iRBCs for MIP1α, MIP1β, GM-CSF, IL-6, IL-8, and TNFα (Figure 3A–F), b) *Pf*sEVs only for MCP1, IL-10 and IL-17α (Figure 3G–I) and c) *Pf*iRBCs only for IFNγ and IP-10 (Figure 3J–L). Notably, the levels of IFNγ and IP-10 induced by *Pf*iRBCs were significantly higher than the levels induced by *Pf*sEVs (Figure 3J–K), while the concentration of IL-17α induced by *Pf*sEVs tended to be higher than that induced

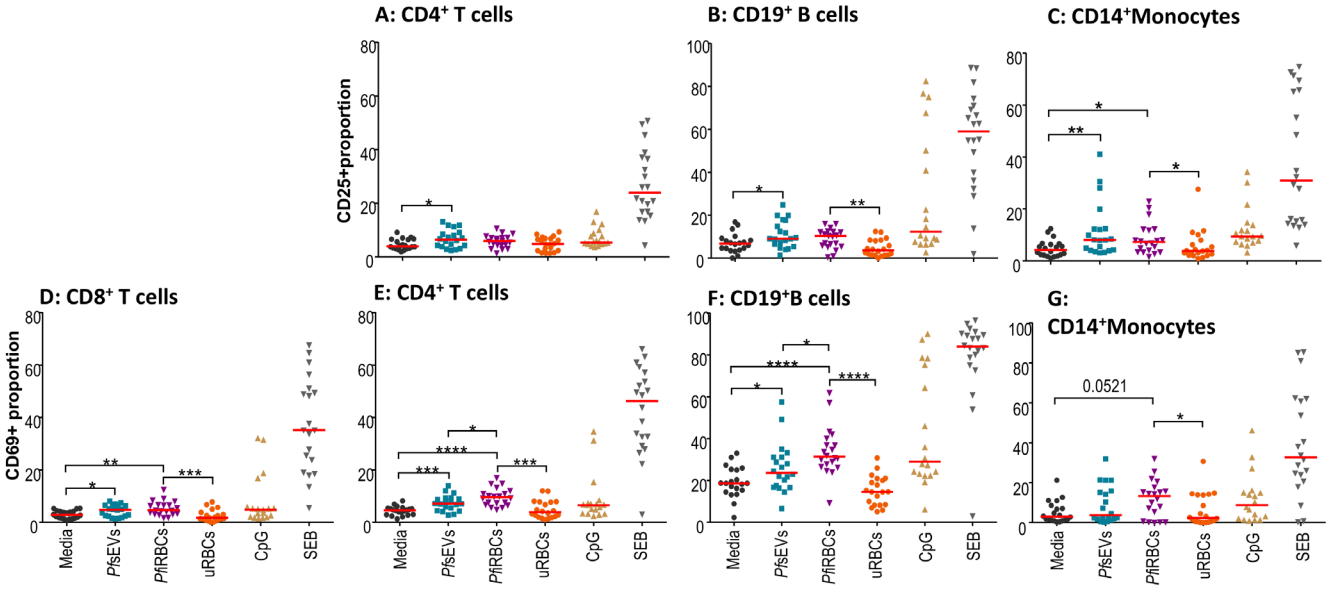

Figure 2. Proportion of T cells, B-cells and monocytes expressing the activation markers CD25 and CD69 under the different stimulation conditions. (**A–G**) Proportions of CD4+ T-cells, CD8+ T-cells, CD19+B-cells, and CD14+ Monocytes expressing the activation markers CD25 (upper panel) and CD69 (lower panel) following PBMC co-culture with *Pfs*EVs or *Pfi*RBCs. unstimulated PBMC (media) and uRBCs were included as negative control conditions while CpG and SEB were included as positive control conditions. Both *Pfs*EVs and *Pfi*RBCs conditions was compared to the background media condition. *Pfi*RBCs was also compared with uRBCs condition. (*P-value = 0.01-0.05; **P-value = 0.001-0.01; ***P-value = 0.0001-0.001; ****P-value <0.0001; the red horizontal lines indicates the median. P-value was calculated using Mann-Whitney U-test).

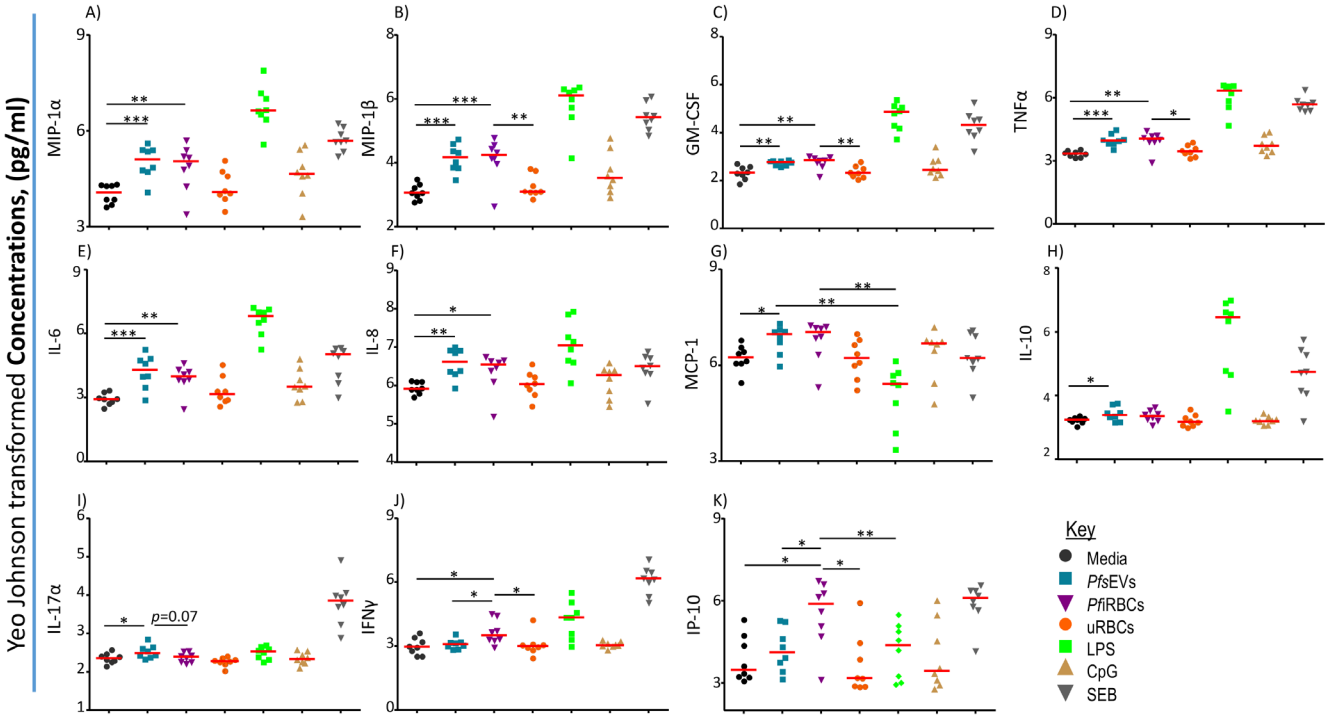

Figure 3. Cytokines/chemokines significantly induced after PBMC co-culture with *Pfs*EVs or *Pfi*RBCs. Cytokine/chemokine level was determined only in the supernants of 8 out of the 20 PBMC samples co-cultured with the different stimulants. The 8 samples were part of the 12 samples where LPS was included as an additional positive control. *P-value = 0.01-0.05; **P-value = 0.001-0.01 and ***P-value = 0.0001-0.001. The red horizontal lines indicate the mean. P-value was calculated using two-sided t-test.

by *Pf*iRBCs (p=0.07, Figure 3I). Interestingly, in contrast to most cytokines/chemokines, the level of MCP-1 induced by both *Pf*iRBCs and *Pf*sEVs was uniquely higher than that induced by LPS (Figure 3G). Additionally, the level of IP-10 induced by *Pf*iRBCs was also significantly higher than that induced by LPS (Figure 3K). Overall, both *Pf*sEVs and *Pf*iRBCs were able to induce various chemokines and cytokines but *Pf*sEVs seems to be better in inducing secretion of IL-17α while *Pf*iRBCs was better in inducing IFNγ and IP-10 suggesting that they might have differential abilities in inducing Th17 and Th1 T cell responses, respectively.

Exposure to malaria tends to tolerise monocytes responses to *Pf*sEVs

Given that malaria exposure has been shown to induce immunological tolerance[37–39], we related the cell surface activation data to each donor's IgG response to crude schizont extract using Spearman's rank correlation. As shown in Figure 4, CD25 expression on $CD14^+$ cells ($CD14^+CD25^+$) following stimulation of the PBMC samples with *Pf*sEVs or *Pf*iRBCs decreased with the level of pre-existing anti-schizont IgG in the plasma of each PBMC donor and this association reached significance for *Pf*sEVs (rho=-0.51, p=0.02, N=20). Furthermore, anti-schizont IgG level explained 19% of the variation in CD25 expression on $CD14^+$ monocytes when anti-schizont was used as an explanatory variable in a linear regression model predicting $CD14^+CD25^+$ (coeff(95%CI)=-0.28(-0.52,-0.03), p=0.03, adjusted R^2=19%, N=20). This result suggest malaria exposure tolerizes the host innate immune response to *Pf*sEVs. The cytokine/chemokine data was not subjected to the same analysis due to small sample size (N=8).

Discussion

In this study we investigated 1) whether *Pf*sEVs can induce PBMC activation *in vitro*, specifically by measuring cell surface activation markers and cytokines/chemokines secreted into the culture media following co-culture with PBMC; 2) how the PBMC activation induced by *Pf*sEVs compares with that of *Pf*iRBCs; and 3) whether prior exposure to malaria among the PBMC donors influences the level of the induced PBMC activation markers.

We showed that both *Pf*sEVs and *Pf*iRBCs induced T-cells, B-cells, and monocytes to express at least one of the surface activation markers examined relative to the negative control condition. *Pf*sEVs showed relatively stronger induction of CD25 expression while *Pf*iRBCs preferentially induced CD69 expression, particularly on B-cells. At the cytokine/chemokine level, both induced secretion of several cytokines/chemokines but they also showed differential ability to induce secretion of some cytokines. Notably, *Pf*sEVs induced secretion of higher levels of IL-17α relative to the background media and tended to be higher than that induced by *Pf*iRBCs (Figure 3I). IL-17α is known to be secreted by Th17 CD4+ T cells and the cytokines, IL-6 and TGF-β have been shown to be able to induce differentiation of naïve CD4 T-cells into Th17 cells *in vitro*[40]. Interestingly, *Pf*sEVs also induced significantly higher levels of IL-6 (Figure 3E) but not TGF-β (*Extended data*, Figure S3)[36]. On the other hand,

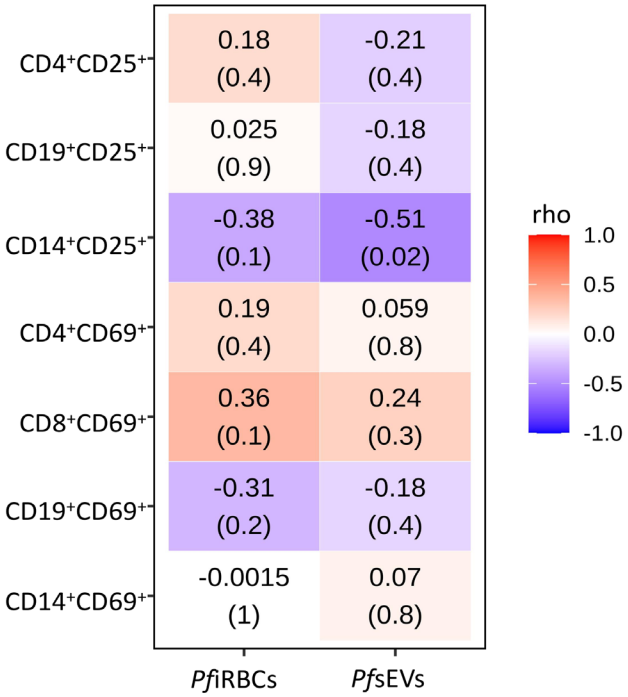

Figure 4. The impact of prior malaria exposure on PBMC response to *Pf*iRBCs and *Pf*sEVs. Correlations between anti-schizont IgG response in the plasma of each PBMC donor and the expression of the activation markers (CD25 and CD69) on $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells and $CD14^+$ monocytes, following co-culture of each donor's PBMC sample with *Pf*iRBCs or *Pf*sEVs was determined using Spearman's rank correlation. Shown is the rho and p-value (in bracket). Increasing colour intensity indicate stronger association.

*Pf*iRBCs showed superior ability to induce secretion of IFNγ and IP-10 relative to the background uRBCs and even to *Pf*sEVs condition when co-cultured with PBMC (Figure 3J–K. IP-10 secretion is driven by a pro-inflammatory cytokine milieu including IFN-γ[41]; thus, it is plausible that high levels of IFN-γ led to the IP-10 secretion that we identified in response to *Pf*iRBCs stimulation.

Hence our interpretation is that *Pf*sEVs and *Pf*iRBCs can induce differentiation of CD4+ T cells into Th17 and Th1 cells respectively. In a recent study[42], *Pf*iRBCs were shown to induce NK cells to secrete IFNγ while *Pf*mEVs could not, which is consistent with the observation made in this study with *Pf*sEVs and *Pf*iRBCs co-cultured with PBMC. This finding warrants further characterization of the T cell subsets activated by *Pf*sEVs, including γδ T-cells known to be activated by phosphoantigens in *P. falciparum* culture medium[43].

We observed *Pf*iRBCs could induce significantly higher MCP-1 (CCL2) and IP-10 (CXCL10) compared to LPS when co-cultured with PBMC (Figure 3C, K). This result is consistent with observation previously made with co-culturing of *Pf*iRBCs with purified dendritic cells[44]. However, in the previous study[44], co-culture of *Pf*iRBCs with purified dendritic cells could not induce secretion of inflammatory cytokines such as TNF-α and IFN-γ[44]. This difference might be explained by the presence of cells such as T cells in PBMC, which could be the source of the TNF-α and IFN-γ.

Previously, persistent exposure to malaria infection have been shown to tolerizes T and B cell response to malaria antigens[38,39]. In this study we showed, albeit with a small sample size, that the degree of previous malaria exposure (determined by the level of IgG response to crude schizont extract in the plasma of each PBMC donor) was negatively associated with monocyte's response to *Pf*sEVs (Figure 4). *Pf*sEV interaction with monocytes *in vitro* have been shown to induce inflammatory response[26], potentially contributing to malaria pathogenesis. Therefore tolerance to *Pf*sEVs following frequent malaria infection may be part of the naturally acquired anti-disease immunity[37].

We showed that uninfected red blood cells (uRBCs) do not produce sEVs (exosomes) containing quantifiable amount of proteins using a Bradford assay. During the isolations, we used culture conditioned media (CCM) from varied uRBCs ranging from fresh (processed for culture within 1 hour after phlebotomy) to 2 weeks old cultures. By contrast, the mEV fraction from uRBC CCM repeatedly contained quantifiable amount of proteins. This might indicate that uRBCs release sEVs, but with very low levels of packaged proteins; however, it could alternatively mean that uRBCs primarily release mEVs (microvesicles). The latter interpretation is consistent with a previous study that showed developing red blood cells release sEVs during earlier stage of haematopoiesis, but mature RBCs do not[35], as they have lost the endocytic pathway that is essential for the biogenesis of sEVs. Other studies have described exosomes from uRBCs[29], but this discrepancy might be explained by a difference in methodology as our protocol involves a filtration step at 0.2 μm that excludes the majority of mEVs from the sEV fraction, a step that was omitted from the earlier reported work.

While we used multiple PBMC donors to generate these results, this study used only one *P. falciparum* isolate and, therefore, we cannot conclusively determine if the results we obtained will remain similar if the number of isolates was increased for diversity. Different parasites have been shown to display a difference in virulence with studies demonstrating that this virulence can be transferred to non-virulent parasite phenotypes via secreted EVs[18,19]. Drawing analogy from these experiments, it would be interesting to see if the phenomena we observe are consistent across isolates of *P. falciparum* with different levels of virulence, and whether any differences can be correlated with differences in protein or RNA content within the sEVs. More functional, proteomic and transcriptomic analysis of the *Pf*EVs is clearly needed.

Data availability

Underlying data

Harvard Dataverse: Replication Data for: Impact of Plasmodium falciparum small-sized extracellular vesicles (PfsEVs) on host peripheral blood mononuclear cells. https://doi.org/10.7910/DVN/QXUFQ7[36].

This project contains the following underlying data:

- Folder 1_Flow cytometry fsc files_PfEV_PBMC_paper_anon.zip. (FCS files generated from flow cytometry experiments.)
- SMwangi_PfsEV_Data_files_anon.zip. (XLSX files containing raw data from cytokine analysis and ELISA experiments).
- SMwangi_PfsEVs_Readme.txt. (README file.)
- SMwangi_PfsEVs_Codebook.pdf. (Dataset codebook.)

Extended data

Harvard Dataverse: Replication Data for: Impact of Plasmodium falciparum small-sized extracellular vesicles (PfsEVs) on host peripheral blood mononuclear cells. https://doi.org/10.7910/DVN/QXUFQ7[36].

File 'supplementary_material_PfEV_PBMC_paper.pdf' contains the following extended data:

- Figure S1: Gating strategy.
- Figure S2: Gel electrophoresis image after PCR to test for Mycoplasma contamination in P. falciparum cultures and isolated *Pf*EVs.
- Figure S3: Cytokines/chemokines not significantly induced following PBMC co-culture with *Pf*sEVs or *Pf*iRBCs .

Acknowledgements

We are grateful to the adult volunteers who donated the blood samples that was used to isolate the PBMC samples used in this study. This paper was published with the permission of the Director of Kenya Medical Research Institute.

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Carmenza Spadafora
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Authors have used a *Plasmodium falciparum* clinical isolate to expand it in the lab and study the immune stimuli that infected erythrocytes from this isolate, or their released small exovesicles, produce on PBMCs from donors.

First, I have two comments on the methodology section. I find it lacks information for others to replicate. Particularly, I note that the description of culture of *P. falciparum* parasites only lists the “ingredients” without specifying their concentrations. If the authors are following the exact recipe of Haynes and Moch or that of Trager and Jensen they should add a reference. If not, then give your own concentrations. The second important petition I have for the group is a better characterization of their sEVs. Even if the methodology of subsequent ultracentrifugations has proceeded well other times, and there is a list of results that the group has obtained from those experiments, it does not mean that in THESE experiments the isolation of EVs was successful. Every time you report a new finding on EVs, you must prove that what you experimented with are indeed EVs. The same holds true for anything that anyone isolates.To expand on this: on the one hand, the authors, on the referenced publication, show a TEM image of the isolated EVs. In that image there is a number of vesicles of the same size but the morphology inside is different. A minority of them have the doubled – layered membrane that characterizes EVs. There is discussion in the literature about other particles and structures that could have similar sizes. On the other hand, I find it inadequate to give, as a sole proof of the isolation, a silver-stained protein gel showing an enrichment of proteins on a fraction of a given step of the centrifugation methodology that worked in that occasion. It is necessary that some markers of EVs are provided, of the many there are (Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles*. 2018;7(1):1535750[1]).

Another issue I have with this manuscript is that there is relevant work that has not been cited.

There are other general functions reported for EVs and a significant amount of literature focused precisely on the immune response to EVs from Plasmodium spp. that the authors should have mentioned in the introduction and then discussed in the conclusions, given that these reports show how PBMCs have been found to respond to plasmodial microparticles, with a direct role in inflammation caused by the disease. Examples are:

- Extracellular vesicles carrying lactate dehydrogenase induce suicide in increased population density of *Plasmodium falciparum in vitro. Correa et al, 2019*[2]
- Circulating red cell-derived microparticles in human malaria. *Nantakomol et al, 2011*[3].
- Parasite-derived plasma microparticles contribute significantly to malaria infection-induced inflammation through potent macrophage stimulation. *Couper et al, 2010*[4]
- Augmented plasma microparticles during acute Plasmodium vivax infection. *Campos et al. 2010*[5]
- Exosomes from Plasmodium yoelii-infected reticulocytes protect mice from lethal infections. *Martin-Jaular et al. 2011*[6].
- Gillrie MR, Ho M. Dynamic interactions of Plasmodium spp. with vascular endothelium. Tissue Barriers 2017;5:e1268667[7].

What can the authors say about the above reports and their results? Do they compare? What are the limitations of using PBMCs *in vitro* and not *in vivo* settings where different surrounding cells would also be releasing their own microparticles and most probably communicating with the infected erythrocytes? Would they foresee a change in the results obtained? How would the immune stimulus change when EVs are signaling a collective suicide? I lack in the discussion precisely that, more of a discussion. For example, what could be the meaning of having the early activation marker CD69 not expressed in monocytes in the presence of infected erythrocytes/sEVs but then having CD25 highly expressed in them when around infected erythrocytes?

Another issue: did not the authors find odd that they could not isolate sEVs from uRBCs given the reports that show how they obtain them, even after filtration with a 0.2 um membrane? (check Correa et al, 2019[2]). Why did they not use silver stain to visualize the proteins in uRBCs as they did with iRBCs or sEVs, but rather chose the much less sensitive Bradford stain for that visualization? Would they have found sEVs from uRBCs had they stained with silver? I believe so.

See Thayer et al, 2019 (Procoagulant activity of red blood cell-derived microvesicles during red cell storage[8]), Kuo et al, 2017 (Red blood cells: a source of extracellular vesicles[9]) or Nowbouossie et al, 2020 article in Blood (Red blood cell microvesicles activate the contact system, leading to factor IX activation via 2 independent pathways[10]).

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parasitology, Plasmodium falciparum Exovesicles, Drug Discovery, Biophysics of Plasmodium falciparum

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 10 November 2020

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I have read the article titled Impact of *Plasmodium falciparum* small-sized extracellular vesicles on host peripheral blood mononuclear cells. The key findings of this work is that there is differential induction of immune response on peripheral blood mononuclear cells by extracellular vesicles derived from malaria infected red blood cells and the plasmodium falciparum infected red blood cells. This differential immune response induced is exerted by the Th17 and Th1 actions which is yet to be fully characterized. In addition the paper details the decline in the capacity of the extracellular vesicles to activate monocytes.

The introduction of the paper was well written with relevant references cited. However, the opening sentence seems to be missing some texts. The authors need to check this out.
The methods used were well described but since the aim of the work was to compare the functional impact of *Pfs*EVs and the autochthonous *Pf*iRBCs of a clinical isolate adapted to short-term culture (<70 cycles) on human peripheral blood mononuclear cells (PBMC). Were there no other methods that could have been employed in the study eg rt-qPCR? I am not sure it is clear what the authors meant by "probably enriched".

The results are clear but it could be more interesting if the authors could demonstrate why infected RBCs release more extracellular vesicles.

The discussion provides a summary of the findings of the study. However, the authors did not provide a clarity on why the difference in the induction of immune response as shown by the level of expression of the activation markers was observed and a justification for the observation. The significance of their observation is not clearly detailed and how this can be provide an insight to an in vivo condition. Do the authors think that different isolates of the *Plasmodium falciparum* could give a different observation from their study findings?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria, Extracellular vesicles, Brain endothelial cells, Microglia

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.