ASSESSMENT OF PHAGOCYTOSIS AND CYTOKINE SECRETIONS BY MONOCYTES IN THE PRESENCE OF *Plasmodium falciparum*

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percentage
<	Less than
°C	Degree Celsius
µL	Microlitre
µm	Micrometre
α	Alpha
β	Beta
γ	Gamma
APC	Antigen presenting cells
BAFF	B-cell activating factor
CCL	Chemokine (C-C motif) ligand
CCM	Complete culture media
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CM	Cerebral Malaria
CMP	Common myeloid progenitor
CO_2	Carbon dioxide
CR	Complement receptor
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alii* - 'and others'

FcγR	Fc gamma receptor
g	gram
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-monocyte progenitor
GPI	Glycosylphosphatidylinositol
x *g*	Gravity
H_2O	Water
HLA-DR	Human leukocyte antigen DR isotype
HO-1	Heme Oxygenase-1
HSC	Hematopoietic stem cells
ICAM	Intercellular adhesion molecule
ICCM	Incomplete culture media
IFN	Interferons
Ig	Immunoglobulin
IL	Interleukin
iRBC	Infected red blood cell
IUGR	Intrauterine growth restriction
LPS	Lipopolysaccharide
LSM	Lymphocyte separation medium
M	Molar
MACS	Magnetic-activated cell sorting
MDP	Macrophage/dendritic cell progenitor
MEP	Megakaryocyte-erythrocyte progenitor
mg	Milligram
MHC	Major histocompatibility complex

MIP-1β	Macrophage inflammatory protein-1 beta
mL	Millilitre
MSP	Macrophage stimulating protein
N_2	Nitrogen
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK cells	Natural killer cells
NO	Nitric oxide
O_2	Oxygen
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
P. falciparum	*Plasmodium falciparum*
PfEMP-1	*P. falciparum* erythrocyte membrane protein-1
pg	Picogram
pH	Power of hydrogen
PM	Placental malaria
PRR	Pattern Recognition Receptor
RBCs	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SMA	Severe malarial anemia
TGF-β	Transforming growth factor beta
Th1	T helper 1

TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
uRBCs	Uninfected red blood cells
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

PENILAIAN FAGOSITOSIS DAN PENGHASILAN SITOKIN OLEH MONOSIT DENGAN KEHADIRAN *Plasmodium falciparum*

ABSTRAK

Malaria merupakan salah satu penyakit yang paling biasa menjangkiti manusia di seluruh dunia. Di kawasan endemik, malaria merupakan penyebab utama morbiditi dan kematian serta mengakibatkan beban sosioekonomi yang besar kepada mereka yang dijangkiti penyakit ini. Monosit adalah sebahagian daripada sistem imun untuk mengawal beban parasit dan melindungi pesakit daripada jangkitan malaria. Monosit memainkan peranan dengan melindungi terhadap malaria melalui fagositosis, pengeluaran sitokin dan persembahan antigen. Walaupun monosit sangat penting untuk mengawal jangkitan malaria, monosit juga terbukti boleh menyebabkan hasil klinikal yang buruk. Objektif kajian ini adalah untuk menentukan morfologi *P. falciparum*, menilai keupayaan fagosit monosit manusia terhadap sel darah merah yang dijangkiti oleh parasit dan mengukur lebih lanjut penghasilan sitokin oleh monosit setelah proses fagositosis dengan menggunakan ELISA. Dalam kajian ini, monosit diasingkan daripada darah yang dikumpulkan daripada individu yang sihat manakala *Plasmodium falciparum* (3D7) dikultur dalam keadaan optimum. Aktiviti fagositosis dan penghasilan sitokin oleh monosit kemudian dinilai secara *in vitro* setelah kultur sel monosit dan sel darah merah dijangkiti *P. falciparum* selama 1 dan 2 jam. Kajian ini telah menunjukkan bahawa monosit melakukan fagositosis terhadap sel darah merah yang dijangkiti *P. falciparum* dan indeks fagositosis meningkat dengan masa inkubasi yang lebih lama iaitu 8.2% pada waktu inkubasi 1 jam menjadi 10.4% ($p < 0.05$) pada waktu inkubasi 2 jam. Selepas fagositosis, sel-sel

ini menghasilkan TNF-α, memulakan tindak balas imun semula jadi untuk membantu pengawalan parasit. Data yang diperolehi menunjukkan bahawa kultur monosit merembeskan tahap tertinggi TNF-α selama 0 dan 1 jam inkubasi, sementara kultur monosit dan sel darah merah yang dijangkiti *P. falciparum* menghasilkan TNF-α yang tertinggi setelah 2 jam inkubasi. Perbandingan trend antara tiga kumpulan, kesemua set menunjukkan peningkatan tahap TNF-α pada jam pertama, tetapi kepekatannya menurun dengan ketara pada jam kedua. Sebagai kesimpulan, hasil kajian ini menunjukkan bahawa monosit memainkan peranan penting dalam jangkitan malaria dengan melakukan fagositosis parasit dan menghasilkan TNF-α, seterusnya memulakan tindak balas imun untuk pembasmian malaria.

ASSESSMENT OF PHAGOCYTOSIS AND CYTOKINE SECRETIONS BY MONOCYTES IN THE PRESENCE OF *Plasmodium falciparum*

ABSTRACT

Malaria remains one of the most common human infections worldwide. In endemic areas, malaria is a leading cause of morbidity and mortality and it causes significant socioeconomic burdens to the affected people. Monocytes are part of the immune system to control parasite burden and to protect host against malaria infection. Monocytes play their protective roles against malaria via phagocytosis, cytokine production and antigen presentation. Though monocytes are crucial for clearance of malaria infection, they also have been shown to cause adverse clinical outcomes. The objective of this study was to determine the morphology of *P. falciparum*, to assess phagocytic capability of infected red blood cells by human monocytes and further measure the cytokine secretions of monocytes following phagocytosis by using ELISA. In this study, monocytes were isolated from whole blood collected from healthy individuals while *Plasmodium falciparum* (3D7) was cultured under optimal conditions. Phagocytotic activity and cytokine production by the monocytes following malaria infection were assessed *in vitro* by co-culturing the monocytes and *P. falciparum*-infected red blood cells for 1 and 2 hours. The present study demonstrated that the monocytes phagocytosed the *P. falciparum*-infected red blood cells and the phagocytosis index increased with longer incubation time, from 8.2% at 1 hour incubation time to 10.4% ($p<0.05$) at 2 hours incubation time. Following phagocytosis, the monocytes produced TNF-α, initiating innate immune response to help in the clearance of parasite. The data have shown that monocytes cultured alone expressed the highest level of TNF-α during 0 and 1 hour of

incubation, while co-culture of monocytes with *P. falciparum*-infected red blood cells produced the highest level of TNF-α after 2 hours of incubation. Comparing the trend among monocyte control, parasite control and co-culture, all showed an increase in the level of TNF-α produced in the first hour, but the concentration decreased significantly in the second hour. As a conclusion, these findings suggest that monocytes play an important role in malaria infection by phagocytosing the parasites and producing TNF-α for the removal of parasites, thereby initiating an immune response for malaria eradication.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Malaria is one of the most common mosquito-borne diseases worldwide. In 2019, an estimated 229 million cases of malaria were reported worldwide with approximately 409,000 number of deaths. Children less than the age of five are the most vulnerable population, contributing for 67% of all malaria deaths worldwide in 2019 (CDC - Centers for Disease Control and Prevention, 2021). In addition to children, pregnant women and travellers or foreign workers are at a higher risk of being affected by malaria. Malaria affects the poor disproportionately with higher morbidity and mortality due to lack of access to effective treatment. Almost 60% of malaria deaths worldwide occur in the poorest 20% of the population (WHO, 2019). According to the World Health Organization (WHO), Africa accounted for 94% of malaria cases and deaths in 2019. Malaria also imposes a major financial and social burden on many regions of the world (WHO, 2021).

Malaria is caused by a small protozoon belonging to the *Plasmodium* species, which comprises of many subspecies. *Plasmodium* is an intracellular amoeboid parasite that accumulates malaria pigment (an insoluble metabolite of haemoglobin). Malaria is a life-threatening infectious disease spread by bites of infected female *Anopheles* mosquitoes. There are 172 *Plasmodium* species, however only five *Plasmodium* species that can cause malaria in humans, which are *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Sato, 2021). *P. falciparum* is the most common and the

most lethal malaria infection worldwide, being responsible for the majority of malaria-related deaths, especially in tropical and sub-tropical regions (Zekar and Sharman, 2021). Patients with severe *P. falciparum* malaria may have liver and kidney failure, convulsions, and coma. This infection has an adverse effect on the brain and central nervous system, and the patient can have cerebral malaria.

During malaria infection, the host immune response is generally classified into two arms, innate and adaptive immunity. Innate immunity aids in the control of parasitemia during the acute phase of infection and the initiation of adaptive immunity. While *Plasmodium*-specific antibodies are implicated in the clearance and resolution of the chronic phase of malaria infection. Notably, during early stages of malaria infection, innate immunity which includes monocytes and other immune cells is vital to limit the growth rate of parasites (Mandala *et al*., 2016). Monocytes perform three key functions which are phagocytosis, antigen presentation, and cytokine production (Ortega-Pajares and Rogerson, 2018). When triggered by antigens, monocytes can establish first line of defense immediately and trigger the adaptive immune response by secreting chemokines and cytokines that recruit and activate other immune cells. In response to parasite intake, monocytes phagocytose infected red blood cells (iRBCs) (Turrini *et al*., 1992) and produce both pro-inflammatory and anti-inflammatory cytokines, which may aid in parasite clearance as well as limiting inflammation (Nagao *et al*., 1996). Circulating human monocytes exist as different subsets which can be distinguished by their expression of CD14 (the co-receptor for Toll-like receptor 4 (TLR4) recognition of bacterial lipopolysaccharide) and CD16 (FcγRIIIa: a receptor for IgG). Until now, three subsets of human monocytes have been identified, which are the classical

($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and nonclassical ($CD14^{+}CD16^{++}$) monocytes (Ziegler-Heitbrock *et al*., 2010). However, the role of distinct monocyte subsets in malaria infection remains unknown.

1.2 Problem Statement

Malaria is the leading cause of morbidity and mortality in endemic areas, and it creates a major social and economic burden. Although the disease is relatively rare in developed countries, where it mostly affects travellers returning from endemic areas, it is nevertheless one of the most common human infections globally. The effort to eradicate malaria began in the 1950s globally. Despite the fact that the majority of malaria can be cured effectively with antimalarials, morbidity and mortality due to malaria are still increasing (Talapko *et al*., 2019). This problem has emerged due to resistance of mosquitoes to the insecticides administered, resistance of malaria parasite to the medicines given during treatment, the prevalence of asymptomatic but infective carriers, reduced funding, and an increase in the number of non-immune tourists and refugees (Deroost *et al*., 2016). These have become some of the major challenges in controlling malaria over recent years and present efforts to control the disease focus on reducing malaria-associated morbidity and mortality (Tizifa *et al*., 2018).

Research into discovering and developing new antimalarial drugs, as well as a potential vaccine, is still ongoing. Despite decades of vaccine development research, an effective antimalarial vaccine with efficacy higher than 50% has yet to be discovered (Draper *et al*., 2018). Developing a vaccine is extremely difficult since the malaria parasite is a complicated organism with a complex life cycle that can

escape the immune system. *Plasmodium* goes through morphological changes and antigenic modifications as it progresses through its life cycle. *Plasmodium* proteins are extremely polymorphic, with their conflicting roles (Arama and Troye-Blomberg, 2014). Until today, many experts around the world are still trying to create an effective vaccine (Laurens, 2018).

Phagocytic cells are an essential first line of defense against malaria and circulating monocytes help to regulate and eliminate infection by clearing iRBCs (Urquhart, 1994). However, the molecular processes through which these cells recognize iRBCs is not well understood. Most researches have focused on phagocytosis of iRBCs, but it is unclear what role does monocytes play during phagocytosis of iRBCs and cytokine production in innate immune system during malaria infection. The functional effect of monocytes in malaria is lacking. The main monocyte subsets that facilitate phagocytosis, as well as the underlying mechanisms are also poorly understood. This knowledge could be useful in developing highly protective vaccines. Moreover, in order to further reduce morbidity and mortality, towards the goal of elimination, a deeper insight into the mechanisms that control the immune system response to parasite clearance or severe consequences is crucial (Deroost *et al*., 2016). In summary, the current data regarding host immune response following malaria infection is still lacking. Therefore, our study was to assess phagocytic capability of monocytes to phagocytose iRBCs and their cytokine production when infected by *P. falciparum in vitro*.

1.3 Significance of the Study

Malaria burden is expected to increase due to the development of drug-resistant parasites and a lack of regular immunological boosting. Eradication of malaria infection seems to be a challenging goal to achieve unless newer prevention and treatment approaches are discovered. One way toward this goal would be to gain mechanistic knowledge into the mechanisms involved in malaria pathogenesis and immune responses (Mbengue *et al*., 2016). Although the failure of immune system to control rapid parasite proliferation and the resulting excessive inflammatory responses are thought to have a role in malaria immunopathology, the underlying mechanisms are unknown (Clark *et al*., 2006). The aim in this field now is to figure out how these early responses play a role in the development of protective innate responses that eliminate malaria-infected red blood cells (iRBCs). Here we investigated whether the isolates of monocytes phagocytose iRBCs and the cytokine level following exposure to malaria parasite. Therefore, our study may provide insights into the role of monocytes in malaria infection by understanding how phagocytosis of iRBCs and subsequent cytokine production by monocytes facilitates parasite clearance *in vitro*. This provides a better understanding of the mechanisms of malarial clearance which may facilitate the development of effective vaccines and new therapeutic interventions for treating malaria.

1.4 Hypothesis

It is hypothesized that monocytes phagocytose *Plasmodium falciparum* followed by induction of cytokine secretions.

1.5 Research Questions

1. What is the phagocytosis index of monocytes when co-cultured with *P. falciparum*-infected red blood cells?
2. What is the level of cytokines following co-culture of monocytes with *P. falciparum*-infected red blood cells?

1.6 Objectives

1.6.1 General Objective

To determine the role of monocytes in *P. falciparum* infection.

1.6.2 Specific Objectives

1. To determine the morphology of different stages of *P. falciparum.*
2. To assess phagocytic capability of human monocytes on *P. falciparum*-infected red blood cells.
3. To measure cytokine secretions of monocytes following phagocytosis by *P. falciparum*-infected red blood cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Burden of Malaria

According to the latest World Malaria Report (2020), there were an estimated 229 million malaria cases in 2019. Global malaria case incidence decreased by 27% between 2000 and 2015 (Talapko *et al.*, 2019), but there has been an increase in the number of cases between 2015 and 2019. The number of malaria cases in 2019 has risen to 229 million, compared to 214 million cases in 2015 and 219 million cases in 2017. In 2019, the WHO African Region, with an estimated 215 million cases, accounted for about 94% of cases, followed by the South-East Asia (3.4%) and Eastern Mediterranean Region (2.1%) (Rahim, Munajat and Idris, 2020). In developed countries, malaria is an imported disease (Kain *et al*., 2001). The WHO Western Pacific Region which includes Malaysia, reported the second lowest malaria incidence among all WHO Regions in 2019. Globally, malaria mortality has decreased substantially, declining from 736,000 in 2000 to 409,000 in 2019 (World Malaria Report, 2020). The number of countries reporting less than 10,000 malaria cases is increasing, from 37 countries in 2010, to 44 in 2016, to 46 in 2017. Moreover, the number of countries having less than 100 local malaria cases has increased from 15 countries in 2010 to 26 countries in 2017 (WHO-World Health Organization, 2018).

Almost 40% of the people worldwide lives in malaria-endemic zones. Malaria mainly affects the poor most probably due to a lack of proper treatment. Almost 60%

of malaria deaths worldwide occur in the poorest 20% of the population. Of all the five *Plasmodium* species that cause human infection, *Plasmodium falciparum* is the most prevalent malaria parasite and has the highest mortality rate especially in the WHO African Region, representing 99.7% of estimated malaria cases in 2017, as well as in the South-East Asia (62.8%), the Eastern Mediterranean (69%) and the Western Pacific region (71.9%) (WHO, 2020).

In addition to children, pregnant women and nonimmune travellers or foreign workers are at the highest risk of developing severe infection. However, all age groups may be at risk of severe disease during malaria epidemics. These happen when there are changes in the physical environment for example climatic changes, agricultural projects or mining which increase the capability of mosquitoes to spread the disease, or when population migrations due to natural disasters or war which expose nonimmune populations to infection (Suh, Kain and Keystone, 2004).

In Malaysia, about one-third (32%) of total malaria cases were found in Peninsular Malaysia (Hussin *et al*., 2020) while Malaysian Borneo, specifically the states of Sabah and Sarawak accounted for the remaining 68% of cases (Cooper *et al*., 2020). According to Ministry of Health Malaysia, 3,941 malaria cases were recorded in 2019, with 3,223 (81.8%) cases being zoonotic malaria, 620 (15.7%) cases being imported malaria, and 98 (2.5%) cases being categorized as others (Rashvinjeet S. Bedi, 2020). In summary, 4630 malaria cases were reported in Malaysia in 2018, with 4131 cases of *P. knowlesi* and 499 cases of human malaria. The *Plasmodium* species that causes the most human malaria cases were *P. vivax* (284), followed by *P. falciparum* (182), *P. ovale* (14), *P. malariae* (13) and mixed infection (6).

2.2 Pathogenesis of Malaria

From the 18th century the assumption that malaria was caused by a miasmatic infection (mal, bad; aria, air) was unclear, hence our understanding of the transmission and etiology of malaria has improved markedly (Suh *et al*., 2004). Therefore, a thorough understanding of the *Plasmodium* life cycles and transmission, as well as the pathophysiology of infection is required to understand the disease process.

Plasmodium parasite has a complex life cycle that is divided into two phases: sexual and asexual. The sexual phase of the life cycle of *Plasmodium* parasites occurs in the vectors, the mosquitoes. The asexual phase happens in humans, the intermediate host of malaria. The parasite is transmitted only by night-biting female mosquitoes of the genus *Anopheles*. These mosquitoes tend to transmit the disease during warm climates with high humidity environments and abundant rain which provide ideal breeding conditions, thereby facilitating transmission (Suh *et al*., 2004). Malaria is spread through the bite of a female *Anopheles* mosquito, which injects the parasite in the form of sporozoites into the human skin. Sporozoites enter the peripheral circulation and then circulate in the human bloodstream and, after half an hour of blood circulation, enters the hepatocytes, and invade hepatocytes.

Plasmodium parasite asexual development begins in the hepatocytes and continues in the erythrocytes (Talapko *et al*., 2019). Sporozoites invade hepatocytes and undergo several rounds of replication and mitotic divisions over the next 5 to 8 days, producing a syncytial-like cell known as a schizont. The blood stage starts when mature liver schizonts rupture, releasing merozoites into the systemic circulation

where they invade the red blood cells (RBCs) (Soulard *et al.*, 2015). The release of merozoite marks the end of the pre-erythrocyte cycle and the beginning of erythrocytic phase (Bucşan & Williamson, 2020). Merozoites infiltrate and ingest hemoglobin in RBCs and develop from immature trophozoites into mature trophozoites. Following that, trophozoites rapidly replicate and divide, forming the schizont, similar to the liver stage. At the same time, mature schizonts destroy the integrity of erythrocyte cell membranes, rupture the infected RBCs (iRBCs), causing capillary endothelial adherence and cell lysis. Merozoites will then be released to invade new RBCs and the cycle continues until the parasites are eliminated by the immune system or treatment, or the patient passes away (Ozarslan, Robinson and Gaw, 2019). This stage of infection is characterized by observable clinical signs and symptoms, and the resulting humoral immune response has been linked to protection against severe disease (Bouharoun-Tayoun *et al*., 1990).

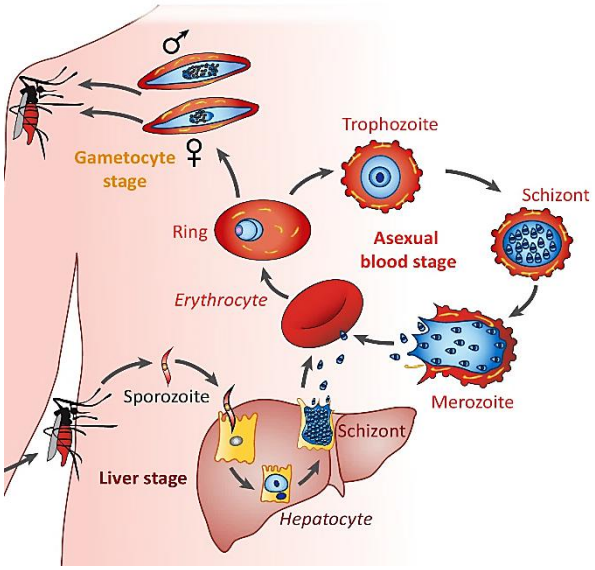

Figure 2.1 Asexual development of *Plasmodium falciparum*. Malaria parasites enter the human bloodstream in the form of sporozoites that are injected by infected female *Anopheles* mosquitoes taking a blood meal. The sporozoites migrate to the liver, where they invade hepatocytes and multiply. Merozoites are formed that are released into the bloodstream, where they invade red blood cells, initiating the asexual multiplication cycle. A fraction of merozoites that are released from infected red blood cells form gametocytes. Mature gametocytes circulate in the peripheral blood, where they can be taken up by mosquitoes. Once ingested by mosquitoes, each gametocyte forms macrogamete or microgametes.

Some trophozoites develop into male and female gametocytes during the sexual stages of the parasite. In *P. falciparum*, sexual differentiation occurs approximately 10 to 12 days where a single merozoite develops into either a male or a female gametocyte (Bousema *et al*., 2010). These gametocytes circulate in the bloodstream and are ingested by the female mosquito when it takes a blood meal. The ingested gametocytes grow into gametes, followed by fertilization and finally a zygote is formed within the mosquito's stomach. The zygotes develop into motile ookinetes that actively burrow through the mid-gut wall of the mosquito. Ookinetes encyst and become oocysts, which produces thousands of active sporozoites. The oocysts eventually burst, releasing their sporozoites which migrate to the salivary glands. When the mosquito bites another person, the human infection cycle starts all over again (Vicki Symington, 2012).

During parasite invasion, the immune system functions to clear the parasites, which leads to several symptoms in the patients. Malaria is characterized by cyclic fever, which occurs in combination with the coordinated release of new merozoites from iRBCs. Trophozoites may reside in the vascular spaces of the central nervous system (CNS), causing cerebral malaria, a leading cause of death in young children. Malaria can cause fever, jaundice, severe anemia, hypoglycemia, acute renal failure, chills, headache, vomiting, respiratory failure, complications of pregnancy, including preterm birth and low birth weight, coma and death, depending on the severity of the infection (Crutcher and Hoffman, 1996). These clinical symptoms often correspond with the rupture of infected erythrocytes and the release of erythrocyte and parasite debris, including malarial pigment (hemozoin) and glycophosphatidylinositol, the

putative malaria toxins, which activate peripheral blood mononuclear cells and stimulate the release of cytokines (Mawson, 2013).

The severity of clinical disease is influenced by a number of factors. High parasite burdens together with the ability of infected erythrocytes to bind to the host endothelium results in severe malaria symptoms (Cooke, Coppel and Wahlgren, 2000). A strong cytokine response to parasite proteins produced during schizont rupture can also lead to adverse clinical consequences (Jakobsen *et al*., 1995). Infections with *P. falciparum* have been known to relapse, resulting in a rapid increase in parasitemia and subsequent erythrocyte destruction. Apart from this, antibodies such as Pfs230, Pfs48/45, and Pfs47 are produced against gametocytes. These antibodies have been demonstrated to inhibit transmission in the mosquito midgut (Acquah *et al*., 2019), but a selective protective immune response targeted specifically at immature intraerythrocytic gametocytes in the human host has not been described (Dantzler *et al*., 2019).

When choosing for an antimalarial treatment, the factors such as the infecting *Plasmodium* species, the severity of disease and the drug susceptibility of the infecting parasites must be considered. The most common antimalarial drugs include chloroquine and artemisinin-based combination therapies (ACTs). However, evidence of resistance to these treatments was reported, and the drugs may be ineffective (Ouji *et al*., 2018).

2.3 Peripheral Blood Mononuclear Cells (PBMCs)

2.3.1 Origin and components of PBMCs

Human peripheral blood mononuclear cells (PBMCs) are any blood cell with a round nucleus isolated from peripheral blood. These include lymphocytes, monocytes, natural killer cells (NK cells) and dendritic cells. PBMCs are a variety of specialized immune cells important in the immune system, as they assist the body in defending against harmful pathogens by destroying foreign substances and tumour cells. PBMCs are widely utilized in research and clinical applications and is a useful tool for studying various aspects of pathology and biology *in vitro* such as immunology, infectious disease, haematological malignancies and vaccine development.

PBMCs are derived from hematopoietic stem cells (HSCs) in the bone marrow. HSCs are the basic components of all blood cells in the immune system. They form two major lineages which are myeloid and lymphoid lineage as they go through a process called hematopoiesis and differentiate into specific cell types. PBMCs include some myeloid and lymphoid cells especially those with a single round nucleus. In humans, the frequencies of these cells differ by individual, but lymphocytes normally account for 70–90%, monocytes are about 2–10%, and dendritic cells represent approximately 1–2% of the cells (Tera Muir, 2021).

Lymphocytes make up the majority of a human PBMC sample which play a crucial role in fighting against infections. Lymphocytes can be further divided into CD3+ T cells, CD4+ helper T cells, CD8+ killer T cells, B cells and Natural killer (NK) cells. Monocytes are the largest (in size) type of PBMCs and express CD14 receptor on their surface. Monocytes can develop into macrophages or dendritic cells which

help in eliminating foreign or dead cells when they are activated. Dendritic cells are the third type of PBMCs. Dendritic cells are highly specialized antigen presenting cells (APCs) that can engulf an antigen and presenting the antigen to the immune system, resulting in activation of T and B lymphocytes and initiation of immune response. Table 2.1 below shows the components of PBMCs and their respective functions.

Table 2.1 Components of PBMCs

Cell type	**Frequency (%)**	**Markers**	**Function**
$CD4^+$ T cells	25–60	$CD3^+CD4^+$	Coordinate adaptive immunity through activation and regulation of other immune cells
$CD8^+$ T cells	5–30	$CD3^+CD8^+$	Destroy cancer cells or cells that are infected or damaged
B cells	5–10	$CD19^+$	Secrete antibodies as part of humoral immune response
NK cells	10–30	$CD56^+$ $CD3^-$	Trigger lysis or apoptosis of infected cells
Monocytes	2–10	$CD14^+$	Take up foreign antigens via phagocytosis, perform antigen presentation, and produce cytokines
Dendritic cells	1–2	$CDllC^+HLA\text{-}DR^+$	Process and present antigen materials to T cells

*Modified from Tera Muir, 2019.

2.3.2 Isolation of PBMCs

Peripheral blood and umbilical cord blood are the primary sources of PBMCs. Usually, whole blood is collected through venepuncture and PBMCs are isolated from whole blood. Ficoll® density gradient centrifugation is the most common method used for isolating PBMCs from whole blood (Harris and Ukaejiofo, 1970). The cell fraction corresponding to red blood cells and granulocytes such as neutrophils, basophils and eosinophils is removed from whole blood via this method. The Ficoll-Hypaque solution has a density of 1.077 g/mL. Differential migration during centrifugation results in the separation of cell types into four different layers by a gradient medium (Ivan J *et al*., 2009). The cells that have a density higher than the Ficoll will pass through the Ficoll layer and sediment at the bottom layer, whereas cell with a lower density will accumulate at the plasma-gradient boundary. Thus, the bottom layer consists of red blood cells which have a high density. A diffuse layer immediately above the bottom layer contains mostly granulocytes (Figure 2.2). The lymphocytes including the monocytic PBMC components appeared as a characteristically white and cloudy layer, sediment at the interface between the granulocytes and top plasma layer due to a slightly lower density (Kleiveland, 2015).

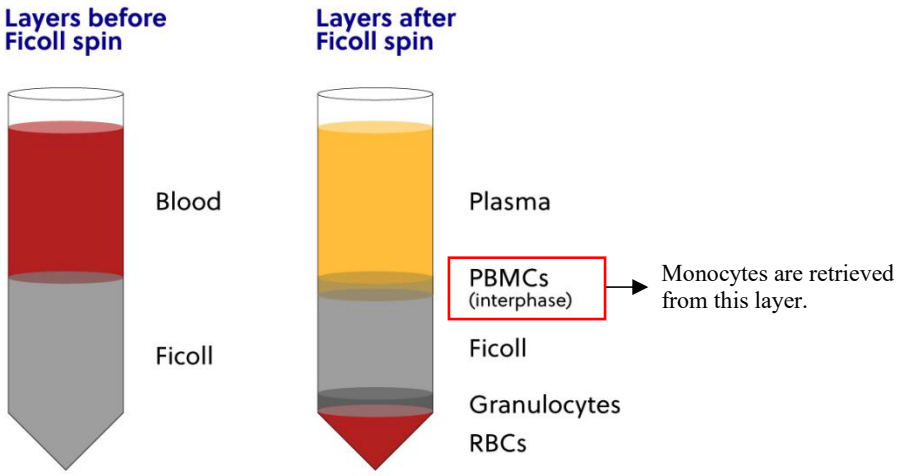

Figure 2.2 Isolation of PBMCs from whole blood. Ficoll-Hypaque solution is layered at the bottom of whole blood followed by centrifugation at 1500 rpm for 20 minutes. After centrifugation, blood components are separated into four different layers based on their density. (Adapted from Z. Lin *et al*., 2014)

2.4 Human Monocytes

2.4.1 The Biology of Monocytes

Circulating monocytes are a diverse and dynamic cell population made up of several subsets that differ in phenotype, size, morphology, and transcriptional profiles (Mildner *et al*., 2013). Monocytes are generated from bone marrow that circulate in the blood for a few hours or days before being recruited into tissues (Patel *et al*., 2017). The expression of chemokine receptors and cell adhesion molecules on their surfaces allows them to leave the bone marrow and enter the bloodstream to carry out their immunological functions (Geissmann *et al*., 2010). Monocytes are the biggest cells in normal peripheral blood with a diameter of 14–20 µm. They have unique morphological characteristics including an irregular cell shape, an oval or kidney-shaped nucleus, cytoplasmic vesicles, and a high nucleus to cytoplasm ratio, approximately 3:1 (Qu *et al*., 2014). Under normal conditions, monocytes make up between 2% and 10% of the circulating cell population and their numbers increase in

response to infection. The life span of circulating monocytes is relatively short, and most of them undergo apoptosis after approximately 24 hours.

Monocytes are formed in the bone marrow from specific hematopoietic progenitors (Álvarez-Errico *et al*., 2014). Monocytes originate from hematopoietic stem cells in the bone marrow through a series of commitment steps and intermediate progenitor stages, including the common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), and macrophage/DC progenitor (MDP) stages (Auffray, Sieweke and Geissmann, 2009). Each of these differentiation processes decides the fate of these cells that limit their developmental potential. Hematopoietic stem cells (HSC) produce CMP and common lymphoid progenitor (CLP) in the bone marrow. Some of the CMP differentiates into either megakaryocyte-erythrocyte progenitor cells (MEP) or granulocyte-macrophage progenitor cells (GMP), which are important for the development of megakaryocyte erythrocyte and granulocyte macrophage lineage cells respectively. GMP give rise to monocyte/macrophages and DC precursors (MDP), MDPs subsequently differentiate to monocytes (Figure 2.3).

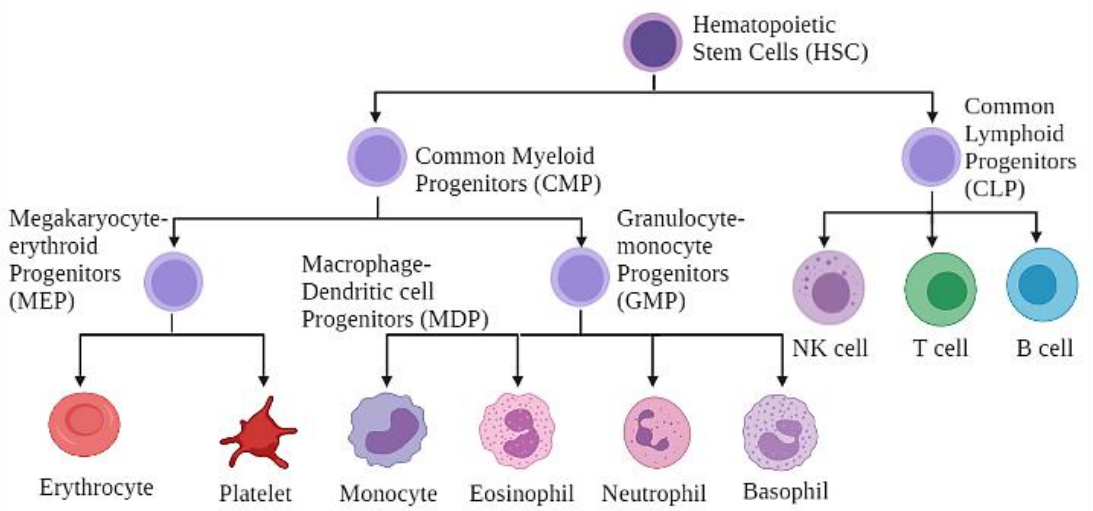

Figure 2.3 Origin of monocytes through the process of haematopoiesis. HSCs form two major lineages which are common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). These cells will then differentiate into specific cell types. PBMCs include myeloid and lymphoid cells which have a single round nucleus. These include lymphocytes, monocytes and dendritic cells. Monocytes are derived from GMP that give rise to MDP and subsequently differentiate into monocytes.

2.4.2 The Function of Monocytes

Monocytes play a crucial role in the innate immune system. Monocytes have two important roles in the immune system. Firstly, they regenerate resident macrophages and dendritic cells under normal conditions, and secondly, they travel to infection sites in the tissues and differentiate into macrophages and dendritic cells to induce an immune response in response to inflammation signals. Monocytes are important in killing pathogens as well as facilitate healing and repair process (Espinoza and Emmady, 2020). Approximately half of the body's monocytes are stored in the spleen as a backup. The circulating monocytes can be divided into two types, one that is believed to ‘guard' vessel walls and maintain endothelial cells (Auffray *et al*., 2007), and another can transmigrate over the endothelium and infiltrate tissues in response to specific signals (Jakubzick *et al*., 2013). Circulating monocytes can either maintain as monocytes in the bloodstream, obtain antigen-presenting ability, or even

mature into macrophages or dendritic cells (Tamoutounour *et al*., 2013). Monocytes are a key component of the innate immune system serving three main immunological functions including phagocytosis, antigen presentation and inflammatory cytokine production (Burdo, Lackner and Williams, 2013).

Monocytes in the peripheral blood function as phagocytes ingest material for two reasons: to remove waste and debris and to kill invading pathogens. After 20 to 40 hours in the bloodstream, most monocytes leave the bloodstream and migrate to tissues and organs, where they develop into macrophages or dendritic cells depending on the signals received (Silva, 2010). Monocytes can phagocytose pathogens by binding to them directly through pattern-recognition receptors (PRR), or by using intermediary (opsonising) proteins such as antibodies or complement, which coat the pathogen. Monocytes may also use antibody-dependent cell-mediated cytotoxicity to destroy infected host cells. Monocytes function as phagocytes and antigen-presenting cells in the peripheral blood to ingest and remove microorganisms, foreign material, and dead or damaged cells.

Microbial pattern-recognition receptors recognize pathogen-associated molecular patterns (PAMP), stimulate monocytes to become activated in order to kill invading parasites. An example of PRR is the mammalian Toll-like receptor (TLR), which recognizes variety of microbial pathogens and their products (Gowda and Wu, 2018). Monocytes express more TLRs than neutrophils. When specific ligands bind, TLRs activate nuclear factor kappa B (NF-κB) and stimulate the production of proinflammatory cytokine from monocytes through a pathway involving the adaptor

protein myeloid differentiation factor 88 (MyD88) (Dale, Boxer and Conrad Liles, 2008).

Monocytes may produce cytokines, which attract more cells and proteins to the infected region, resulting in an activated immune response. In response to parasite ingestion, monocytes secrete both pro-inflammatory and anti-inflammatory cytokines as well as growth factors, which results in parasite removal and minimizing inflammation (Zhou *et al*., 2015). Monocytes secrete pro-inflammatory cytokines such as interleukin (IL) and tumor necrosis factor (TNF), which can activate other leukocytes and endothelial cells to a pro-adhesion, pro-migratory condition and induce secretion of vasoactive substances (Willis *et al.*, 2003).

Tumor necrosis factor-alpha (TNF-α) released by monocytes triggers the release of a cascade of cytokines that play a role in inflammatory responses. TNF-α exerts its effect by binding as a trimer to one of two membrane receptors, tumor necrosis factor receptor 1 (TNFR1) or TNFR2. Cell stimulation by TNF-α triggers the downstream activation of transcription factor NF-κB, which is a mechanism shared by many agonists, including interleukin 1 beta (IL-1β) and lipopolysaccarides (LPS), resulting in systemic inflammatory response (Østerud and Bjørklid, 2003). There are many types of interleukins produced by monocytes, for example, IL-1, IL-6, IL-10, and IL-12. TNF-α has been shown to induce the production of IL-1β by monocytes. IL-1β can resemble the activation signals triggered by TNF-α. Besides that, IL-1β functions as a neutrophil chemoattractant, stimulates production of neutrophil from the bone marrow into the bloodstream, and promotes adherence of leukocytes to the endothelium (Duque and Descoteaux, 2014). In addition, IL-1β enhances

proliferation of endothelial cell and promotes T-cell activation by increasing IL-2 formation and upregulating the IL-2 receptor. IL-10 is an anti-inflammatory cytokine produced by activated monocytes. The anti-inflammatory effects of IL-10 on the vascular system are most likely due to inhibition of leukocyte-endothelial cells interaction and proinflammatory cytokine and chemokine formation by monocytes (Østerud and Bjørklid, 2003). IL-12 stimulates interferon gamma (IFN-γ) in NK cells and T cells, causing T-cell differentiation to shift toward a T helper 1 (Th1) response, promoting differentiation of activated NK cells, $CD8^+$ T cells, and $CD4^+$ T cells.

Monocytes are a type of specialized antigen-presenting cell that play a key role in the recruitment and activation of innate immune cells. Monocytes may also transmit co-stimulatory signals to activate naive T cells, stimulating adaptive immune responses. Thus, they serve as the bridge between the innate and adaptive immune systems (R. Lin *et al*., 2016). Lymph node-trafficking monocytes are weak antigen presenters and instead serve as antigen transporters that deliver antigen to draining lymph nodes. Other studies, on the other hand, has suggested that monocytes process and present antigen similarly to classical dendritic cells (cDCs) (Larson *et al*., 2016). It is likely that the methodological differences are the reason for the variation of results. Both *in vivo* and *ex vivo* studies demonstrated that monocytes play a significant role in the presentation of antigen to T cells and the induction of particular T cell subsets (Claudia, Gwendalyn and Peter, 2017).

2.5 Monocyte subsets

Monocytes in humans are heterogeneous. They consist of three subsets based on expression of CD14 and CD16. Circulating human monocytes consist of the $CD14^{++}CD16^{-}$ classical monocytes, $CD14^{++}CD16^{+}$ intermediate monocytes and $CD14^{+}CD16^{++}$ non-classical monocytes (Ziegler-Heitbrock, 2015). The monocytes initially develop in the bone marrow before being transported as $CD14^{+}$ classical monocytes into the circulatory system. Gradually, classical monocytes give rise to non-classical monocytes through an intermediate phase of intermediate monocytes (Kapellos *et al*., 2019). Classical monocytes are the most predominant subset, accounting for around 80% of the total circulating monocytes population (Dobbs, Crabtree and Dent, 2020). The remaining 20% of monocytes are the non-classical monocytes and intermediate monocytes. These subsets vary in their differentiation properties, migratory capabilities, and cytokine productions.

2.5.1 Classical Monocytes

The classical inflammatory monocytes include $CD14^{++}CD16^{-}$ in humans. The classical monocyte is characterized by high expression of the CD14 cell surface receptor. Classical monocytes were found to be primed for phagocytosis, innate sensing/immune responses and migration. Classical monocytes also known as inflammatory monocytes, have a more pro-inflammatory nature, with the ability to infiltrate tissues and produce soluble inflammatory cytokines and differentiate into dendritic cells and inflammatory macrophages, linking between innate and adaptive immune responses. Classical monocytes express many PRRs and are involved in removing microorganisms and dying cells through phagocytosis (Sprangers, Vries and Everts, 2016). Classical monocytes in humans are distinguishable from the other

two subsets by additional markers such as CD36 and CD64, and they participate in antimicrobial mechanisms such as endothelium adherence, migration, and phagocytosis (Kapellos *et al*., 2019).

The classical monocytes which make up the majority of blood cells, react strongly to bacterial products through TLR4 and infiltrate inflammatory sites in response to the chemokine C–C motif chemokine ligand 2 (CCL2). They are more efficient in producing reactive oxygen species (ROS) (Weber *et al*., 2000). These monocytes proliferate in the bone marrow in response to infection or injury. They are released into bloodstream in a C-C chemokine receptor type 2 (CCR2)-dependent manner, and migrate to the targeted site with the aid of chemokines. For example, during bacterial infection, these monocytes migrate to the infection site, phagocytose pathogens, and produce a variety of chemokines that attract other immune cells, and present antigen through major histocompatibility complex (MHC) class II. These monocytes may leave the blood vessels and survey the tissue microenvironment without further differentiation before exiting through the lymphatics (Chiu and Bharat, 2016). The $CD14^{+}$ classical monocytes express high levels of chemokine receptors such as CCR1, CCR2, CCR5, C-X-C motif chemokine receptor 1 (CXCR1), and CXCR2, indicating their ability to migrate to signals arising from injured or inflamed tissues (Wong *et al*., 2011), but they are also distinguished by their ability to secrete pro-inflammatory molecules such as IL-6, IL-8, CCL2, CCL3, and CCL5 (Cros *et al*., 2010). Classical monocytes are able to differentiate into monocyte-derived macrophages and dendritic cells (DCs) (Menezes *et al*., 2016) and they play an important role in regulating inflammation and tissue recovery.

2.5.2 Non-Classical Monocytes

Non-classical monocytes are the $CD14^{+}CD16^{++}$ in humans. The non-classical monocytes show low expression of CD14 and additional co-expression of the CD16 receptor. $CD14^{+}CD16^{++}$ non-classical monocytes may protect blood vessel walls and react to viral ligands by TLR7/8. They have high fractalkine receptor (CX3CR1) expression for their own survival but they also migrate in response to variety of chemokines (Idzkowska *et al*., n.d.). Non-classical monocytes are involved in endothelium intraluminal monitoring, complement and fragment crystallizable gamma (Fcγ)-mediated phagocytosis of damaged endothelium, trans-endothelial migration, neutrophil recruitment to the injury site and anti-viral responses. These monocytes are able to detect and respond to circulating nucleic acids and viruses via TLR7 signaling, and trigger an innate immune response by secreting cytokines and chemokines (Gerhardt and Ley, 2015). However, the exact role of the non-classical monocytes is still debatable.

In humans, the non-classical subset has been shown to have a proinflammatory effect. The non-classical monocytes skew towards a more anti-inflammatory subset that suppresses inflammation by releasing IL-10 (Karlmark, Tacke and Dunay, 2012). They have antigen-processing abilities (Schmidl *et al*., 2014), but they differ from the classical monocytes by involving in wound healing processes (Krzyszczyk *et al*., 2018). Moreover, they have antagonizing functions to classical monocytes and promote neutrophil adhesion at the endothelial interface via the secretion of TNF-α (Chimen *et al*., 2017) but they do not produce pro-inflammatory cytokines at the same levels as the classical monocytes (Boyette *et al*., 2017).