THE IMPACT OF *PLASMODIUM FALCIPARUM* SMALL-SIZED EXTRACELLULAR VESICLES (*Pf*sEVs) AND *PLASMODIUM FALCIPARUM*-INFECTED RED BLOOD CELLS (*Pf*iRBCs) ON HOST IMMUNE CELLS *IN VITRO*

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The Impact of *Plasmodium falciparum* Small-Sized Extracellular Vesicles (*Pf*sEVs) and *Plasmodium falciparum*-Infected Red Blood Cells (*Pf*iRBCs) on Host Immune Cells *in vitro*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature…… ……Date……

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This thesis has been submitted for examination with our approval as the university supervisors

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DEDICATION

I dedicate this work to the late Dr. Geoffrey William Griffin (Founder and Director, The Starehe Boys' Centre) for giving me a learning opportunity and paving my way during my formative years.

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ABBREVIATIONS AND ACRONYMS

COVID-19	Corona virus disease 2019
CCM	Culture conditioned media
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EVs	Extracellular vesicles
EV-uRBCs	Extracellular vesicles from uninfected red blood cells
ILV	Intraluminal vesicle
iRBCs	Infected red blood cells
KEMRI	Kenya Medical Research Institute
KWTRP	KEMRI-Wellcome Trust Research Programme
mEVs	Medium-sized extracellular vesicles
MVB	Multivesicular body
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
***Pf*EMP1**	*Plasmodium falciparum* erythrocyte membrane protein 1
***Pf*iRBCs**	*Plasmodium falciparum*-infected red blood cells
***Pf*sEVs**	*Plasmodium falciparum* small-sized extracellular vesicles
RBCs	Red blood cells

RNA	Ribonucleic acid
uRBCs	Uninfected RBCs

ABSTRACT

Overly increased immune activation is thought to play a key role in malaria pathogenesis. During blood stage infection, *Plasmodium falciparum* can directly interact with host immune cells through the infected red blood cells (*Pf*iRBCs) but can also do so indirectly via the release of extracellular vesicles (EVs). This study compared the impact of *Pf*iRBCs and *P. falciparum* small-sized EVs (*Pf*sEVs, also known as exosomes) from a clinical parasite isolate (*Pf*KE12) adapted to short-term laboratory culture conditions on host peripheral blood mononuclear cells (PBMC). *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 then co-cultured for 18 hours with the study PBMC. Cellular responses acquired were quantified by quantifying the cell surface expression of activation markers (CD25 & CD69) and cytokine/chemokine levels in the supernatant. *Pf*sEVs induced CD25 expression on $CD4^+$, $CD19^+$ and $CD14^+$ cells while *Pf*iRBCs induced the same on $CD19^+$ and $CD14^+$ cells relative to the background. Both *Pf*sEVs and *Pf*iRBCs induced CD69 on $CD4^+$, $CD8^+$ and $CD19^+$ cells. Additionally, *Pf*iRBCs induced higher expression of CD69 on $CD14^+$ cells. CD69 induced by *Pf*iRBCs on $CD4^+$ and $CD19^+$ cells were significantly higher than that induced by *Pf*sEVs. The secretion of MIP1**α**, MIP1β, GM-CSF, IL-6, IL-8, and TNF**α** were significantly induced by both *Pf*sEVs and *Pf*iRBCs whereas MCP-1, IL-10, IL-17**α** 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*sEVs. *Pf*sEVs and *Pf*iRBCs showed differential abilities to induce secretion of IL-17**α** and IFN-**γ** suggesting that the former is 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.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria remains a public health challenge in the world and especially in sub-Saharan Africa. In 2020, there was an estimated 241 million cases of malaria and about 627,000 deaths globally (WHO Malaria Report, 2021). Majority of the burden falls on children below five years accounting for 67% of the deaths. Sub-Saharan Africa bears the major brunt of malaria since 95% of the global malaria cases and 96% of deaths were reported in this region (WHO Malaria Report, 2021).

Plasmodium falciparum, one of the five species that infect humans, is the most lethal among the human malaria parasites and accounted for almost all the malaria cases in the African region (WHO Malaria Report, 2021). The other species infecting humans are *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* which was previously thought to only infect primates but recently found to infect humans (White, 2008).

P. falciparum parasites have a complex life cycle involving human and mosquito hosts. In the human host, the parasite goes through two stages, the liver, and blood stages and it is the blood stage that is responsible for the pathology of malaria. The exact pathogenesis of severe malaria remains unknown although several factors including host inflammatory responses (Cunnington et al., 2013), parasite burden (Dondorp et al., 2005) and sequestration of infected red blood cells (Taylor et al., 2004) are thought to play major roles. In severe form of the disease, infected erythrocytes have been shown to stick to epithelial cells to escape splenic clearance. This leads to blockage of microvasculature and exacerbated disease (Taylor et al., 2004; Turner et al., 2013). This binding/sticking is through endothelial protein C receptor (EPCR) and is thought to induce inflammation (Turner et al., 2013), but parasite factors independent of cytoadhesion have also been suggested to induce widespread inflammation and endothelial activation (Abdi et al., 2014; Hanson et al., 2015; Tripathi et al, 2009).

In endemic areas, people acquire natural immunity that initially protects against severe malaria and finally against mild malaria. Naturally acquired immunity to *P. falciparum* parasite infection has been shown to develop slowly; requiring long and continued exposure to the pathogen, often never sterile and in an age-dependent manner (Langhorne et ., 2008; Struik & Riley, 2004). Antigenic variations are thought to play a role in the slow development of the naturally acquired immunity but parasite secreted effectors that are able to manipulate the host immune response may also have a role (Abdi et al., 2017; Das et al., 2013; Hanson et al., 2015; MacDonald et al., 2001; Sun et al., 2012; Tripathi et al., 2009).

One way in which secreted effector molecules are released from cells is through extracellular vesicles (EVs). EVs are double-layered membrane-bound molecules that are released by cells. They are usually classified into two major subgroups; the small-sized and medium-sized extracellular vesicles (Théry et al., 2018) often termed as exosomes and microvesicles respectively (Raposo & Stoorvogel, 2013; Théry et al., 2009). The small-sized extracellular vesicles (sEVs) are vesicles with a diameter ranging between 30-150 nm. They are generated through inward budding of the limiting membrane of late endosomes resulting in the formation of intraluminal vesicles (ILVs). During the inward budding of the endosomal membrane, cytosolic proteins such as those involved in glycolysis, RNA, lipids, and metabolites are sorted into the ILVs. Late endosomes containing many ILVs are called multivesicular bodies (MVBs) which then fuse with the cell membranes and release ILVs into the extracellular space as exosomes or sEVs (Raposo & Stoorvogel, 2013; Théry et al., 2018). The medium-sized EVs (mEVs) have a diameter range of 100-1000 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 (Raposo & Stoorvogel, 2013). Due to the overlap in their diameters, exosomes and microvesicles are termed as 'extracellular vesicles'.

EVs have been shown to have the ability to transfer packaged effector molecules (including proteins, lipids, nucleic acids, and metabolites) from one cell to another, hence, modifying the properties of the recipient target cell(s) (Kalluri, 2016; Schorey et al., 2015; Théry et al., 2009; Zhao et al., 2016). Research in the pathophysiology

of several diseases like cancer (Costa-Silva et al., 2015; Hodgson et al., 2015; Kalluri, 2016) and infectious diseases (Mantel et al., 2013; Marti & Johnson, 2016; Neta Regev-Rudzki et al., 2013; Szempruch, Sykes, et al., 2016; O Twu et al., 2013) have demonstrated a role of EVs in mediating intercellular interactions (Mantel et al., 2013; Regev-Rudzki et al., 2013; Szempruch et al., 2016; Théry et al., 2009; Twu et al., 2013). 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 (Buck et al., 2014; Filipazzi et al., 2012; Lambertz et al., 2012; Swatler et al., 2020; Szempruch et al., 2016).

In the context of malaria, *P. falciparum* EVs (*Pf*EVs) have a role in intercellular communication as well as in inducing sexual commitment (Mantel et al., 2013; Regev-Rudzki et al., 2013). Extracellular vesicles reflect the molecular phenotype of their cells of origin (Kalluri, 2016). To this end, 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 (Abdi et al., 2017; Mantel et al., 2013) and have been shown to induce inflammation (Mantel et al., 2013) and endothelial activation (Mantel et al., 2016). However, to date, all studies on the impact of *Plasmodium* EVs on host immune response have been performed using either rodent malaria (Demarta-Gatsi et al., 2019; Martin-Jaular et al., 2011) or using long-term laboratory-adapted *P. falciparum* isolates (Mantel et al., 2013; Sisquella et al., 2017). This current study is the first to use a *P. falciparum* clinical isolate. The quantity and repertoire of *Pf*EVs from clinical isolates appear to be different from that of long-term laboratory-adapted parasite isolates (Abdi et al., 2017) and this may affect their functional impact on the host immune cells. This study aimed to compare the functional impact of *Pf*sEVs and the autochthonous *Pf*iRBCs of a clinical isolate adapted to short-term culture (<70 cycles) on human peripheral blood mononuclear cells (PBMC).

1.2 Statement of the Problem

Malaria disease remains one of the major public health concerns worldwide despite the huge investments made in its fight. In 2020, for instance, the total funding for malaria control and elimination stood at about US$ 3.3 billion. Despite this, there were over a quarter million cases and more than half a million deaths recorded (WHO Malaria Report, 2021). Additionally, majority of these cases and deaths occur in the tropics. Sadly, many of the countries in the tropics are faced with a huge burden of infectious diseases and malaria only exacerbates the public health challenge. In 2020, for example, 95% of global malaria cases and 96% of global deaths recorded in the sub-Saharan Africa with the under-fives bearing the major brunt of the disease (WHO Malaria Report, 2021). Majority of deaths from malaria are due to a severe form of the disease called severe malaria. Severe malaria is a complicated multi-syndromic disease condition whose pathogenesis remains to be fully understood. It often manifests as cerebral malaria, severe malaria anaemia and respiratory distress (WHO Malaria Report, 2021).

The cause(s) of severe malaria remains to be fully understood. However, parasite sequestration (found in infected erythrocytes), endothelial dysfunction, and host inflammatory responses are thought to play major roles. Induction of inflammation and endothelial activation may involve the interaction of *P. falciparum*-infected erythrocytes with host endothelia through endothelial protein C receptor (EPCR) but it may also involve factors independent of cytoadhesion (Abdi et al., 2014; Hanson et al., 2015; Tripathi et al., 2009). These factors include *P. falciparum* extracellular vesicles released by the parasite (Mantel P.Y. et al., 2013). There remains, therefore, a need to further understand the interaction between the malaria parasite and the human host. This study investigated the impacts of *Pf*sEVs from a *P. falciparum* clinical isolate and its purified infected erythrocytes on host immune cells. The host immune cells were obtained from consented malaria-exposed and non-exposed volunteers to test whether prior/recent exposure to malaria modifies the host immune response to parasite.

1.3 Justification of the Study

Malaria is still a major cause of morbidity and mortality especially in the sub-Saharan African region with *P. falciparum* as the most lethal malaria parasite (WHO Malaria Report, 2021). The exact pathogenesis of severe malaria remains to be elucidated. Moreover, different people respond differently to the invading pathogen to end up with severe, uncomplicated, or asymptomatic malaria. Apart from infected erythrocytes/RBCs, several factors, including virulence of the invading parasite, are thought of to be the reasons for the severity of the disease (Gonçalves et al., 2014). Recently, *P. falciparum* extracellular vesicles (*Pf*EVs) packaged with effector molecules have been shown to be released by infected erythrocytes and involved in intercellular communications (Mantel et al., 2013; Regev-Rudzki et al., 2013). To further understand the interaction between the malaria parasite and the human host and the outcome thereof, this study was necessary. The knowledge gained from this understanding will add onto efforts towards designing products such as vaccines designed to stop the parasite from attacking its human host. The study, therefore, hypothesized that *Pf*sEVs and infected RBCs (*Pf*iRBCs) from *P. falciparum* clinical isolate will have varying potency in inducing activation on PBMCs. Additionally, there was an interest to elucidate whether continued exposure to the parasite factors (*Pf*iRBCs and *Pf*sEVs) may have any bearing on response of PBMC.

1.4 Research Question

1. Do *Plasmodium falciparum* small-sized extracellular vesicles (*Pf*sEVs) and *Plasmodium falciparum* infected red blood cells (*Pf*iRBCs) activate peripheral blood mononuclear cells (PBMC)?
2. What are the effects of *Plasmodium falciparum* small-sized extracellular vesicles (*Pf*sEVs) and *Plasmodium falciparum* infected red blood cells (*Pf*iRBCs) on T cell proliferation?
3. What inflammatory cytokines/chemokines are released by PBMC upon exposure to *Plasmodium falciparum* small-sized extracellular vesicles (*Pf*sEVs) and *Plasmodium falciparum* infected red blood cells (*Pf*iRBCs)?

1.5 Objectives

1.5.1 General Objective

To determine the impact of *Plasmodium falciparum* small-sized extracellular vesicles (*Pf*sEVs) and *Plasmodium falciparum* infected red blood cells (*Pf*iRBCs) on host immune cells *in vitro.*

1.5.2 Specific Objectives

1. To determine whether *Pf*sEVs and *Pf*iRBCs activate PBMC
2. To determine the effect(s) of *Pf*sEVs and *Pf*iRBCs on T cells' proliferation
3. To elucidate the level of selected inflammatory cytokines released by PBMC upon exposure to *Pf*sEVs and *Pf*iRBCs

1.6 Scope

This thesis describes and discusses *in vitro* experiments and results obtained in a lab-based *P. falciparum* parasite culture and cellular assays.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria epidemiology

Malaria is caused by a *Plasmodium* parasite that is transmitted through the bite of an infected female Anopheles spp mosquito. There are five known species of *Plasmodia* that infect humans: *Plasmodium malariae, P. falciparum, P. ovale, P. vivax* and the recently described *P. knowlesi* as the fifth human malaria parasite (White, 2008).

Malaria is majorly found concentrated in the world tropics that are hot and humid with undulating high and low malaria transmission seasons. *P. falciparum* contributes the largest burden of global malaria followed by *P. vivax* (Snow et al., 2005). Infections due to *P. falciparum* parasite are majorly concentrated in the sub-Saharan region (WHO Malaria Report, 2021) while *P. vivax* infection is more common in South-East Asia and South America (Price et al., 2007; WHO Malaria Report, 2021). Both species though are found occurring in South-East Asia. The other species are uncommon and only found in the endemic regions, for example, the sub-Saharan region for *P. malariae* and southern parts of Asia (mostly Thailand) for the *P. knowlesi* species (White, 2008).

There has been a global decline in the incidence rates of malaria between 2000 and 2020 from 81 to 59 cases/1000 people at risk. The slight increase in 2020 compared to 56 in 2019 was due to service disruptions during the COVID-19 pandemic (WHO Malaria Report, 2021).

2.2 Malaria cases and deaths

In 2020, there were an estimated 241 million cases of malaria worldwide compared to 227 million cases in 2019, an increase brought by the COVID-19 pandemic and disruption of services. Out of these, the World Health Organization (WHO) African region accounted for 95% of all the cases with the rest occurring in South-East Asia (2%) and elsewhere (3%) (WHO Malaria Report, 2021).

Additionally, WHO estimated the 2020 global mortality from malaria to be 627,000 deaths compared with 558,000 deaths the previous year, and 562,000 deaths in 2015. The 12% increase in deaths from the 2019 figures was attributed to COVID-19. From these, the African region accounted for 96% of all deaths while the rest were in the South-East Asia, the Americas, and the Mediterranean regions. The under-fives remain the most vulnerable group and accounted for 77% of all the 2020 malaria deaths (WHO Malaria Report, 2021).

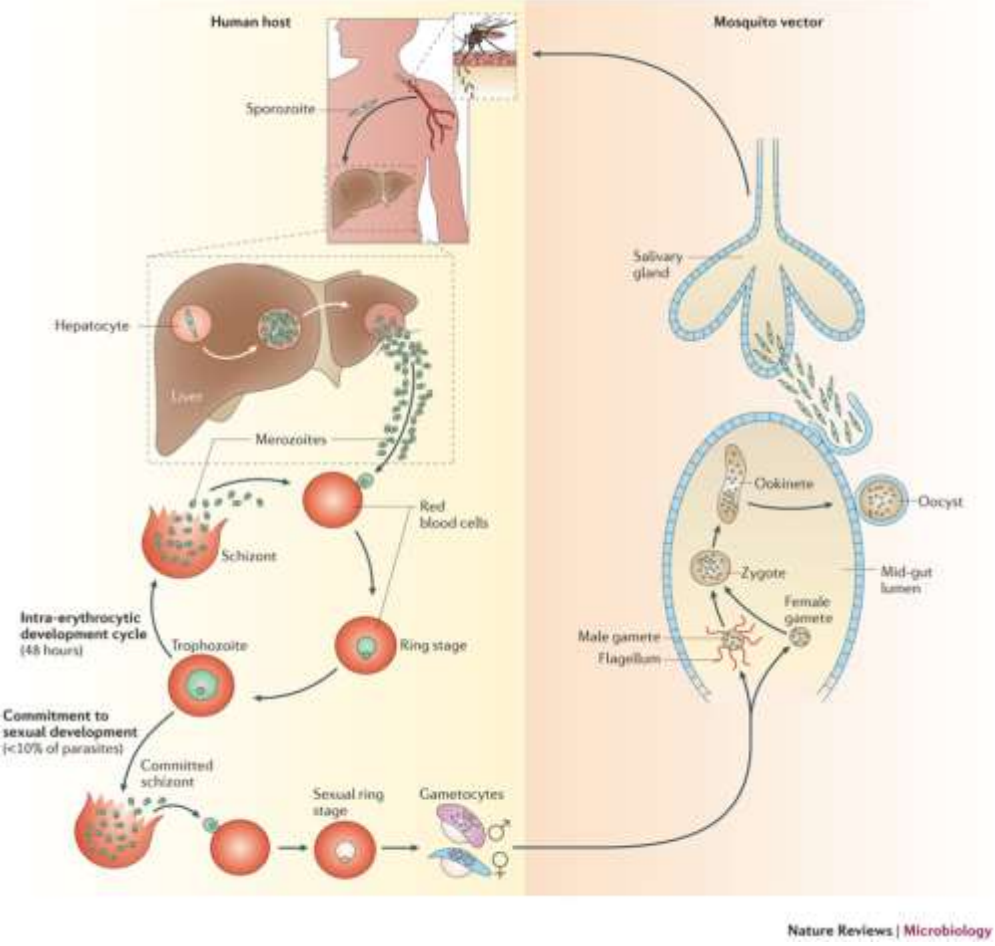
P. falciparum remains the most prevalent parasite in the WHO African region and accounted for most of the estimated malaria cases and consequently majority of the deaths in 2020 (WHO Malaria Report, 2021). The pathogenesis of this *P. falciparum* malaria is linked to the life cycle of the parasite. Below, I will briefly describe the life cycle of *P. falciparum* as the study described in this thesis focuses on this species.

2.3 The life cycle of Plasmodium falciparum malaria parasite

P. falciparum has a complex life cycle that alternates between its mosquito vector and the human host. The human infection starts when a malaria infected female Anopheline mosquito injects between 1 to 100 mature sporozoites from her salivary glands into the bloodstream of a human host during a blood meal. About 5-10% of the injected sporozoites travel through the bloodstream and infect hepatocytes (Ferreira et al., 1986) from where they undergo mitotic cell division and mature into schizonts. Each schizont forms about 30,000 merozoites (Jones & Good, 2006) which are released as merozomes when a hepatocyte ruptures after about 6-7 days. This is termed as the exoerythrocytic schizogony and is an asymptomatic stage in the host. The parasites then enter the erythrocytic cycle which constitutes asexual multiplication. The released merozoites from ruptured hepatocytes invade erythrocytes in the blood. After the invasion, the merozoites will then mature and divide to form schizonts that contain between 8 and 32 merozoites. The infected erythrocyte will eventually rupture to release daughter merozoites into the bloodstream which can then invade fresh red blood cells and the cycle starts all over again (Figure 2.1). The entire erythrocytic cycle from invasion to rupturing of red

blood cells takes around 48 hours for *Plasmodium falciparum* leading to an exponential increase in the number of parasites. It is suggested that the intermittent release of merozoites is responsible for the rise in the symptoms of malaria that is observed in the host (Langhorne et al., 2008). This, in turn, is responsible for the recurrent fevers and clinical manifestations of malaria disease in humans.

Some parasites while in the erythrocytes will differentiate into male and female gametocytes which constitutes the sexual erythrocytic cycle. The male (microgametocytes) and female (macrogametocytes) gametocytes will then be picked up and ingested by a female Anopheles mosquito during a blood meal. Once ingested, the gametocytes fuse and travel to the mosquito's midgut and mature to form zygotes. The zygotes will then become polar and elongate to form a motile and invasive stage called ookinete. The ookinete transverses the mosquito's midgut wall into the hemoceal and develop into oocysts by undergoing several rounds of mitotic cell divisions. These oocysts will then grow and divide to produce thousands of haploid forms called sporozoites. After the sporogonic phase, the oocysts rupture releasing sporozoites into the mosquito's body cavity which then find their way into the mosquito's salivary glands from where they can be transmitted during a blood meal in a human host (Figure 2.1) starting the cycle all over again (Josling & Llinás, 2015).



The life cycle of the *P. falciparum* oscillates between the human host consisting of the exo-erythrocytic and the intra-erythrocytic cycles and the mosquito vector linked by the gametocytes (Josling & Llinás, 2015).

Figure 2.1: *Plasmodium falciparum* life cycle

2.3 Severe malaria pathogenesis

Severe malaria (SM) is a complicated and multi-faceted disease whose exact pathogenesis remains unknown. However, unique characteristics of cerebral malaria (CM)/impaired consciousness, severe malarial anaemia, and respiratory distress (RD) define this condition (Cunnington et al., 2013; WHO Malaria Report, 2021). Each of the different forms of SM has some defining properties that are used to stratify them. Parasite sequestration of infected red blood cells (mainly in the brain microvasculature) has been described as the major cause of cerebral malaria (White

et al., 2014). This sequestration phenomenon is unique to *P. falciparum* and, hence, distinguishes this parasite from other Plasmodium species causing malaria. This also makes *P. falciparum* the deadliest among the Plasmodia species. The manifestation of CM is, however, different in experimental mouse models making it challenging to translate findings to human studies (Craig et al., 2012).

An additionally associated characteristic of severe malaria is the host immune response marked by excessive production of cytokines. These cytokines include tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10). The inflammatory responses are higher in CM and RD than in mild or uncomplicated malaria (Cunnington et al., 2013). Though that, the cause of this immunopathology in humans remains to be fully established although parasite sequestration is thought to be a major cause.

Parasites (iRBCs) sequestration is the process of infected erythrocytes (IEs) attaching themselves to the surfaces of endothelial cells that make up the inner wall of blood vessels as a strategy to avoid splenic clearance. The highly polymorphic *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family of proteins that are encoded by the *var* gene are responsible for binding of these IEs to the endothelial surfaces. The IEs have been shown to bind via endothelial protein C receptor (EPCR) (Turner et al., 2013). This binding has been suggested to hinder the interaction of the EPCR with its activated protein C (APC) (Turner et al., 2013), which is key in maintaining the integrity of vascular endothelial structure (Bouwens et al., 2013). Consequently, lack of such structural integrity has been hypothesized to be one of the factors that induce inflammation and endothelial activation in the host (Moxon et al., 2013; Turner et al., 2013). However, other studies have shown that inflammation/endothelial activation is induced in a process independent of cytoadhesion (Tripathi et al, 2009) ranging from microvascular obstruction (Hanson et al., 2015) to antigenic variation and *Pf*EMP1 expression (Abdi et al., 2014). Taken together, findings from these other studies suggest that other parasite factors independent of cytoadhesion are in part responsible for the observed widespread inflammation/endothelial activation associated with severe malaria.

2.4 Immunity to malaria

The immune system forms an integral part of the human body with a mechanism for recognizing and responding to foreign antigens from pathogens and fighting off infections. These immune responses can be through the innate and adaptive arms of the immune system or both. Cytokines, chemokines and other inflammatory immune mediators are responsible for activating and recruiting immune cells to sites of infection resulting in the elimination of the invading pathogen(s) (Schorey et al., 2015). Lymphocytes are the most important group of these immune cells. They include B cells, T cells, monocytes, macrophages, dendritic cells, natural killer cells, among others. B and T cells are especially key in the establishment of the adaptive immune system. Macrophages and dendritic cells are also useful as antigen presenting cells (APCs) and in linking-up the innate and adaptive arms of the immune system.

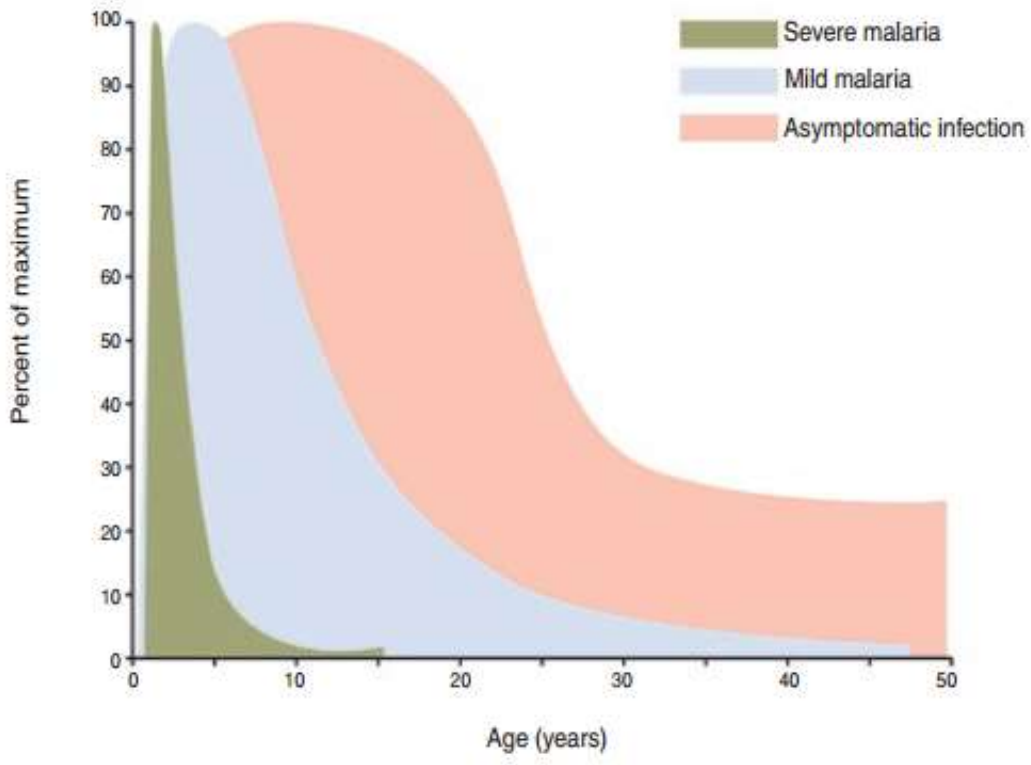
The type of immune response mounted is dependent on the nature of the invading parasite. The response can thus be either cellular; similar to that of natural killer cells and cytotoxic T cells or can be humoral through the production of antibodies and cytokines. The main function of B cells is the production of antibodies to specific antigens presented by APCs and retention/establishment of a memory repertoire of the various antigens encountered by the immune system. The subset of lymphocytes that remain as antigen-specific memory cells can mount a faster and targeted immune response upon re-infection by the same antigen.

T cells, on the other hand, give ‘help’ to B cells through production of cytokines so that those B cells can produce quality antigen-specific antibodies. Part of these T cells also forms a group of those lymphocytes that remain as antigen-specific memory cells upon clearance of the invading pathogen.

The invading pathogens have unique pathogen-associated molecular patterns (PAMPs) which elicit the immune responses experienced during an infection. These PAMPs are of diverse structures including proteins, lipids, carbohydrates, and genetic material (DNA and RNA). Different pathogens display diverse set(s) of PAMPs. Conversely, host immune cells have pattern recognition receptors (PRRs)

on their surfaces that interact with PAMPs. B cells' activation, for example, happens via B cell receptor (BCR), CD40, Toll-like receptors (TLRs), and cytokines' receptors among other PRRs. Hence, an immune response is initiated when there is an interaction between PAMPs and PRRs on immune cells leading to a cascade of reactions and cell signaling (Schorey et al., 2015). Pathogens can either be intracellular (such as *P. falciparum*) or extracellular (like Toxoplasma) with specific types of PRRs for each. Depending on the type of PAMPs (whether from an intracellular or extracellular pathogen), the immune system has a way of responding to each accordingly.

Immunity to malaria has been the subject of research for a long time with many researchers first trying to understand the concept of natural immunity and how it is formed. Natural immunity is that which comes about without inducement of a vaccine but through an infection and recovery from the infection. The memory lymphocytes that remain after the infection is cleared are critical regarding how the immune system responds to a subsequent infection from a similar pathogen. Naturally acquired immunity to *P. falciparum* parasite infection has been shown to be slow to develop, often never sterile and is age-dependent (Figure) (Langhorne et al., 2008; Portugal, Pierce, & Crompton, 2013; Struik & Riley, 2004). Despite that, individuals who grow up in malaria endemic area acquire immunity first to severe malaria, then mild, and never against asymptomatic infection (Langhorne et al., 2008).



Children often suffer from severe malaria but as they grow older above 5 years, they suffer from mild malaria due to continued exposure to the parasite and acquired natural immunity. Older children and adults only get asymptomatic infections due to some levels of protection from naturally acquired immunity (Langhorne et al., 2008).

Figure 2.2: Malaria natural immunity increases with age

As previously mentioned, children are the most vulnerable to malaria (WHO Malaria Report, 2021) compared to the older children who often get either mild or asymptomatic infections. Acquisition of natural immunity is, therefore, determined by age and exposure (Weiss et al., 2010) (Figure). Those people living in malaria-endemic areas tend to tolerate infections which result in effective priming of anti-blood stage immune responses against the parasites. These responses finally confer significant levels of humoral and cellular immunity. During subsequent infections, there is a decline in parasite density, prevalence, and severity of malaria in those individuals. This points to the development of a degree of natural immunity and anti-parasitic effector mechanisms (Langhorne et al., 2008). This concept of natural immunity to malaria was first demonstrated in the early 1960s. Passive transfer of

serum from naturally immune adults to children with clinical malaria was shown to reduce disease severity and parasitemia (Cohen, McGregor, & Carrington, 1961). Several factors are thought to contribute to the slow development of natural immunity against malaria. These include clonal antigenic variation (Takala & Plowe, 2009), alternate allelic forms, and polymorphism within parasite populations (Cunnington et al., 2013), implying one requires accumulating variant-specific immunity in order to achieve a 'fairly' protective immunity (Illingworth et al., 2013). Another possible mechanism could be due to direct parasite interference with the host immune system in a way that supports parasite survival (Abdi et al., 2017).

Both B and T cells are key in naturally acquired immunity to *P. falciparum* malaria. As earlier demonstrated, antibodies and $CD4^+$ T cells (Cohen et al., 1961) are key in protection against blood-stage *Plasmodium* parasite infections. Further to this, it was established that chronic infections drove T cells to exhaustion and dysfunction thus rendering suboptimal functions (Horne-Debets et al., 2013; Illingworth et al., 2013). This phenomenon is similarly seen in chronic viral infections (Day et al., 2006; Wherry et al., 2007) as well as in parasitic diseases. Indeed, repeated exposure to malaria antigens drives the expression of T cell inhibitory receptors including programmed cell death-1 receptor (PD-1) and lymphocyte-activation gene 3 (LAG-3) (Illingworth et al., 2013) that lead to poor effector function (Horne-Debets et al., 2013). Consequently, these poor T cell effector functions could be one of the major reasons for poor natural immunity against malaria (Wykes et al., 2014). In a recent finding, Bediako and colleagues found that $CD4^+$ T cells of continuously exposed individuals to malaria parasite proliferated less when stimulated with *P. falciparum* iRBCs than those of 'historically exposed'. This points to changes that are instituted by continued exposure to *P. falciparum* parasite (Bediako et al., 2016).

Malaria parasites may also evade humoral immunity through dysregulation of B cell responses (Portugal et al., 2013; Weiss et al., 2009). Further studies suggest that this dysregulation could also be through subversion of their normal functions by making them 'weak' or 'exhausted' (Illingworth et al., 2013). A similar phenomenon was demonstrated in patients suffering from HIV and Hepatitis B and C infections (Moir & Fauci, 2009; Wherry, 2011). Illingworth and colleagues termed this 'exhausted'

memory B cell phenotype as ‘atypical’ in relation to malaria immunity (Illingworth et al., 2013). Consequently, key B cell responses like proliferation, cytokine production, and antibody secretion are impaired, hence, reduced effector function and inefficient acquisition of effective humoral immunity to malaria (Portugal et al., 2015).

Therefore, the malaria parasite (*P. falciparum*), just like other pathogens is thought to fine-tune the host immune responses in a way that promotes its survival in that host. Secretions from cells infected by these pathogens, in addition to apoptotic bodies and cell debris, have been shown to have the potential to deliver their pathogenic material to host cells or tissues in their vicinity (Ashida et al., 2011). One of such delivery processes is by extracellular vesicles whose study has gained momentum in the last three decades.

2.5 Extracellular Vesicles

Extracellular vesicles (EVs) is a collective term used to define membrane-bound vacuoles released from cells under different physiological conditions for different functions (Raposo & Stoorvogel, 2013). They are found in many biological fluids including urine, saliva, nasal fluids, amniotic fluids, serum, plasma and seminal fluids, (Caby et al., 2005; Keller et al., 2011; Pisitkun et al., 2004) among others. Due to this, EVs have different origins, functions, physical characteristics, and compositions often dependent on the cellular source, state, and environmental conditions (Buzas et al., 2015).

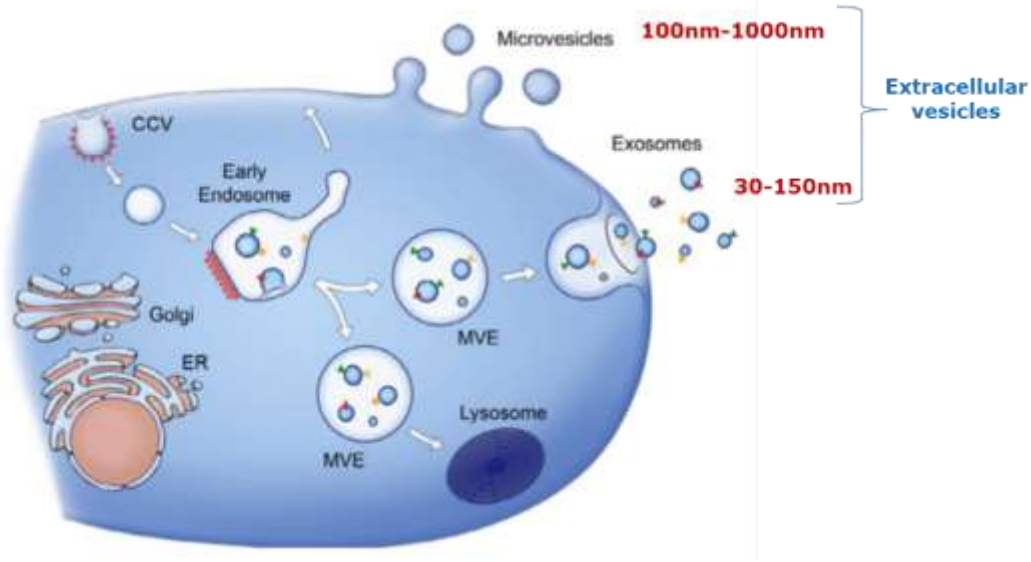
EVs are characterized by size, floatation density and the presence of proteins (used as markers) with divergent functions on their surface. Majority of these proteins are used for transport and fusion. These include GTPases, annexins, Rab, flotillin, components of the endosomal sorting complex required for transport (ESCRT) such as Alix, heat shock proteins (HSPs), tumor susceptibility gene 101 (TSG101) and tetraspanins [e.g. CD63 and CD81] (Chaput & Théry, 2011; Record et al., 2011; Simons & Raposo, 2009; Théry et al., 2009).

As earlier mentioned, EVs are majorly classified into two major sub-groups; small-sized and medium-sized EVs; often termed as exosomes and microvesicles, respectively (Théry et al., 2018). This thesis will focus on the small-sized EVs (sEVs) that are less than 200nm and that fall within exosomes but may also contain some microvesicles.

2.5.1 Small-sized EVs (sEVs)/Exosomes

Exosomes are small membrane/endosomal-derived nanovesicles that were identified over three decades ago (Harding et al., 1983) during studies of transferrin receptor trafficking in reticulocytes' maturation process and later in other cells (Théry et al., 2009). These transferrin receptors function to mediate intracellular iron uptake necessary for hemoglobin synthesis. During reticulocyte maturation, reticulocytes were described as having large 'sacs' enclosing other small sacs or vesicles and these vesicles were termed "exosomes."

Exosomes have a diameter range of 30-150nm and have an endocytic cellular origin. They result from the inward invagination of the limiting membrane of the late endosomes which leads to the formation of intraluminal vesicles (ILVs) (Abels & Breakefield, 2016). In the process, different molecules including cytosolic proteins, lipids and RNA are normally sorted into these ILVs. Multiple ILVs contained in the late endosomes are called multivesicular bodies (MVBs) and once they fuse with the cellular plasma membrane release the ILVs as exosomes into the extracellular space (Figure) (Colombo, Raposo, & Théry, 2014; Harding et al., 1983; Schorey & Bhatnagar, 2008; Théry, Zitvogel, & Amigorena, 2002). The density of these exosomes ranges between 1.13 to 1.19g/ml and pellet at 100,000×g (Raposo & Stoorvogel, 2013).

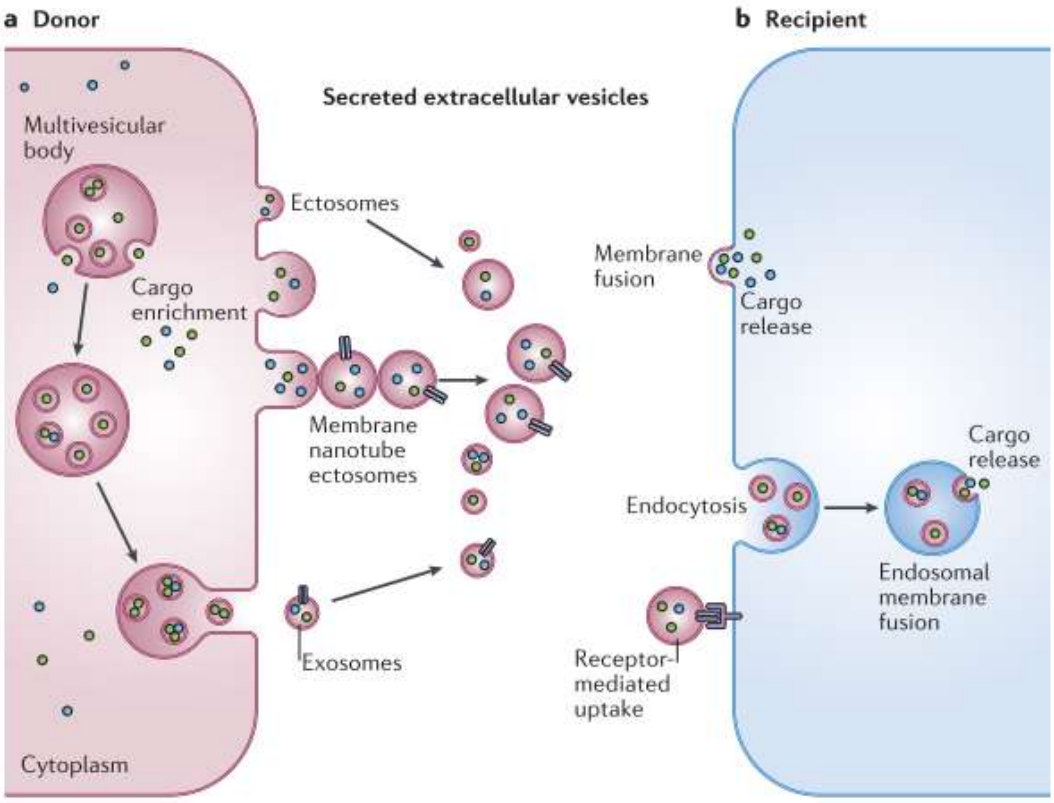


Exosomes formed as ILVs collect in early endosomes and are released extracellularly when MVBs (or MVEs-multivesicular endosomes) fuse with the plasma membrane. Other MVEs fuse with lysosomes. Microvesicles bud directly from the plasma membrane. The different shapes on released exosomes represent various membrane-associated molecules [(figure adapted with modifications from (Raposo & Stoorvogel, 2013)].

Figure 2.3: Formation and release of extracellular vesicles

2.5.2 Uptake mechanisms of excreted EVs

Phagocytosis is thought to be the most common form of uptake of EVs (Christianson et al., 2013; Mantel et al., 2013; Morelli et al., 2004) with macrophages and monocytes being described as the main targets. Three mechanisms of these EVs' interaction with the recipient cells have been suggested to be: receptor-mediated binding, membrane-membrane fusion and entry through the endocytic pathway (Figure.) (Cocucci & Meldolesi, 2015; Szempruch et al., 2016; Théry et al., 2009). Once bound, the EVs may remain in association with the plasma membrane, dissociate, fuse with that target cell(s) or be internalized (Raposo & Stoorvogel, 2013; Szempruch et al., 2016; Théry et al., 2009).



EVs may be released by the donor cell (a) either as ectosomes/microvesicles that 'pinch off' from the surface of the plasma membrane with receptor proteins or they can be exosomes that are released once the MVBs fuse with the plasma membrane and empty their ILVs as exosomes extracellularly. Released EVs will then interact with the recipient cell (b) through plasma membrane fusion, endocytosis or by receptor-mediated uptake (Szempruch, Dennison, et al., 2016).

Figure. 2.4: Mechanism of EVs' interactions between the donor and recipient cells.

2.6 Role of extracellular vesicles

2.6.1 Bacterial extracellular vesicles

Both gram-negative and gram-positive bacteria produce extracellular vesicles (mainly termed 'outer membrane vesicles, OMVs) (Lee et al., 2009). This is an essential and a highly conserved biological function, and especially in the gram-negative bacteria. These OMVs seem to be involved in several functions including molecular transport, the formation of biofilms, mediating of stress responses, as well

as interactions with their hosts (Manning & Kuehn, 2011; McBroom & Kuehn, 2007).

In transport and delivery, bacteria use OMVs as trafficking vehicles for molecules key in intracellular communications as well as quorum sensing (Mashurn-Warren, Mclean, & Whitley, 2008; Mashburn & Whiteley, 2005). Additionally, OMVs can facilitate the horizontal transfer of antibiotic resistance between bacteria(Mashburn-Warren & Whiteley, 2006) or the delivery of toxins and virulence factors (Rivera et al., 2010).

As pointed out earlier, EVs may be produced by cells during stressful conditions, and this is a key function exploited by bacteria. To this end, they use OMVs to sequester and export away stressors like heat shock proteins (McBroom & Kuehn, 2007), to protect themselves against complement factors as well as against anti-bacterial peptides (Manning & Kuehn, 2011). For example, EVs released from *Mycobacterium tuberculosis* were shown to modulate immune responses via IFN-γ (Singh et al., 2011) and through inducing varied cytokine responses with an inhibitory role in T cell activation (Yang et al., 2012).

Bacterial biofilms, on the other hand, are key in the survival, protection, and persistence of the bacterial colony/community. OMVs are thus key both in the formation and maintenance of the integrity of extracellular matrix found in bacterial biofilms. These OMVs are involved in the transport of molecules within the matrix as well as antibiotic resistance thus enhancing survival of the bacteria (Inagaki et al., 2006; Schooling et al., 2009).

Moreover, bacterial OMVs have a vital function of maintaining interactions with their hosts. This applies to both pathogenic and symbiotic interactions which are crucial in physiological balance thus ensuring maintenance of epithelial barrier integrity. Human bacterial flora, for example, is found colonizing different organs including the skin where they interact with different cell types. These bacteria use OMVs which can cross the epithelial tissues to interact and exert their effects especially on immune cells (Zhang et al., 2013).

2.6.2 Extracellular vesicles in cancer

The immunological functions of EVs in the context of tumors vary and range with emphasis on tumor antigen presentation and modulation of tumor immunity (Greening et al., 2015). As such, cancer cell-associated exosomes have been shown to activate natural killer (NK) cells and play a role in activating B and T cells via dendritic cells' (DC) differentiation. These exosomes may also be involved in promoting immune subversion through impairment of DC maturation (Kalluri, 2016) and independent of IL-10 (Deaglio et al., 2007).

Muller and colleagues also demonstrated that tumor-derived exosomes (TEX) target immune response-regulating genes and induce changes in transcriptional profiles of T cells (Muller et al., 2016). In yet another experiment, these exosomes were also described to induce activation and expansion of human regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) both *in vitro* and *in vivo* (Iero et al., 2008).

Collectively, tumor cell-associated exosomes are thought to suppress immune cell functions such as inhibiting functions of $CD8^+$ T lymphocytes through induction of apoptosis (Kim et al., 2005) and suppressing cytolysis by constraining functions of NK cells (Whiteside, 2013). This downregulation of key immune cells' functions promotes tumor progression and regulation of peripheral tolerance in cancer patients (Whiteside, 2013). However, other studies reported that exosomes released by tumor-activated DCs also had anti-tumor effects as well as activation of naïve CD4 T cells (Théry et al., 2002).

2.6.3 Extracellular vesicles in parasitic infections

Extracellular vesicles have been shown to play a role in pathogenic protozoans which, overall, contributes to their development, disease progression and pathology while in the host. In context, they have been demonstrated to modulate the immune systems of their hosts by eliciting varied inflammatory responses (Szempruch et al., 2016). Various emerging roles of EVs in Kinetoplastids that include Leishmania *spp*, *Trypanosoma cruzi* and *Trypanosoma brucei* as well as the Apicomplexa

Toxoplasma gondii and *Plasmodium falciparum* have been described (Marti & Johnson, 2016; Szempruch et al., 2016).

T. brucei brucei are non-infective in humans since they are readily eliminated by the host serum factors called trypanosome lytic factors (TLFs). EVs from *T. brucei rhodesiense,* an infective species in humans, have been shown to transfer serum-resistance protein (SRA) to the non-lethal *T. brucei brucei* when co-cultured thus rendering them lethal and infective in the host (Szempruch et al., 2016). This might be a pointer to the mixed human trypanosome infections which in turn promote the parasite survival in their hosts (Stephens & Hajduk, 2011) as well as causing host anemia (Marti & Johnson, 2016; Szempruch et al., 2016; Szempruch et al., 2016). *T. cruzi* have also been shown to release EVs that are enriched with immunogenic proteins and tRNA-derived small RNAs (tsRNAs) (Garcia-Silva et al., 2014). These tRNAs are also transferred between cells in a similar manner as well as the released EVs causing differentiation of *T. cruzi* and promoting infection susceptibility of host cells (Garcia-Silva et al., 2014).

Leishmania parasites have also been shown to release EVs that suppress immune responses and modulate pro-inflammatory responses including changes in interleukin levels (Silverman et al., 2010). The downregulation of pro-inflammatory genes and suppression of macrophage activation are thought to promote their survival in the host.

Trichomonas vaginalis likewise secrete EVs that contain proteins and small RNAs as is the case in other parasites (Twu et al., 2013). These EVs are described to increase parasite adherence to host cells *in vitro* of the poorly adhering G3 parasite strains (Twu et al., 2013). Other *T. vaginalis* strains with preferential binding to prostrate rather than vaginal epithelial cells can also transfer the phenotype in those strains lacking the preferential binding properties. In addition, *T. vaginalis* EVs have been demonstrated to modulate host immune responses by dampening IL-8 responses while inducing an IL-6 response (Twu et al., 2013). Reduced IL-8 production (key in anti-parasite activity) means enhanced parasite survival through adherence to epithelial walls and reduced clearance due to remodeled/dampened immune

responses (Marti & Johnson, 2016). Helminths have also displayed similar immune modulation properties (Chaiyadet et al., 2015) that promote their survival.

2.7 Role of EVs in malaria infection

Infection with malaria triggers the release of EVs from various cell types including endothelial cells, platelets, and red blood cells (Marti & Johnson, 2016). Plasmodium infection (both in human and murine models), and especially *P. falciparum* infection (Pankoui et al., 2010) leads to an overall increase in circulating EV numbers resulting from host erythrocytes as well as other cell types (Campos et al., 2010; El-Assaad et al., 2014; Nantakomol et al., 2011). Coincidentally, this increase in EVs production correlates with the severity of malaria disease both in rodent malaria model and in humans (Campos et al., 2010; El-Assaad et al., 2014; Nantakomol et al., 2011).

EVs contain packaged effector molecules with the ability to transfer them from one cell (donor) to another (recipient) and, hence, modifying the properties of those recipient/target cells (Kalluri, 2016; Schorey et al., 2015; Zhao et al., 2016). *Pf*EVs were described to have a key role in parasite-parasite communication including inducing sexual commitment (Mantel et al., 2013; Regev-Rudzki et al., 2013) as well as contributing to increased inflammation and inflammatory responses in the host.

These EVs released during such Plasmodia infections have been shown to result to pro- and anti-inflammatory responses (Mantel & Marti, 2014; Marcilla et al., 2014; Marti & Johnson, 2016). These increased pro-inflammatory responses are helpful to the parasites for their survival within the host. Consequently, they may help in endothelial cell activation and dysfunction which promotes IEs/iRBCs' sequestration to the body's microvasculature and related pathologies (Mantel et al., 2013; Marti & Johnson, 2016). In rodent malaria, for example, EVs from *P. berghei*-infected mice with cerebral malaria co-cultured with macrophages were shown to cause increased expression of tumor necrosis factor (TNF) and CD40. These EVs caused cerebral lesions in addition to adhering to blood vessels in the brain when transferred to naïve mice indicating a role in malaria pathology (El-Assaad et al., 2014).

As earlier opined, the exact pathogenesis of severe malaria remains to be established although parasite sequestration is thought to be a major cause as it leads to inflammation and endothelial activation (Moxon et al., 2013; Turner et al., 2013). However, widespread endothelial activation and sequestration have been found to be independently associated with severe malaria (Abdi et al., 2014; Hanson et al., 2015). This suggests that other parasite factors that are independent of the cytoadhesion property may be partly responsible for the observed inflammation/endothelial activation observed with severe malaria.

*Pf*EVs released by *Pf*iRBCs contain packaged effector molecules and by extension represents their cells (iRBCs) of origin. Severe malaria is manifested in unique characteristics including cerebral malaria/impaired consciousness, severe anemia and respiratory distress (Cunnington et al., 2013) all of which point to the possibility that parasites may release factors (contained in *Pf*EVs) with varied characteristics hence the different forms of severe malaria. It is at the backdrop of this information that this thesis aimed to establish the effects of *Pf*EVs from a *P. falciparum* clinical isolate when used to stimulate PBMCs from both 'malaria-naïve' and 'malaria-exposed' individuals.

CHAPTER THREE

METHODS

3.1 Study design

This was an *in vitro* laboratory-based study that compared the effects of *Plasmodium falciparum* small-sized extracellular vesicles (*Pf*sEVs) and *Plasmodium falciparum* infected red blood cells (*Pf*iRBCs) on host immune cells.

3.2 Ethical approval

The ethical approval for this study was obtained from the 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 conducted in accordance with the approved guidelines.

3.3 Sourcing of PBMCs

The PBMCs used in this study were obtained from twenty (20) individuals who were either 'malaria naïve' (eight) or 'malaria exposed' (twelve). 'Malaria naïve' group were consented adults volunteers who lived close to the KWTRP facilities in Kilifi (a region of low malaria transmission) and grew up in non-malaria endemic regions of Kenya and with no history of known clinical malaria episodes in their lifetime. The 'malaria exposed' group were Junju adults from the 2016 annual cross-sectional bleed which is part of a malaria surveillance cohort at KWTRP. Junju is a malaria-endemic region (Bejon et al., 2007) located south of the Kilifi creek in the larger Kilifi Health and Demographic Surveillance System (KHDSS) area at the Kenyan coast (Scott et al., 2012). These number of PBMC donors were arrived at based on availability of resources to conduct the planned experiments.

3.4 P. falciparum parasite culture

The *P. falciparum* isolate used in this study was a clinical parasite isolate that been adapted to *in vitro* culture and used in previous studies (Pieper et al., 2017; Tan et al.,

2016). This isolate was known as 9215 based on Kilifi clinical isolate naming system and had been obtained from a child admitted at Kilifi County Hospital with respiratory distress (Marsh et al., 1995). The 9215 isolate has been renamed *Pf*KE12 in unpublished genome data (Otto et al., 2019) and will be referred to as such in this thesis.

*Pf*KE12 was cultured under standard culturing conditions (Trager & Jensen, 1976). The frozen culture vial was taken out of liquid nitrogen and thawed quickly by rubbing between gloved hands. The culture was then transferred into a 50ml falcon tube (Greiner .Bio-One), in sterile conditions, where a series of sodium chloride solutions in reducing concentrations (12%, 1.8%, and 0.9% respectively) were systematically added. The culture was then washed twice with incomplete culture media (Table 3) by centrifugation at 440×g for 5 minutes at room temperature. The thawed culture was resuspended in an appropriate volume of complete *P. falciparum* parasite culture media (90% incomplete media + 10% Albumax-II [Gibco]) ready for transfer into the culture incubator.

Table 3.1: *Plasmodium falciparum* parasite culture: Incomplete medium

Ingredients	Volume added (ml)
Hepes (1M) [Gibco]	18.75
L-Glutamine (200mM) [Gibco]	5.0
Gentamicin (10mg/ml) [Gibco]	1.25
D-Glucose (20%) [Gibco]	5.0
Sodium hypoxanthine (8.4mg/ml) [Gibco]	3.0
RPMI 1640 [Sigma]	500

A 200µl aliquot from the resuspended culture in complete culture medium was taken for mycoplasma contamination testing. Another 10µl aliquot was taken to prepare a thin smear on a glass slide, fixed with absolute methanol for 30 seconds and stained with 10% Giemsa (Sigma) solution for 15-20 minutes. This was then observed under a light microscope using the ×100 oil-immersion objective lens to determine the general appearance of the thawed culture. Finally, the culture was gassed (92% Nitrogen, 5% CO_2, and 3% O_2; Kenya BOC Limited) and incubated at 37°C.

3.4.1 D-Sorbitol synchronization of *P. falciparum*-infected iRBCs

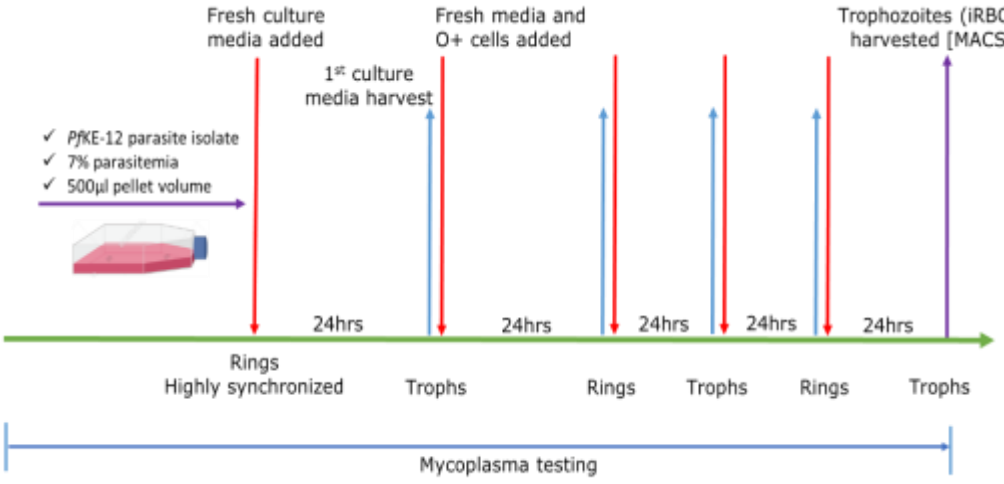
After the parasite started growing with a predictable growth rate, the parasite culture was tightly synchronized by D-sorbitol (Sigma) treatment as described below.

Five percent (5%) of sorbitol was freshly prepared by dissolving D-sorbitol in deionized water according to required proportions. The solution was then filter-sterilized using a 0.20µm filter (FiltoSpur S, Sarstedt). The sorbitol solution was then pre-warmed in a 37°C water bath for 20 minutes prior to use. The parasite culture was transferred into a 50ml falcon tube (Greiner Bio-One) and centrifuged at 440×g for 5 minutes at room temperature. The supernatant was then aspirated out and the pellet resuspended in ten volumes of 5% D-sorbitol solution. The mixture in a 50ml falcon tube was transferred into a 37°C water bath and incubated for 20 minutes with intermittent gentle shaking at least thrice during the incubation period. This was then subjected to centrifugation at 440×g 5 minutes at room temperature followed by two washes with 10mls of incomplete parasite culture media per wash at the same centrifugation conditions. The supernatant from the final wash step was aspirated out and the pellet resuspended in an appropriate volume of complete parasite culture media. A 10µl was taken to make a thin smear as previously described to check and ascertain the synchronization process. The culture was then transferred into new T75 culture flasks (BD), gassed for 30-60 seconds, and incubated at 37°C.

3.4.2 Processing of culture conditioned media (CCM)

The tightly synchronized culture as described above was bulked up until six flasks, each containing 500µl packed cell volume, at 7% parasitemia were obtained. The cultures were grown in 40 ml of complete parasite culture media (incomplete culture media as shown in Table 3 supplemented with 10% Albumax-II [Gibco] depleted of sEVs by ultracentrifugation at 150,000×*g* for 2 hours). The complete culture media added at an early ring stage was harvested after 24 hours when the parasites were in the trophozoite stage (herein referred to as the rings-to-trophozoite or the RT sample. Fresh culture media was then added at this stage and harvested after another 24 hours of incubation (herein referred to as the trophozoite-to-rings or the TR sample). This

was repeated until three sets of each culture-conditioned media (CCM) type (RT and TR) were obtained (Figure).



Highly synchronised ring stage culture at 7% parasitaemia was continuously monitored and CCM harvested after every 24 hours. Fresh culture media was replenished after every CCM harvesting point while freshly washed O^{+ve} RBCs were added at each trophozoite stage. Infected RBCs (iRBCs) were finally harvested via MACS as trophozoites. Mycoplasma testing was conducted throughout the culturing process.

Figure 3.1: *Plasmodium falciparum* parasite culture media harvesting.

In this study, the CCM from the RT sample was harvested as previously described (Abdi et al., 2017) and shown in Figure The parasite culture was transferred from the T75 culture flasks (BD) into a 50ml falcon tube and centrifuged at 360×g for 5 minutes to pellet down RBCs and the supernatant transferred to a new 50ml 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 50ml falcon tube. This was followed by centrifugation twice at 2,000×g for 10 minutes to remove dead cells and large cellular debris, then once at 3,600×g for 10 minutes to remove cellular membranes, hemozoin, and any other debris. The final centrifugation was at 15,000×g for 30 minutes to pellet down medium-sized extracellular vesicles (mEVs) or microvesicles (MVs).

The mEVs pellet was then resuspended in 1×phospate buffered saline (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 0.2µm filter (FiltoSpurS, Sarstedt) and stored at -80°C until use.

During the trophozoite stages, the cultures were appropriately diluted (Figure) with freshly washed, malaria-negative O^{+ve} red blood cells such that the culture was maintained at 7% parasitemia and at 500µl total pellet volume before being gassed and returned to the 37°C incubator.

3.4.3 Isolation of 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 (Table 3) and the supernatant aspirated out. Appropriate volume of glycerolyte was added to the pellet and the trophozoite samples stored at -80°C in small aliquots until use. Uninfected RBCs (uRBCs) that were cultured for CCM harvesting (as explained below) were washed twice in incomplete culture media (after CCM harvesting) and stored in appropriate volumes of glycerolyte in -80°C until use.

3.5 Culture of uninfected red blood cells

Uninfected red blood cells (uRBCs) were cultured in a similar way to the *Pf*iRBCs. A 500µl of packed cell volume of uninfected red blood cells were incubated with 40ml of complete parasite culture media and the media harvested after 24 hours of culture. The cell pellet was resuspended in 40ml of fresh complete culture media and incubated for another 24 hours. This was repeated three times leading to 3×6×40ml of CCM that were processed as detailed in section 0 and then stored at -80°C until use.

After harvesting the final CCM, the uninfected red blood cells were frozen in glycerolyte to use as a control for the *Pf*iRBCs. The same batch of O^{+ve} red blood cells was used for both *Pf*iRBCs and uRBCs' cultures. Both malaria parasite and uRBCs' cultures were tested for mycoplasma contamination throughout the culturing period as described in section 0 below.

3.6 Mycoplasma testing

Both *Pf*iRBCs and uRBCs cultures were regularly tested for mycoplasma contamination. Deoxyribonucleic acid (DNA) extraction from the parasite culture aliquots (200µl) was extracted with QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was then subjected to polymerase chain reaction (PCR) using conditions tabulated in Table and

Table 3.2. Positive controls used were DNA from *Mycoplasma pirum, Acholeplasma laidlawii* and DNA from two *P. falciparum* isolates with confirmed mycoplasma contamination.

Table 3.2: Mycoplasma PCR master-mix

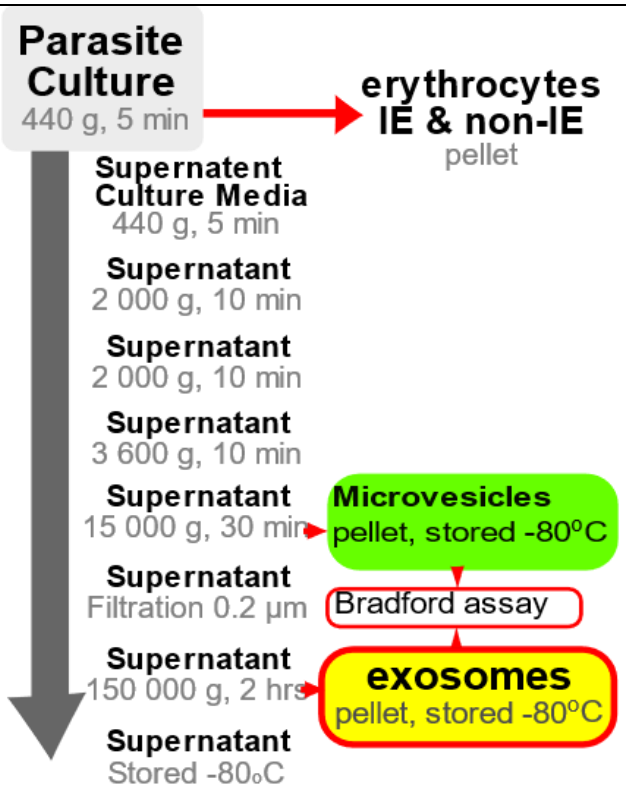
PCR reagent	**×1 reaction (µl)**
10× Ammonium buffer	2.5
50mM Magnesium chloride	0.75
40mM dNTPs	0.625
Primers (PCR 1 & PCR 2)	0.56
Taq™ polymerase	0.5
DNA template	1
PCR water	19
Total	25µl

Table 3.3: Mycoplasma thermocycling program

Stage	Temperature (°C)	Time
Hold	95°C	5 min
	95°C	30 sec
	58°C	40 sec
35 Cycles	72°C	1 min
Hold	4°C	5 min

3.7 Isolation of PfsEVs from culture-conditioned media

The 0.20µm-filtered CCM supernatant stored at -80°C was thawed at room temperature and transferred under sterile conditions into quick-seal ultracentrifuge tubes (Beckman Coulter cat# 343322). These were then ultracentrifuged using Optima XE-90 ultracentrifuge in a pre-cooled (4°C) 70.1Ti fixed-angle rotor (Beckman Coulter) at 150,000×*g* for 2 hours at 4°C (Figure). This approach is likely to enrich for exosomes, based on their size and the 0.20µm filter cutoff, but because there is no clear way to distinguish between exosomes and microvesicles, the resulting pelletted material is, henceforth, referred to as *Pf*sEVs. The pellet was then washed twice by resuspending in cold 1× PBS and ultracentrifuged at 150,000×*g* for 2 hours and 4°C between washes. The final *Pf*sEVs pellet was resuspended in 400µl of 1× PBS and stored in aliquots at -80°C until use (Figure). Bradford™ protein assay was used to determine protein concentration in the *Pf*sEVs following the manufacturer's instructions. The isolated *Pf*sEVs were also assessed for mycoplasma contamination. The processed uRBCs' CCM was subjected to a similar isolation process to obtain the equivalent of *Pf*sEVs from uninfected RBCs, i.e., uRBCs_sEVs. Bradford™ protein assay was also used to check for the protein concentration in the isolated uRBCs_sEVs.



Parasite culture was first centrifuged to remove erythrocytes/iRBCs and other cellular debris. The CCM was then sequentially centrifuged to obtain microvesicles and exosomes whose protein concentration was quantified via the Bradford assay and the EV pellets stored at -80˚C until use (Mwangi et al., 2020).

Figure 3.2: Isolation of EVs from malaria parasite culture media

3.8 Processing of PBMCs

3.8.1 PBMC isolation

Fresh heparinized whole blood (~20-30mls) was obtained from each consented adult donor (both malaria naïve and malaria exposed) for plasma and PBMC isolation. The blood was centrifuged at 440×g for 5 minutes at room temperature to remove plasma that was aspirated and 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 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 PBMCs' layer

between the Lymphoprep and media was carefully aspirated out into a 50ml falcon tube and washed twice in R0 by centrifuging at 440×g for 5 minutes and 4°C.

The isolated PBMCs were then resuspended in 1ml of complete culture media, R10 (Table). The cell count and viability were determined using a Vi-CELL XR 2.03 counter (Beckman Coulter, USA) and/or hemocytometer chamber. After the counting, the PBMCs were then either used fresh in the stimulation assay or resuspended in a cold freezing media (10% dimethyl sulfoxide [DMSO] in fetal calf serum [FSC]; all from Gibco) at a final concentration of 1×10^6 PBMCs/ml. The PBMCs were then transferred into Nunc cryovials (Thermo Scientific) and stored overnight at -80°C in Mr. Frosty cryocontainers (Thermo Scientific) before being transferred to liquid nitrogen until use in *in vitro* assays.

Table 3.4: Complete PBMCs culture medium (R10)

Ingredients	Volume added (ml)
L-Glutamine (200mM)	5.0
RPMI 1640	450
Penicillin-Streptomycin (100×)	5.0
FCS [heat-inactivated]	50

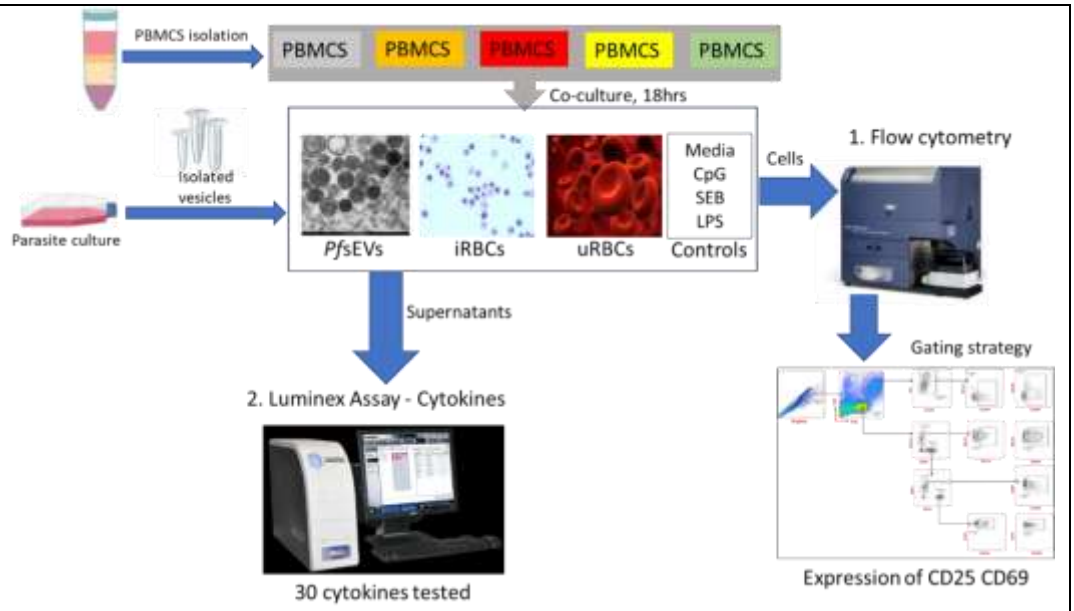
3.8.2 Thawing of cryopreserved PBMCs

Cryopreserved vials of PBMCs were taken out of liquid nitrogen and quickly thawed by immersing the vials in a 37°C water bath. Partially thawed PBMCs were then transferred into 50ml falcon tubes containing pre-warmed complete (R10) PBMCs culture media (Table). The cells were then washed twice with R10 at 360×g for 5 minutes at room temperature. The PBMCs were then resuspended in 1ml of R10 media and rested for 1 hour before being used in the assay. An aliquot was taken for cell counting (using Vi-CELL XR 2.03 counter (Beckman Coulter, USA) and/or hemocytometer) to determine the viability and the number of cells present per milliliter to be used. The PBMCs were finally resuspended in appropriate volume of R10 to a final volume of 1×10^6 PBMCs per experimental condition.

3.9 PBMC assays

3.9.1 PBMCs stimulation and measuring of cell surface activation markers

A 1×10^6 PBMCs per stimulation condition were co-cultured with *Pf*iRBCs or *Pf*sEVs 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 (Mantel et al., 2013), and 1×10^6 MACS-purified iRBCs (*Pf*iRBCs). The positive controls were *Staphylococcal* enterotoxin B (SEB) at 2.5µg/ml as a polyclonal activator and CpG-ODN (2.5µg/ml). Lipopolysaccharide (LPS) at 200ng/ml was included as an additional positive control for a subset of experiments that involved PBMCs from 12 donors. Co-culture wells with PBMCs plus cell growth medium only and PBMCs plus 1×10^6 uRBCs were included in each experiment as negative controls. These uRBCs were of the same batch as that used to culture the parasites. The PBMCs were co-incubated with the stimulants for 18 hours in a humidified incubator at 37ºC and 5% CO_2. Following the 18 hours' stimulation, the cells and culture supernatants were harvested. The supernatants were stored at -20°C for cytokine analysis via Luminex assay and/or enzyme-linked immunosorbent assay (ELISA) while the cells were prepared for flow cytometry (Figure).



Isolated PBMCs from different donors were co-cultured with varied stimulants for 18 hours. Cells and supernatants were harvested and used for flow cytometry and Luminex assays, respectively.

Figure 3.3: The stimulation experimental setup

In the flow cytometry analysis, a 200µl of RBC lysis buffer (BD™ FACS™ lysing solution) was first added to the iRBCs and uRBCs culture condition/wells and incubated on ice for 10 minutes. This step was key to avoid interference of RBCs during acquiring of cytometry events. This was followed by addition of 200µl of fraction of crystallization receptor (FcR) blocking reagent (Miltenyi Biotec) to avoid non-specific antibody binding to all the samples and incubated for 10 minutes at 4°C in the dark.

This incubation step was followed by two cell washes in cold fluorescence-activated cell sorting (FACS) buffer (1×PBS + 5% FCS + 0.01% Sodium azide) by centrifuging at 440×g for 5 minutes. The cells were then stained for 30 minutes at 4°C with 30µl of fluorescently-labelled 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 dilutions apart from BV711 anti-human CD25 that was used at a 1:100 dilutions. (Those antibodies without indicated concentrations have either lot-specific concentrations or pre-diluted for use at recommended volume per test).

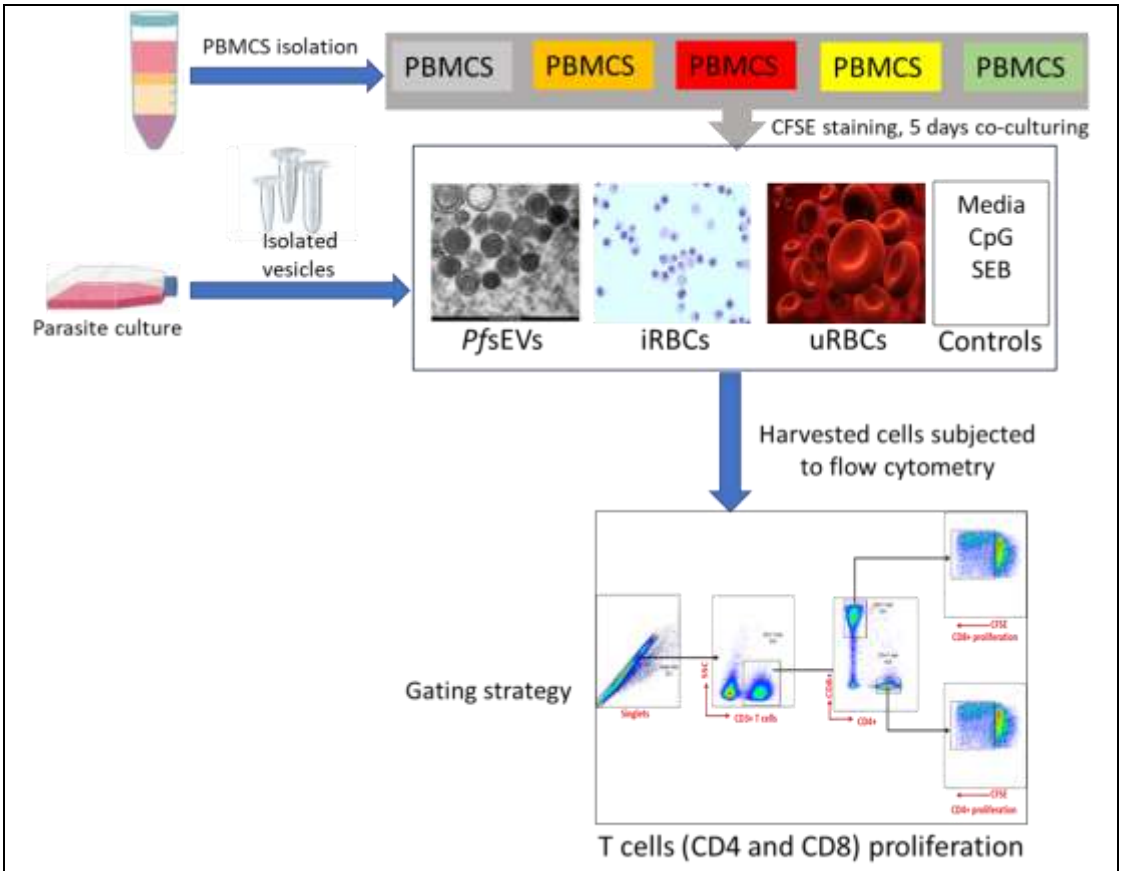
The staining step was followed by two washes with FACS buffer (by centrifuging at 440×g for 5 minutes) before being re-suspended in 300µl of FACS-flow buffer (BD Biosciences). Cells were acquired on the LSRFortessa™ cell analyzer (BD Biosciences). At least 100,000 events were acquired per stimulation 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).

3.9.2 PBMCs' proliferation assay – CFSE staining

Thawed PBMCs (as outlined in section 0 above) were first rested for an hour in R10 media before being labelled with Carboxyfluorescein diacetate succinimidyl ester (CSFE) [BioLegend] staining medium. Labelling entailed resuspension of 1×10^6 cells per culture condition in an appropriate volume of warm (pre-warmed in a water bath at 37°C) CSFE staining medium (1×PBS supplemented with 0.1% bovine serum albumin [BSA]) at a final CFSE concentration of 1.25µM, following the protocol by Quah (Quah et al., 2007). These cells in the CFSE staining media were incubated for 10 minutes at 37°C before being quenched with a five-times volume of ice-cold complete R10 followed by a further 5 minutes' incubation in ice. The cells were then washed twice (centrifugation at 440×g for 5 minutes) with R10 and resuspended in appropriate volume of the complete culture medium, R10.

The CFSE-labelled PBMCs were subsequently incubated in 96-well U-shaped culture plates (Greiner Bio-One) at a density of 1×10^6 PBMCs per culture condition. The culture conditions were *Pf*sEVs (20µg/ml, as used in section 0), 1×10^6 MACS-purified iRBCs (*Pf*iRBCs), CpG-ODN (2.5µg/ml), and SEB (2.5µg/ml) as a positive control. Co-culture wells with (PBMCs + cell culture medium only) and (PBMCs + 1×10^6 uRBCs) were included in each experiment as negative controls. These uRBCs were of the same batch as that used to culture the parasites. The stimulated CFSE-stained PBMCs were cultured for 5 days in a humidified incubator at 37°C and 5% CO_2 (Figure).

After 5 days of co-culture, an aliquot from each culture condition was taken and mixed with trypan blue dye (Sigma) to assess the cells' viability under a light microscope. The cells were then washed once (centrifugation at 440×g for 5 minutes) in FACS buffer before being stained with 30µl of an antibody cocktail containing: PE-Cy5-conjugated anti-CD3, PE-Cy7-conjugated anti-CD4, PE-Texas Red-conjugated anti-CD8, Fluorescein isothiocyanate (FITC) anti-CFSE, and Fixable Viability Dye eFluor® 780 to gate on the live cells only. The stained cells were then incubated for 30 minutes at 4°C. After incubation, the cells were afterwards washed twice with FACS buffer and resuspended in 300 µl of FACS flow (BD Biosciences). At least 100,000 events/culture condition were acquired (~170,000 for uRBCs and iRBCs conditions) using the BD LSRFortessa™ cell analyzer and DIVA software (BD Biosciences, USA). Data were analyzed using FlowJo® software version 10.0 (Tree Star).



Isolated PBMCs from different donors were stained with CFSE and co-cultured with varied stimulants for 5 days. The cells were then harvested and used for flow cytometry to check for T cells' (CD4 and CD8) proliferation.

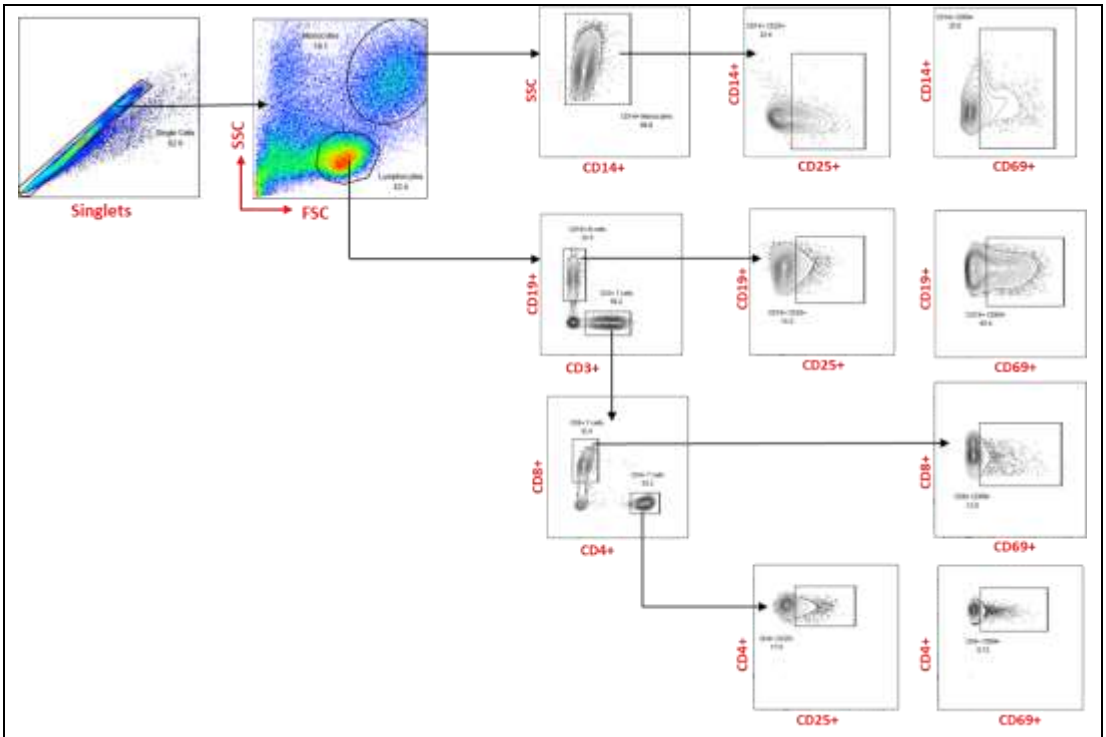
Figure 3.4: The proliferation experimental setup.

3.10 The gating strategies

3.10.1 The PBMC stimulation data

FlowJo software version 10.0 (Tree Star) was used to analyze the FCS files obtained from the DIVA software in the LSRFortessa™ flow cytometer (BD Biosciences). A gating strategy was applied to locate the cells of interest (Figure). Dead cells were first removed using the Fixable Viability Dye followed by gating on live single cells (singlets). From the lymphocytes gate, B and T cells were gated based on the expression of CD19 and CD3, respectively. The T-cell subsets ($CD4^+$ and $CD8^+$) were then gated from the $CD3^+$ gate. The monocyte gate was confirmed based on

CD14 expression. The expression of the activation markers CD25 and CD69 was then determined in the T, B, and monocyte cells (Figure).

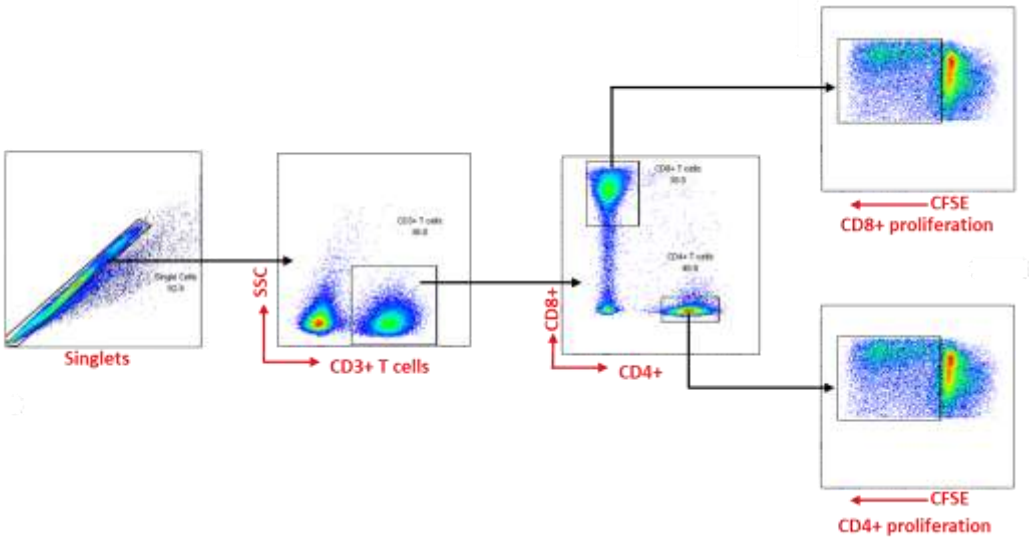


Single cells are gated in from the live cells followed by gating on lymphocytes and monocytes. The lymphocytes are then gated on either B or T cells. The monocytes, B cells and T cells are finally gated depending on the expression of CD25 and CD69 (Mwangi et al., 2020).

Figure 3.5: The stimulation assay gating strategy

3.10.2 The PBMC proliferation data

The proliferation assay gating strategy was like that of the stimulation assay. Dead cells were first removed using the Fixable Viability Dye followed by gating on live single cells (singlets). $CD3^+$ T cells gated in from the singlets were then gated to both $CD4^+$ and $CD8^+$ T cell subsets. The proliferation of each subset was then measured by gating on the reducing CSFE intensity that occurs after every cell cycle (Figure 3.6).



Single cells are gated in from the live cells followed by gating on $CD3^+$ T cells. The $CD3^+$ T cells are then gated to both $CD4^+$ and $CD8^+$ T cells subsets. The T cell subsets are finally gated for proliferation using CSFE.

Figure 3.6: The proliferation assay gating strategy

3.11 ELISA & Luminex assays to characterize stimulation cytokine profiles

Cell free culture supernatants from the stimulation assays were aspirated and stored at -20^{0}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 25µl of the culture supernatant was diluted 1:5 times in assay medium and incubated with 25µl 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 25µl 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 150µL 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. A 100µl of both samples and standards were added into an anti-human TGFβ-precoated plate and incubated for 2 hours at room temperature (RT) with shaking. The plate was washed five times with provided wash buffer using ELx405 Select (BioTek instruments) ELISA washer. A 100µl of biotin-conjugate was then added to all the wells and incubated for 1 hour at RT with shaking. This was followed by five washes, addition of 100µl Streptavidin-HRP (Horse radish peroxidase), and incubation for 1 hour at RT with shaking. The plate was washed five times as in the preceding steps followed by addition of 100µl TMB (tetramethyl-benzidine) substrate solution and incubated for 30 minutes at RT. The reaction was stopped by adding 100µl of stop solution (1M phosphoric acid). The plate was read at 450nm using Synergy 4 (BioTek Instruments) ELISA plate reader, and optical densities (OD) of each sample recorded.

3.12 Anti-schizont antibody ELISA

To determine the levels of plasma IgG responses to crude schizont extract of the PBMC donors, an anti-schizont antibody ELISA was performed. 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 100µl 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 five washes between each step (wash buffer: 1×PBS + 0.05% Tween-20). This was followed by addition of 100µl of the 1:1000 diluted plasma

samples and an overnight incubation. On the following day, the plates were washed four times and then incubated with 100 µl of HRP-conjugated Rabbit anti-human IgG (Thermo Scientific) for 3 hours at room temperature. The washed plates were finally incubated with 100µl of the o-Phenylenediamine dihydrochloride (OPD) substrate for 15 minutes. The reaction was stopped with 25µl of 2M H_2SO_4 and the plates read at 492nm on a Synergy 4 (Bio Tek) plate reader, recording the samples' optical densities (OD).

3.13 Data analysis

The flow cytometry data was collated using FlowJo® Software (Tree Star) v.10.6.2. The resultant collated flow cytometry data and ELISA data were then analysed 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 culture medium (media) was used as the background negative control condition for both *Pf*sEVs and *Pf*iRBCs. For *Pf*iRBCs, relative comparison to uRBCs condition was also shown. To assess 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 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.

CHAPTER FOUR

RESULTS

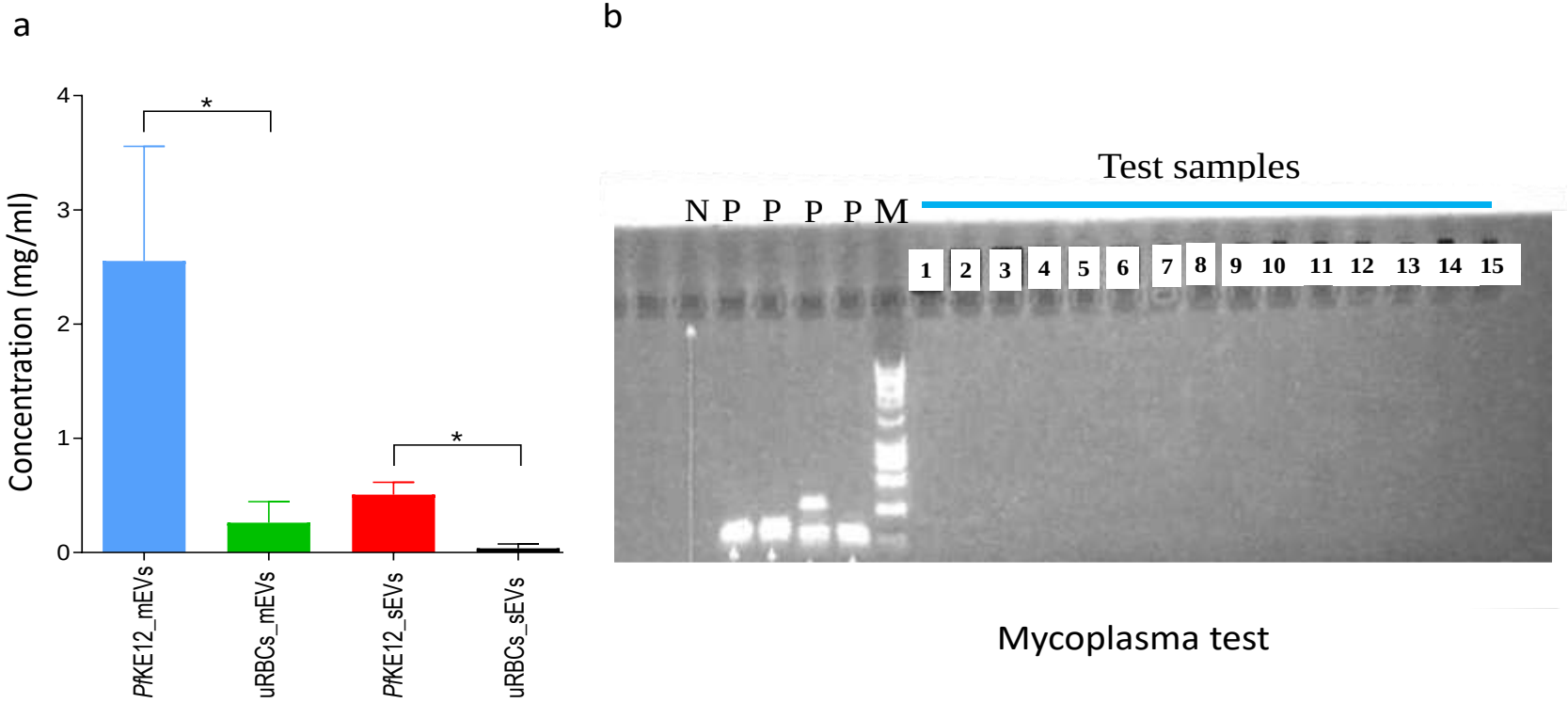
4.1 Comparison of the quantities of small-sized extracellular vesicles (sEVs) released by infected RBCs and uninfected RBCs

The mEVs and sEVs were isolated from culture conditioned media (CCM) of *Pf*iRBCs from a laboratory-adapted clinical isolate (*Pf*KE12) and uRBCs as shown in **Error! Reference source not found.**a. Previous research has demonstrated successful isolation of sEVs from *Pf*iRBCs CCM by Transmission Electron Microscope (TEM) (Abdi et al., 2017) using the protocol described in methods section. In this study, the protein concentrations of the isolated mEVs and sEVs were used as proxies for EV abundance (Théry et al., 2018). As shown in **Error! Reference source not found.**a, the mean protein concentration of mEVs from *Pf*iRBCs CCM (*Pf*KE12_mEVs) was ~4.9 fold higher than that of uRBCs (uRBCs_mEVs) ($p = 0.033$), as would be expected when parasite proteins are packaged in the mEVs.

The mean protein concentration of the sEVs fraction from fresh or aged uRBCs CCM was negligible (**Error! Reference source not found.**a) (p =ns), consistent with previous reports that show mature RBCs primarily release mEVs (microvesicles) but not sEVs (exosomes) (Kuo et al., 2017).

4.2 Test for parasite culture and vesicles contamination

During the parasite culturing, mycoplasma contamination was routinely monitored via PCR method. The cultures remained negative for mycoplasma contamination throughout the culturing period (**Error! Reference source not found.**b; test samples 7-15). To rule out the possibility of low-level Mycoplasma contamination not detectable by PCR analysis in parasite culture samples but might be enriched in the isolated *Pf*sEVs-DNA was extracted from the isolated *Pf*sEVs and PCR analysis performed to detect mycoplasma contamination, which was negative as shown in figure 4.1 (test samples 1-6).



a) The bar graph shows the crude protein concentration of the EVs (both small and medium extracellular vesicles) from uninfected RBCs (uRBCs) and the parasite isolate *Pf*KE12 using Bradford assay. Each bar represents data from at least three biological replicates.

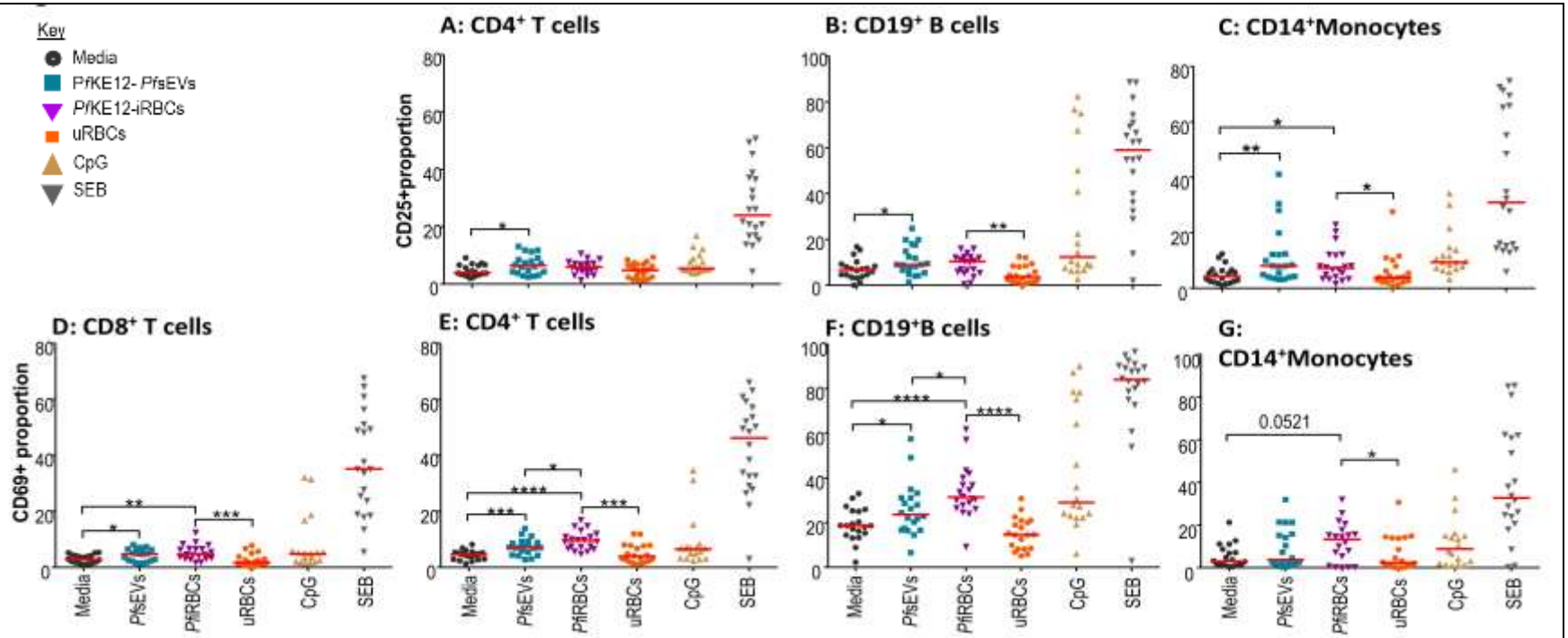
b) Gel electrophoresis image after PCR to test for Mycoplasma contamination in *P. falciparum* cultures and isolated *Pf*sEVs. N-Negative control, P-Positive control, M-Molecular marker, 1-15 are test samples (1-6 are DNA extracted from isolated *Pf*sEVs and 7-15 are DNA from the parasite cultures). [*P-value = 0.01-0.05; the error bars indicate the mean with standard deviation. P-value was calculated using Mann-Whitney U test].

Figure 4.1: EVs protein concentrations and mycoplasma results

4.2 Effects of PfiRBCs and PfsEVs on T cells, B cells and monocytes

*Pf*sEVs induced significantly higher expression of CD25 on $CD4^+$ T cells, $CD19^+$ B cells and $CD14^+$ compared to the background media condition [p =0.0467, =0.0369, and =0.0048, respectively] (Figure A-C). In contrast, the effect of *Pf*iRBCs was apparent only on the antigen presenting cells, $CD19^+$ B-cells [p =0.0042] and $CD14^+$monocytes [p =0.0155] (Figure B&C) with a significantly higher CD25 expression relative to the background media condition or to uRBCs [p =0.0304].

Both *Pf*sEVs and *Pf*iRBCs induced a higher expression of CD69 on $CD4^+$ [p =0.005 and p <0.0001 respectively), $CD8^+$ [p =0.0424 and p =0.0013, respectively] and $CD19^+$ [p =0.0437 and p <0.0001, respectively] relative to the background condition (Figure D-F). Additionally, *Pf*iRBCs induced significant CD69 expression on $CD14^+$ cells (Figure G) relative to the uRBCs [p =0.0345]. Notably, *Pf*iRBCs induced a higher CD69 expression on $CD4^+$ and $CD19^+$ cells relative to that induced by *Pf*sEVs (Figure 4.2).

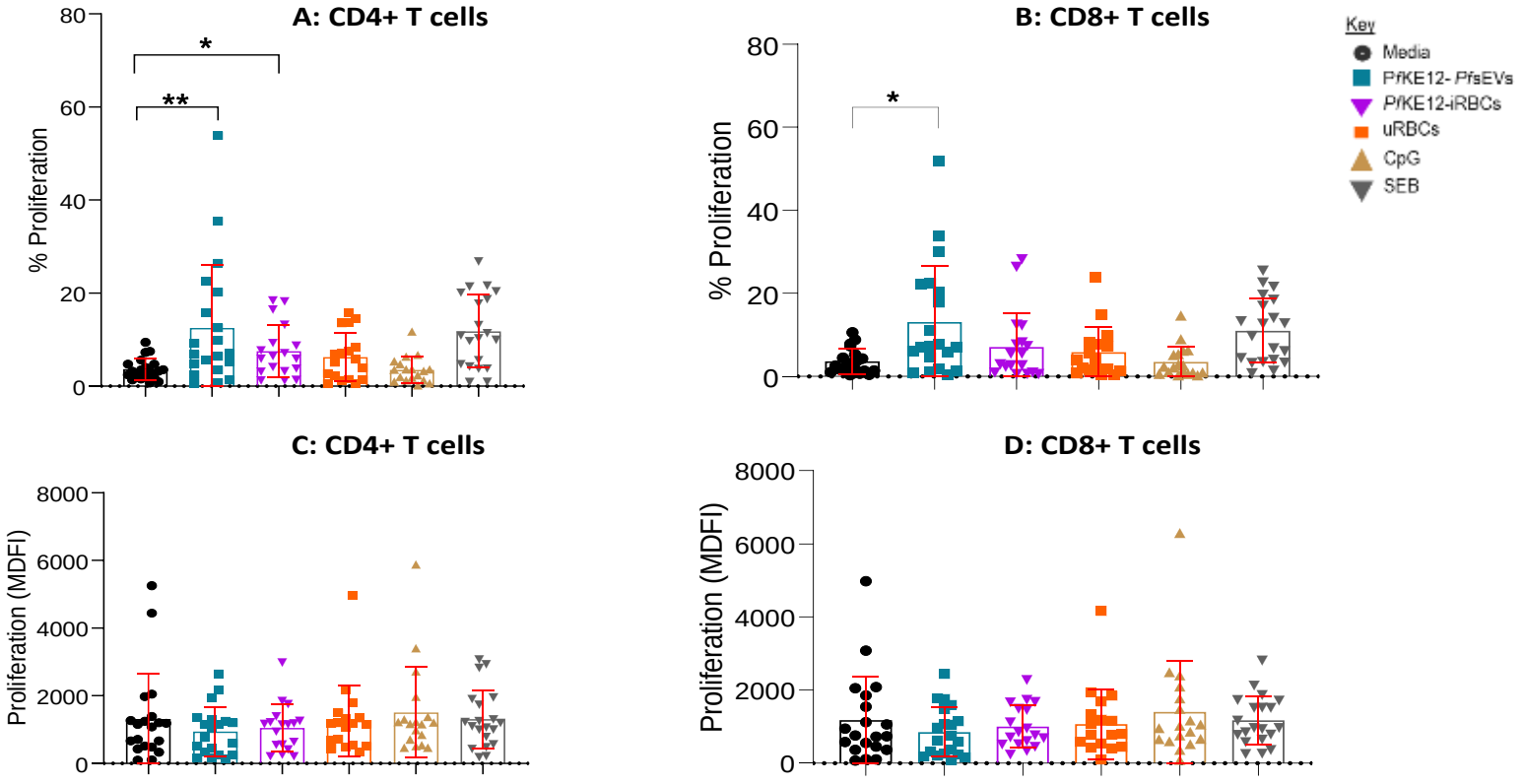


Proportions of $CD4^+$ T-cells, $CD8^+$ T-cells, $CD19^+$ B-cells, and $CD14^+$ Monocytes expressing the activation markers CD25 (upper panel) and CD69 (lower panel) following PBMC co-culture with *Pf*sEVs or *Pf*iRBCs. Unstimulated PBMC (media) and uRBCs were included as negative control conditions while CpG and SEB were included as positive control conditions. Both *Pf*sEVs and *Pf*iRBCs conditions were compared to the background media condition. *Pf*iRBCs 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 indicate the median. P-value was calculated using Mann-Whitney U-test).

Figure 4.2: Expression of CD25 and CD69 under different stimulation conditions

4.3 Impact of PfsEVs and PfiRBCs on T cell proliferation

The second objective of the current study was to assess if *Pf*sEVs and *Pf*iRBCs had any impact on the proliferation of T cells. From the results, *Pf*sEVs induced significantly more T cells proliferation above the background (media/unstimulated) (Figure A&B) as well as above the iRBCs, though not statistically significant for the iRBCs. On the other hand, the iRBCs induced a significantly higher proliferation in $CD4^+$ T cells compared to the background (Figure A) but not in $CD8^+$ T cells (Figure B). However, this observation was lost when proliferation was analysed depending on the median fluorescent intensity (MDFI) (Figure C&D) rather than the percentage number of cells that proliferated (Figure 4.3).



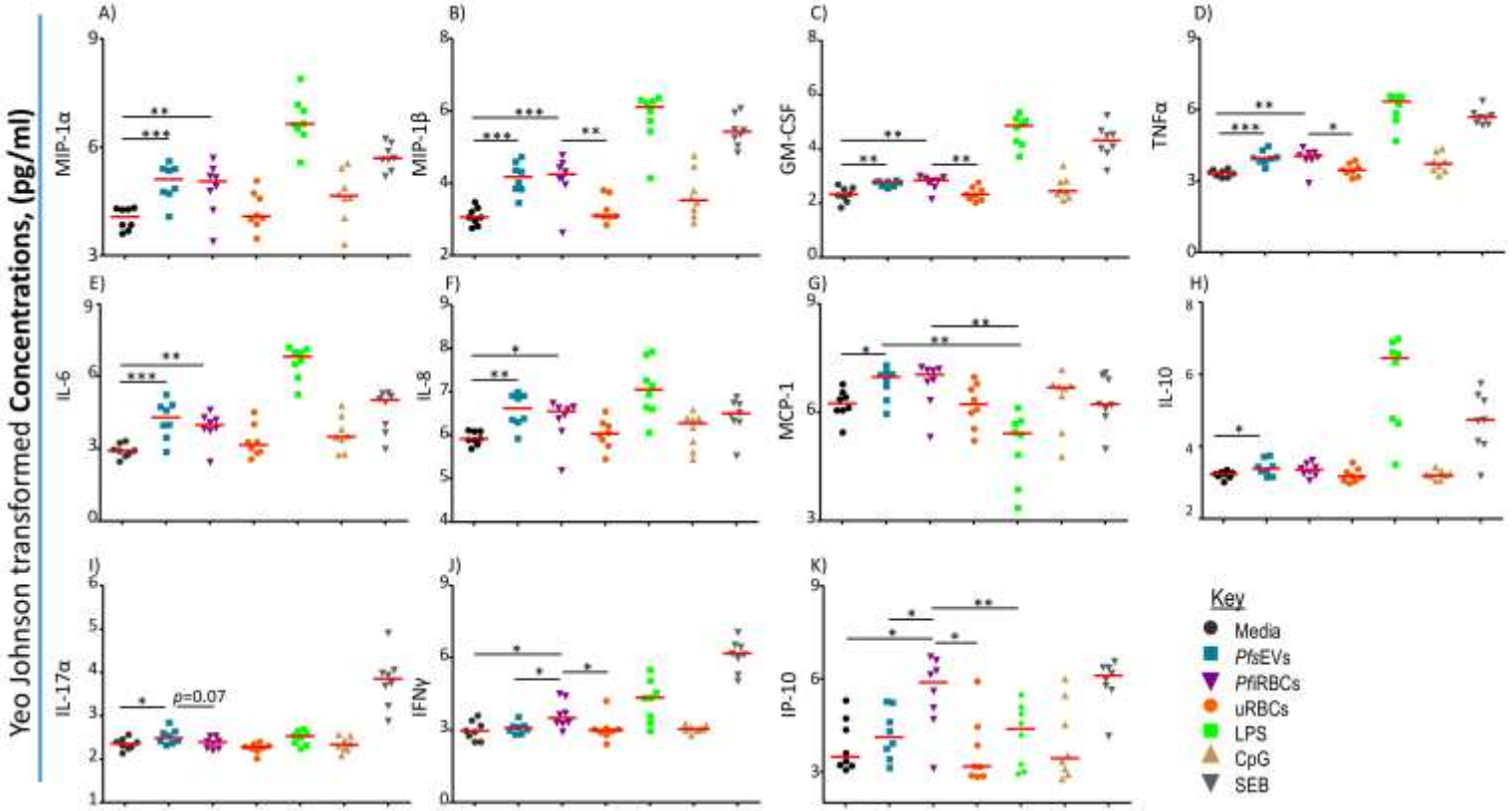
A and B show percentage proliferation while C and D show the median fluorescent intensity (MDFI) of the proliferating cells. *P-value = 0.01-0.05 and **P-value = 0.001-0.0. The red bars indicate mean with standard deviation. P-value was calculated using Mann-Whitney U-test.

Figure 4.3: T cell proliferation

4.4 The cytokine and chemokine profile of PBMC stimulated by PfsEVs and PfiRBCs

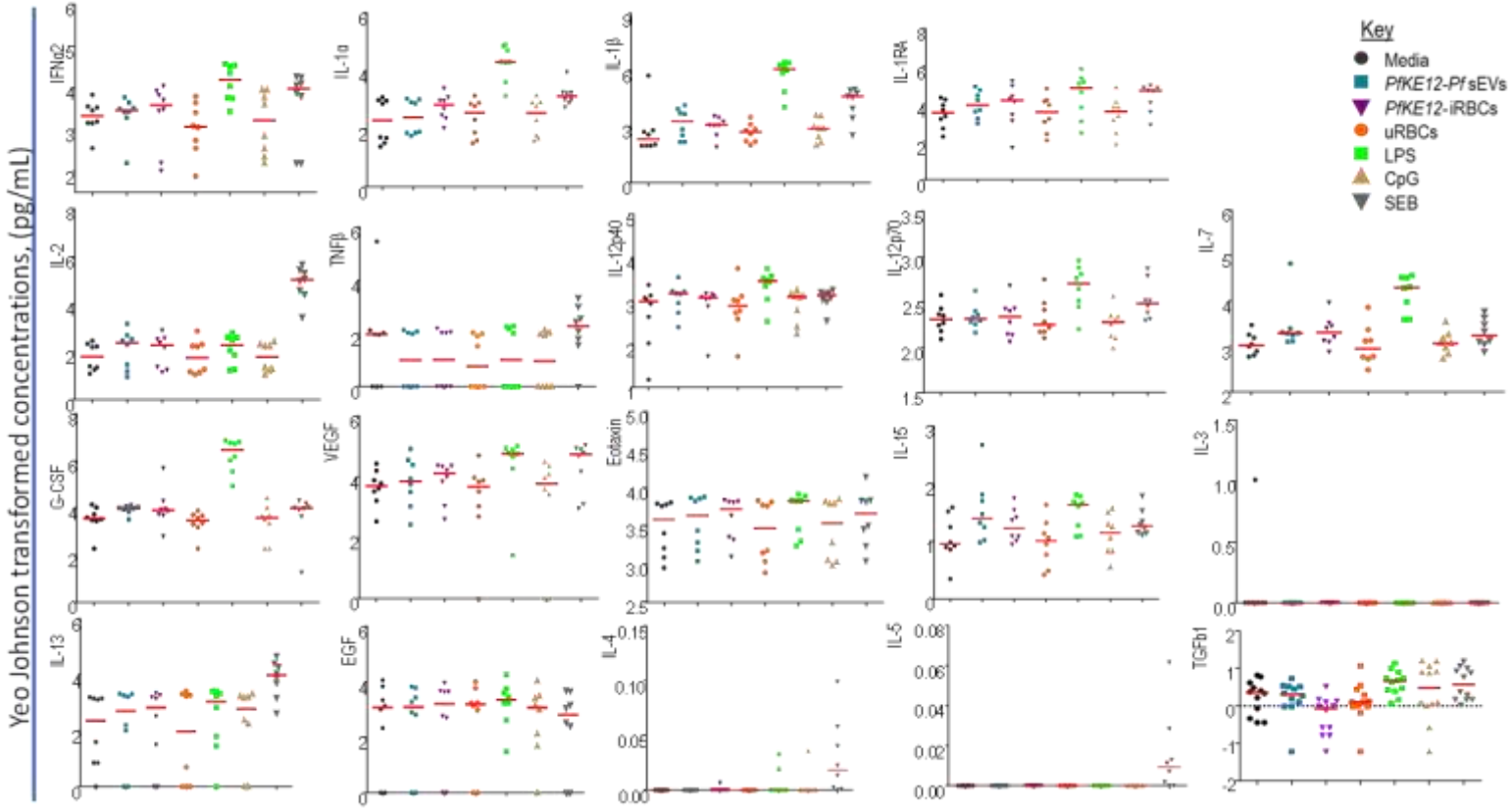
The third objective of the study intended to determine the cytokine profile of the stimulated PBMC. For (eight) 8 out of the twenty (20) PBMC samples, the levels of 29 different cytokines/chemokines in the culture supernatant induced by each of the stimulants were measured using Luminex. TGF-β was additionally measured by ELISA. IL-3, IL-4, and IL-5 levels were below the limit of detection with both *Pf*sEVs and *Pf*iRBCs (Figure). 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 (Figure).

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 MIP1**α**, MIP1β, GM-CSF, IL-6, IL-8, and TNF**α** (Figure A-F), b) *Pf*sEVs only for MCP1, IL-10 and IL-17**α** (Figure G-I) and c) *Pf*iRBCs only for IFN**γ** and IP-10 (Figure J-L). Notably, the levels of IFN**γ** and IP-10 induced by *Pf*iRBCs were significantly higher than the levels induced by *Pf*sEVs (Figure J-K) while the concentration of IL-17**α** induced by *Pf*sEVs tended to be higher than that induced by *Pf*iRBCs ($p=0.07$, Figure I). 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 G). Additionally, the level of IP-10 induced by *Pf*iRBCs was also significantly higher than that induced by LPS (Figure K).



The cytokine/chemokine levels released upon PBMCs' stimulation was only determined 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

Figure 4.4: Significantly induced cytokines/chemokines

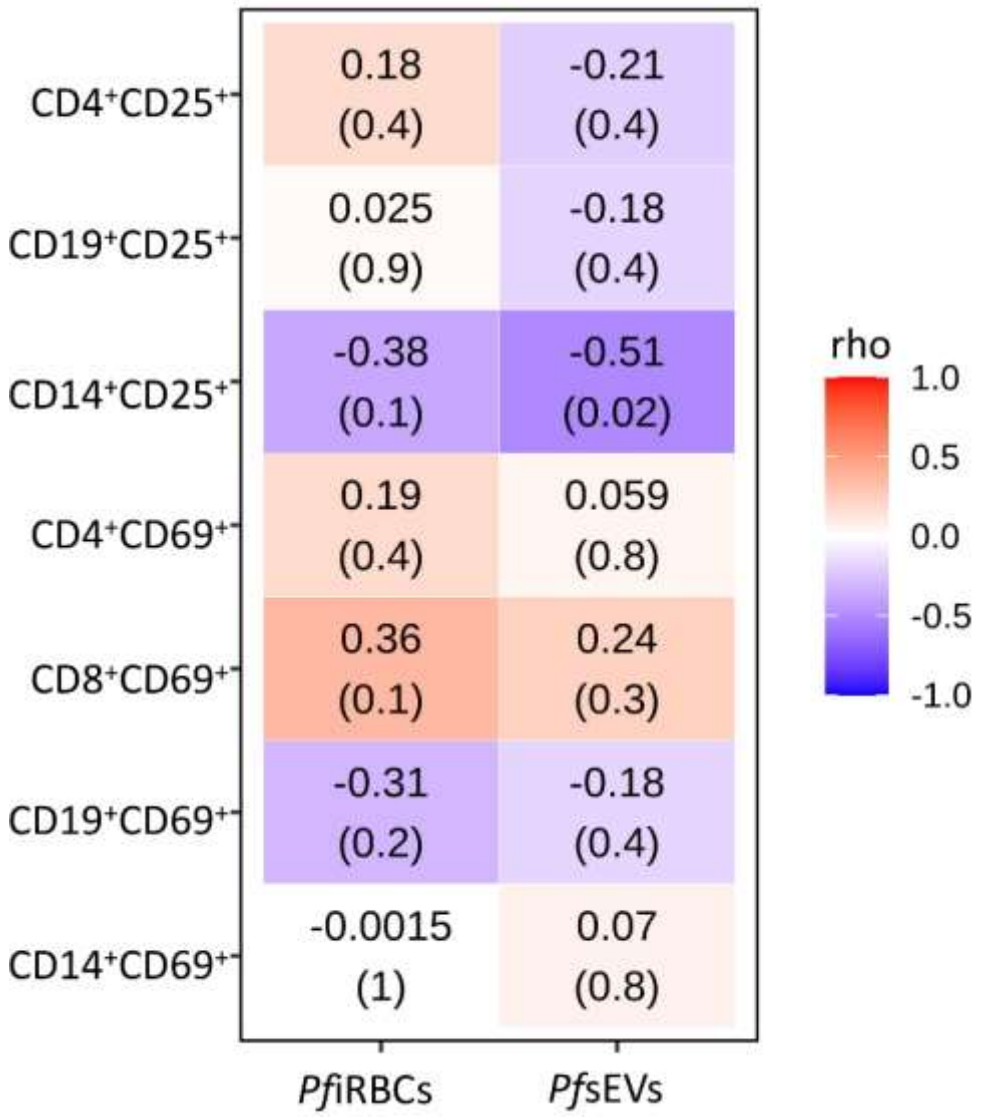


Nineteen out of the thirty cytokines/chemokines that were tested upon stimulation of PBMCs, showed insignificant levels and/or below detection levels such as seen in TGFb1.

Figure 4.5: Cytokines/chemokines with insignificant levels of induction

4.5 Previous exposure to malaria in responses to PfsEVs stimulation

Given that malaria exposure has been shown to induce immunological tolerance (Bediako et al., 2016; Illingworth et al., 2013; Langhorne et al., 2008), a correlation of the cell surface activation data to each donor's IgG response to crude schizont extract was done using Spearman's rank correlation. As shown in Figure, 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).



The 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.

Figure 4.6: The impact of prior malaria exposure on PBMC response to *Pf*iRBCs and *Pf*sEVs

CHAPTER FIVE

DISCUSSION

5.1 Discussion

This study sought to investigate 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) if secreted *Pf*sEVs and *Pf*iRBCs induce proliferation on T cells and in addition 4) whether prior exposure to malaria among the PBMC donors influences the level of the induced PBMC activation markers.

Results indicate 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. In proliferation, the *Pf*sEVs had more impact compared to the infected iRBCs in both T cell subsets while the *Pf*iRBCs were noted to only impact proliferation in $CD4^+$ T cells relative to the background. This relationship, however, changed if we considered the proliferation signal rather than the number of proliferating cells. More experiments are needed to make conclusive remarks regarding *Pf*sEVs considering it has previously been reported that continued exposure to malaria infection negatively impacts (lowers) T cell proliferation (Bediako et al., 2016) in experiments involving infected erythrocytes. This current study could not make substantive measurements with *Pf*sEVs or measure any proliferative cytokines.

At the cytokine/chemokine level, both *Pf*sEVs and *Pf*iRBCs 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-17**α** relative to the background media and tended to be higher than that induced by *Pf*iRBCs. IL-17**α** 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* (Bettelli et al., 2006). Interestingly, *Pf*sEVs also induced significantly higher levels of IL-6 but not TGF-β. On the other hand, *Pf*iRBCs showed superior ability to induce secretion of IFN**γ** and IP-10 relative to the background uRBCs and even to *Pf*sEVs condition when co-cultured with PBMC. IP-10 secretion is driven by a pro-inflammatory cytokine milieu including IFN-γ (Luster & Ravetch, 1987); 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.

This, therefore, maybe interpreted that *Pf*sEVs and *Pf*iRBCs can induce differentiation of CD4+ T cells into Th17 and Th1 cells, respectively. In a recent study (Ye et al., 2018), *Pf*iRBCs were shown to induce NK cells to secrete IFN**γ** while *Pf*mEVs could not, which is consistent with the observation made in this study with *Pf*sEVs & *Pf*iRBCs co-culture with PBMC. This finding warrants further characterization of the T cell subsets activated by *Pf*sEVs including **γ**δ T-cells known to be activated by phosphoantigens in *P. falciparum* culture medium (Guenot et al., 2015).

*Pf*iRBCs were observed to induce significantly higher MCP-1(CCL2) and IP-10 (CXCL10) compared to LPS when co-cultured with PBMC. This result is consistent with observation previously made with co-culturing of *Pf*iRBCs with purified dendritic cells (Götz et al., 2017). However, in the previous study (Götz et al., 2017), co-culture of *Pf*iRBCs with purified dendritic cells could not induce secretion of inflammatory cytokines such as TNF-**α** and IFN-**γ** (Götz et al., 2017). 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 has been shown to tolerizes T and B cell response to malaria antigens (Bediako et al., 2016; Illingworth et al., 2013). This study showed, albeit with 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. *Pf*sEV interaction with monocytes *in vitro*, have been shown to induce inflammatory response (Mantel et al., 2016) potentially contributing to malaria pathogenesis. Therefore, tolerance to *Pf*sEVs following frequent malaria infection may be part of the naturally acquired anti-disease immunity (Langhorne et al., 2008).

The results showed that uninfected red blood cells (uRBCs) do not produce sEVs (exosomes) containing quantifiable amount of proteins using Bradford assay. During the isolations, culture conditioned media (CCM) used varied from uRBCs ranging from fresh (processed for culture within 1 hour after phlebotomy) to 2 weeks old cultures. By contrast, the mEVs 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 but could alternatively mean that uRBCs primarily release mEVs (microvesicles). The latter interpretation is consistent with previous studies that showed developing red blood cells release sEVs during earlier stage of hematopoiesis, but mature RBCs do not (Kuo et al., 2017), as they have lost the endocytic pathway that is essential for the biogenesis of sEVs. Other studies have described exosomes from uRBCs (Sisquella et al., 2017) but this discrepancy might be explained by a difference in methodology as the study protocol involves a filtration step at 0.2µm that excludes the majority of mEVs from the sEVs fraction, a step that was omitted from the earlier reported work.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study set out to find the role(s) that secreted vesicles from *Pf*iRBCs played in the host immune system. The study employed in vitro methods to culture the malaria parasite, harvest the CCM, isolate small EVs from the CCM and test their effects via co-culture with PBMC from varied donors. Results indicate that isolated *Pf*sEVs as well as *Pf*iRBCs had varied abilities to induce activation markers on different subsets of PBMCs, proliferate and induce secretion of varied cytokines.

The study also hinted that prior exposure to malaria affects the ability of *Pf*sEVs to activate monocytes, suggesting a role of the EVs in immune tolerance. These results, however, do not conclusively and exhaustively explain severe malaria disease. Though that, they are a pointer to the potential role that malaria parasite secretomes (for example, *Pf*sEVs in this study) have in driving this phenomenon. Consequently, to unravel the happenings behind severe malaria, more experiments with varied PBMC donors are required.

Altogether, this study is a pointer that *Pf*sEVs have the potential to give more information, not only in cellular assays but also in proteomics and transcriptomics analyses. This is a study of its kind as it successfully used a clinical isolate parasite adapted to lab culture with <70 culture cycles, hence, a closer reference to a human *P. falciparum* malaria parasite that the more widely used 3D7 reference isolate.

6.2 Recommendations

This study showed the ability of *Pf*sEVs from a malaria clinical isolate adapted to lab culture to induce varied outcome on PBMCs. Though that, additional work is needed to complement the results reported in this thesis.

- While PBMC from multiple donors were used to generate these results, this study used only one *P. falciparum* isolate and, therefore, cannot conclusively determine if the results obtained will remain similar if the number of isolates was increased for diversity. Therefore, there is need for more work with an increased number of parasite isolates including a lab isolate such as 3D7 as a control.
- Apart from conducting a similar experiment with an increased number of parasite isolates, varied isolates need also to be included in the expanded study. This is because 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. It would therefore be interesting to see if the phenomena observed will be 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.
- This study also recommends an increase in the number of study participants to increase the study power while drawing conclusions from the stimulation, proliferation, and cytokine assays.
- Finally, more functional, proteomic, and transcriptomic analysis of *Pf*sEVs is clearly needed to fully understand them and ultimately apply the knowledge in malaria vaccine design.

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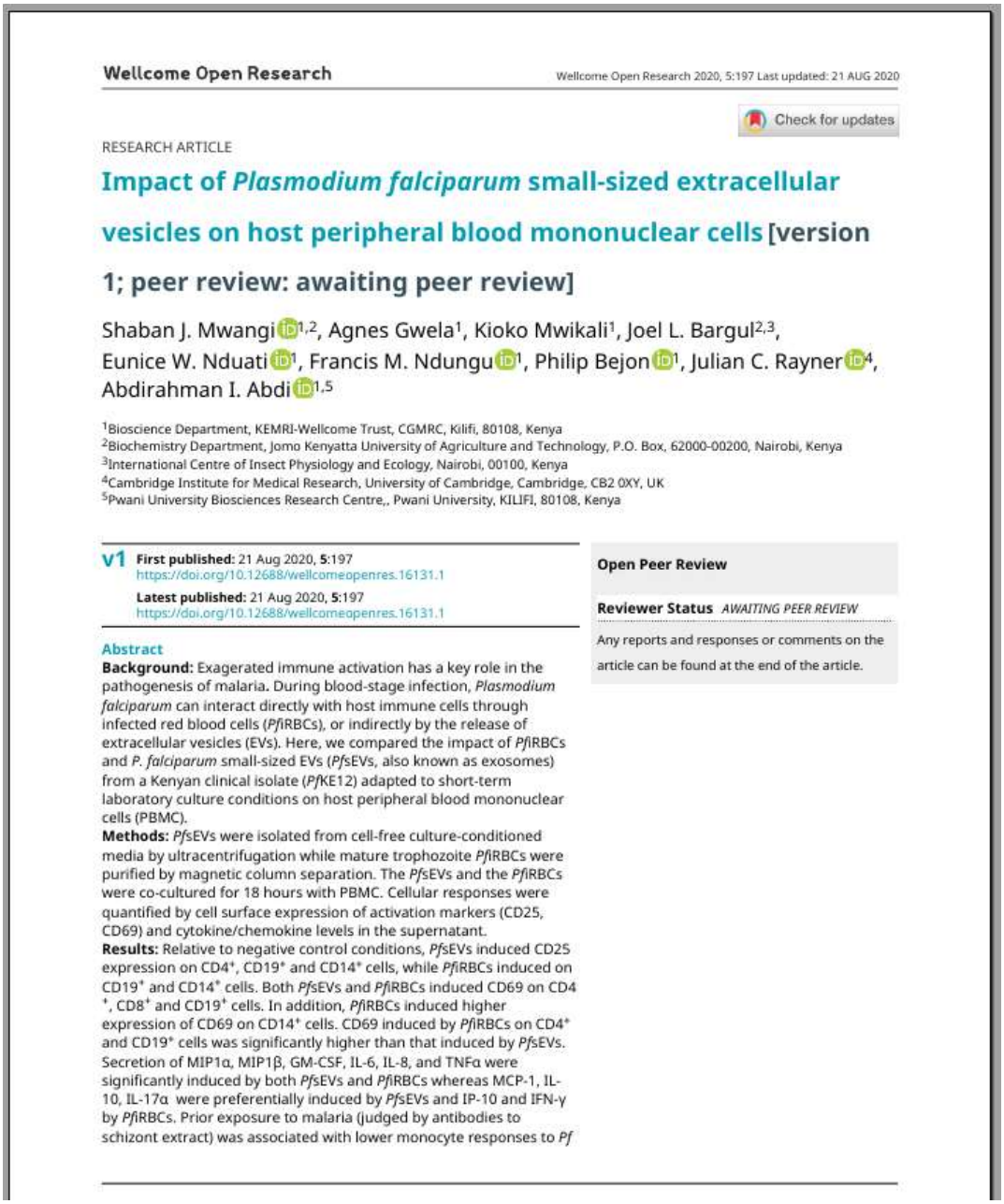
APPENDICES

Appendix I: Accepted research article for peer review



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RESEARCH ARTICLE

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

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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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schizont extract) was associated with lower monocyte responses to *Pf* 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

Sydney, Sydney, Australia

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REVISED **Amendments from Version 1**

Additional text and references was added in response to the reviewers comments. Everything else remain the same as the originally submitted version.

Any further responses from the reviewers can be found at the end of the article

Introduction

Plasmodium falciparum causes almost 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[25] as well as in inducing sexual commitment[16,17]. Extracellular vesicles reflect the molecular phenotype of the cells releasing them[17]. 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[16,26] and have been shown to induce inflammation[16] and endothelial activation[27] and its plasma circulating level to increase with severity of the infection[28]. However, to date, all studies on the impact of *Plasmodium* EVs on host immune response have been performed using either rodent malaria[29–31] or using long-term laboratory-adapted *P. falciparum* isolates[16,32]. 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[26] 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[33] and was adapted to *in vitro* culture (<70 cycles) and used in previous studies[34,35]. Isolate 9215 was renamed to *Pf*KE12 in unpublished genome data[36].

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

*Pf*KE12 was grown under standard culturing conditions[37]. 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 [sigma] + L-glutamine [2mM] + Hepes [37.5mM] + D-glucose [0.2%w/v] + gentamicin [25µg/ml]+ sodium-hypoxanthine [50µg/ml]) (all from Gibco) supplemented with 0.25% 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[26] 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 cultured in six flasks each containing 500µl packed cells and 40ml culture

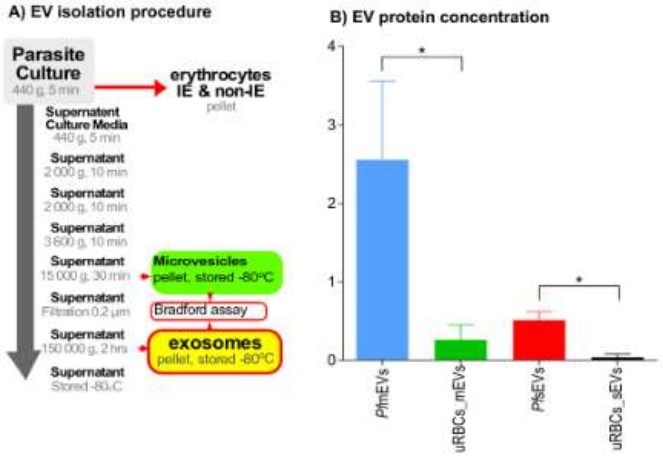

Figure 1. EV isolation procedure and the protein concentration of the isolated EVs. (A) Schematics showing the procedure for EV isolation from culture conditioned media. (B) The bar graphs show the crude protein concentration of medium EVs (mEVs) and small EVs(sEVs) isolated from *P. falciparum (Pf)* 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.

media at 37°C in 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) 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[2mm] and Penicillin/Streptomycin [1U/ml], 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 transformed using Yeo Johnson transformation[38] for the data to conform to normal distribution 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 *Pfs*EVs and *Pfi*RBCs. For *Pfi*RBCs, relative comprison to uRBCs condition was also shown. To test the impact of prior malaria exposure on PBMC response to *Pfs*EVs and *Pfi*RBCs 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 *Pfs*EVs or *Pfi*RBCs. 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 *Pfi*RBCs from a Kenyan clinical isolate (*Pf*KE12) and uRBCs as shown in Figure 1a. We have previously demonstrated successful isolation of sEVs from *Pfi*RBCs CCM by transmission electron microscopy[26] 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[9]. As shown in Figure 1B, the mean protein concentration of mEVs from *Pfi*RBCs CCM (*Pfm*EVs) was ~4.9-fold higher than that of uRBCs (uRBCs-mEVs), perhaps as result of a combination of the additional parasite-derived proteins packaged into the mEVs on top of those derived from the host and increased rate of mEVs release due to the infection.

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)[39].

Both *Pfi*RBCs and *Pfs*EVs 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 *Pfi*RBCs and *Pfs*EVs 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 (6 from high and 6 from low/no malaria transmission residents), 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 *Pfs*EVs 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[40] shows the FlowJo gating strategy to enumerate different cell populations. Raw flow cytometry files are available as *Underlying data*[40].

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

To exclude the possibility of low-level Mycoplasma contamination that may be enriched during *Pfs*EVs isolation, *Pfs*EV-DNA extract was used in a PCR-based test. No contamination was detected (*Extended data*, Figure S2)[40].

Pfs EVs and *Pfi*RBCs induced common and unique cytokine/chemokine profile

For 8 out of the 20 PBMC samples (4 from high and 4 from low/no malaria transmission residents), 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 *Pfs*EVs and *Pfi*RBCs (*Extended data*, Figure S3)[40]. The levels of 16 cytokines/chemokines induced by *Pfs*EVs and by *Pfi*RBCs 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 *Pfs*EVs and *Pfi*RBCs were significantly higher relative to the background conditions (*Extended data*, Figure S3)[40]. Output ELISA files are available as *Underlying data*[40].

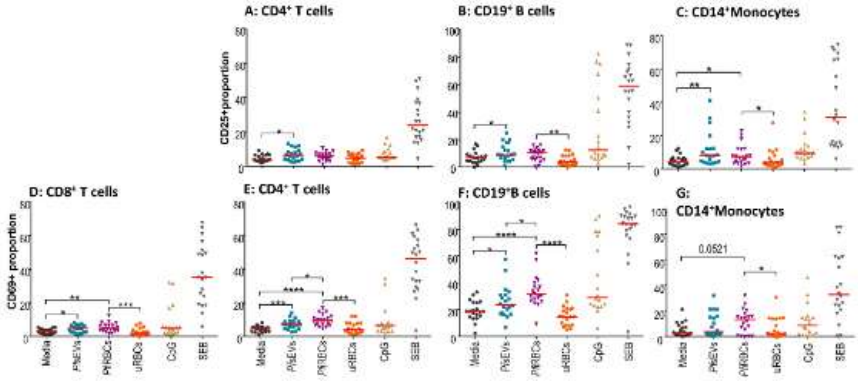

Figure 2. Proportion of T cells, B-cells and monocytes expressing the activation markers CD25 and CD69 under the different stimulation conditions. (**A–G**) Proportions of $CD4^+$ T-cells, $CD8^+$ T-cells, $CD19^+$ B-cells, and $CD14^+$ Monocytes expressing the activation markers CD25 (upper panel) and CD69 (lower panel) following PBMC co-culture with *Pf*sEVs or *Pf*RBCs. unstimulated PBMC (media) and uRBCs were included as negative control conditions while CpG and SEB were included as positive control conditions. Both *Pf*sEVs and *Pf*RBCs conditions was compared to the background media condition. *Pf*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).

Of the remaining 11 cytokines/chemokines, significant induction relative to the negative control background was observed with; a) both *Pf*sEVs and *Pf*RBCs for MIP1α, MIP1β, GM-CSF, IL-6, IL-8, and TNF-α (Figure 3A–F), b) *Pf*sEVs only for MCP1, IL-10 and IL-17α (Figure 3G–I) and c) *Pf*RBCs only for IFN-γ and IP-10 (Figure 3J–L). Notably, the levels of IFNγ and IP-10 induced by *Pf*RBCs 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 by *Pf*RBCs (p=0.07, Figure 3I). Interestingly, in contrast to most cytokines/chemokines, the level of MCP-1 induced by both *Pf*RBCs and *Pf*sEVs was uniquely higher than that induced by LPS (Figure 3G). Additionally, the level of IP-10 induced by *Pf*RBCs was also significantly higher than that induced by LPS (Figure 3K). Overall, both *Pf*sEVs and *Pf*RBCs were able to induce various chemokines and cytokines but *Pf*sEVs seems to be better in inducing secretion of IL-17α while *Pf*RBCs was better in inducing IFN-γ 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[40–43], 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*RBCs 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*RBCs; 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*RBCs 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*RBCs preferentially induced CD69 expression, particularly on B-cells. At the cytokine/chemokine level, both induced secretion of several cytokines/chemokines

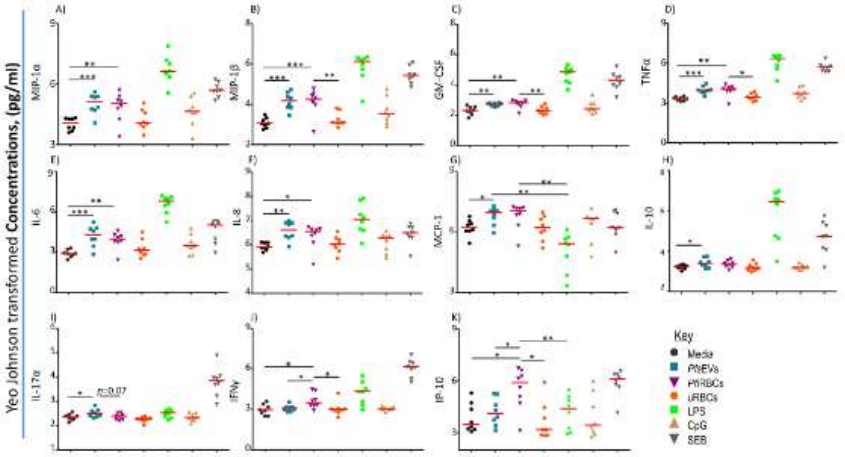

Figure 3. Cytokines/chemokines significantly induced after PBMC co-culture with *Pfs*EVs or *Pfi*RBCs. Cytokine/chemokine level was determined only in the supernants of 8 out of the 20 PBMC samples co-cultured with the different stimulants. The 8 samples were part of the 12 samples where LPS was included 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.

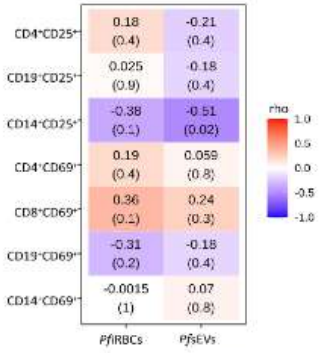

Figure 4. The impact of prior malaria exposure on PBMC response to *Pfi*RBCs and *Pfs*EVs. Correlations between anti-schizont IgG response in the plasma of each PBMC donor and the expression of the activation markers (CD25 and CD69) on CD4+ and CD8+ T cells, CD19+ B cells and CD14+ monocytes, following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

but they also showed differential ability to induce secretion of some cytokines. Notably, *Pfs*EVs induced secretion of higher levels of IL-17α relative to the background media and tended to be higher than that induced by *Pfi*RBCs (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*[44]. Interestingly, *Pfs*EVs also induced significantly higher levels of IL-6 (Figure 3E) but not TGF-β (Extended *data*, Figure S3)[45]. On the other hand, *Pfi*RBCs showed superior ability to induce secretion of IFN-γ and IP-10 relative to the background uRBCs and even to *Pfs*EVs condition when co-cultured with PBMC (Figure 3J–K. IP-10 secretion is driven by a pro-inflammatory cytokine milieu including IFN-γ[45]; thus, it is plausible that high levels of IFN-γ led to the IP-10 secretion that we identified in response to *Pfi*RBCs stimulation.

Hence our interpretation is that *Pfs*EVs and *Pfi*RBCs can induce differentiation of CD4+ T cells into Th17 and Th1 cells respectively. In a recent study[46], *Pfi*RBCs were shown to induce NK cells to secrete IFN-γ while *Pfm*EVs could not, which is consistent with the observation made in this study with *Pfs*EVs and *Pfi*RBCs co-cultured with PBMC. This finding warrants further characterization of the T cell subsets activated by *Pfs*EVs, including γδ T-cells known to be activated by phosphoantigens in *P. falciparum* culture medium[47].

We observed *Pfi*RBCs 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 *Pfi*RBCs with purified dendritic cells[48]. However, in the previous study[48], co-culture of *Pfi*RBCs with purified dendritic cells could not induce secretion of inflammatory cytokines such as TNF-α and IFN-γ[48]. 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[42,43]. 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 *Pfs*EVs (Figure 4). *Pfs*EV interaction with monocytes *in vitro* have been shown to induce inflammatory response[22], potentially contributing to malaria pathogenesis. Therefore, tolerance to *Pfs*EVs following frequent malaria infection may be part of the naturally acquired anti-disease immunity[43].

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 and this is consistent with the findings of multiple studies[26,49]. 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 previous studies that showed developing red blood cells release sEVs during earlier stage of haematopoiesis, but mature RBCs do not[50,51] as they have lost the endocytic pathway that is essential for the biogenesis of sEVs. Despite our view, favoring the possibility of uRBCs not releasing sEVs, we cannot rule out small amount of sEVs released by uRBCs especially reticulocytes contributing to the overall impact of *PfsEVs* on PBMCs which we did not control for.

Our study has several limitations;1) lack of microscopy or NanoSight evidence demonstrating successful isolation of sEVs in according to ISEV2018 recommendation[8]. However, we have previously[26] isolated EVs whose size and density match those of sEVs using the method used in this study and also validated *P. falciparum* protein marker that can be targeted for western blot analysis does not exist currently, 2) 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, 3) *Pfs*EVs impact on PBMC *in vivo* might be different from that observed under *in vitro* experiment since *Pfs*EVs are expected to interact with multiple host cells including endothelial cells leading to a more complex interaction and outcome. 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 (*Pfs*EVs) on host peripheral blood mononuclear cells. https://doi.org/10.7910/DVN/QXUIQ7[40].

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/QXUIQ7[40].

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 *Pfs*EVs or *Pfi*RBCs .

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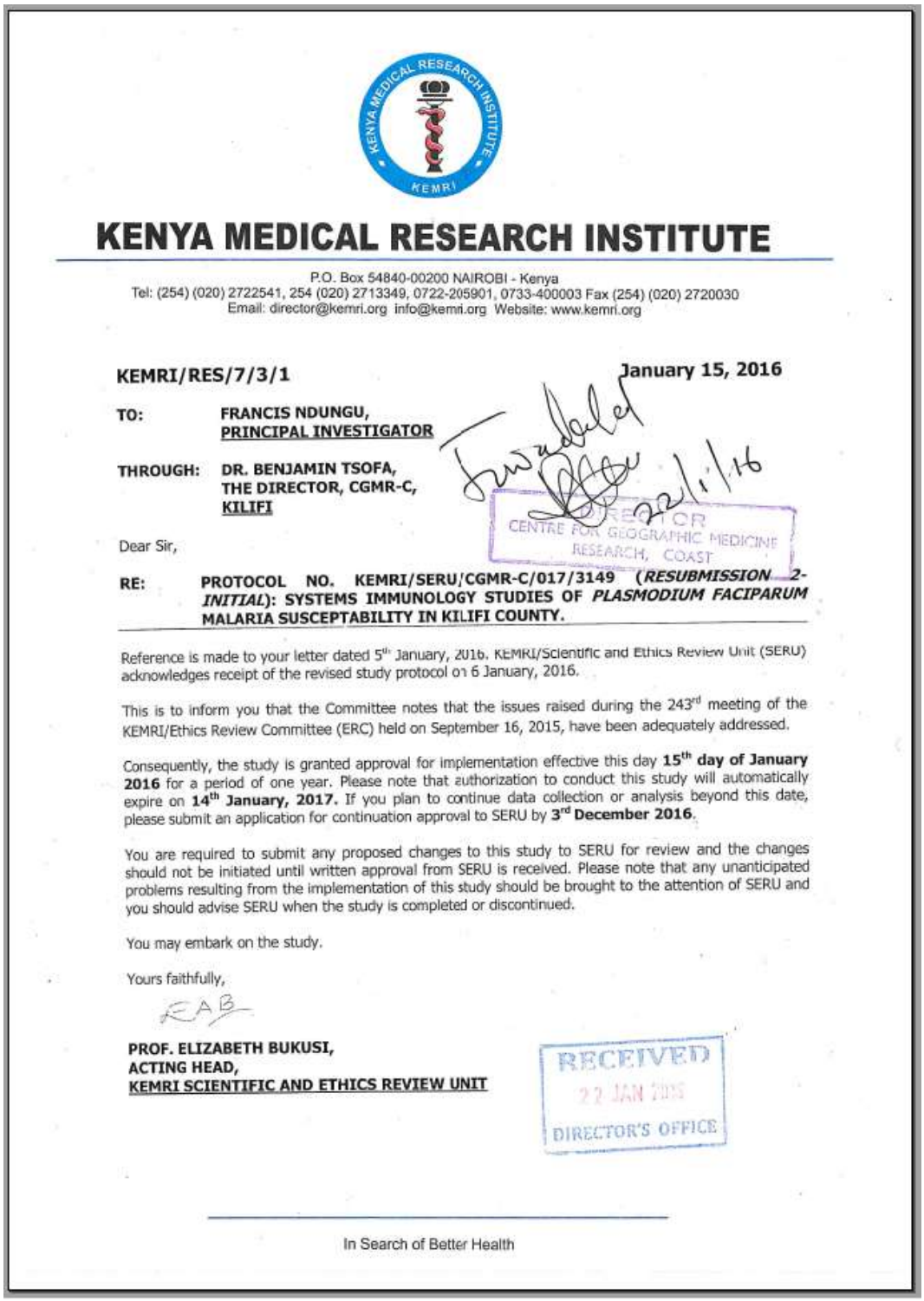
Appendix II: Initial ethics review approval from KEMRI/SERU



KENYA MEDICAL RESEARCH INSTITUTE

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Email: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1 **January 15, 2016**

TO: **FRANCIS NDUNGU,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. BENJAMIN TSOFA,**
THE DIRECTOR, CGMR-C,
KILIFI

DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE RESEARCH, COAST

Dear Sir,

RE: **PROTOCOL NO. KEMRI/SERU/CGMR-C/017/3149 (*RESUBMISSION 2-INITIAL*): SYSTEMS IMMUNOLOGY STUDIES OF *PLASMODIUM FACIPARUM* MALARIA SUSCEPTABILITY IN KILIFI COUNTY.**

Reference is made to your letter dated 5th January, 2016. KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study protocol on 6 January, 2016.

This is to inform you that the Committee notes that the issues raised during the 243rd meeting of the KEMRI/Ethics Review Committee (ERC) held on September 16, 2015, have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day **15th day of January 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **14th January, 2017.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **3rd December 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

RECEIVED
22 JAN 2016
DIRECTOR'S OFFICE

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Appendix III: Updated ethics review approval from KEMRI/SERU

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E-mail: director@kemri.org, info@kemri.org, Website.www.kemri.org

KEMRI/RES/7/3/1 February 27, 2019

TO: **FRANCIS NDUNGU,**
PRINCIPAL INVESTIGATOR,

THROUGH: **THE DIRECTOR, CGMR-C,**
KILIFI.

Received: 7/3/19

DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE RESEARCH, COAST

Dear Sir,

RE: **KEMRI/SERU/CGMR-C/022/3149 (*REQUEST FOR ANNUAL RENEWAL AND PROTOCOL DEVIATION*): SYSTEMS IMMUNOLOGY STUDIES OF *PLASMODIUM FACIPARUM* MALARIA SUSCEPTIBILITY IN KILIFI COUNTY**

Thank you for the continuing review report for the period February 16, 2018 to February 05, 2019.

The Committee noted that a protocol deviation form has been submitted as the request for annual renewal was done after the date of submission required. Measures taken to address deviation are adequate.

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **March 15, 2019** through to **March 14, 2020**. Please note that authorization to conduct this study will automatically expire on **March 14, 2020**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **January 31, 2020.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

Yours faithfully,

ENOCK KEBENEI
ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

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Appendix IV: Funding Statement

‘This work was supported through the DELTAS Africa Initiative [DEL-15-003]. The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)’s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa’s Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust [107769/Z/10/Z] and the UK government. The views expressed in this thesis are those of the author and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government’.