DTECTION OF IFN- γ +874T/A GENE POLYMORPHISM AND Plasmodium falciparum INFECTION IN ARDAMATA IDP CAMP, AL-GENEINA CITY SUDAN

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DTECTION OF IFN- γ +874T/A GENE POLYMORPHISM AND Plasmodium falciparum INFECTION IN ARDAMATA IDP CAMP, AL-GENEINA CITY SUDAN

by

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LIST OF SYMBOLS

Bp	Base pairs
Cm	Centimetre
Et al.	et alii—‘and others’
g	Gram
H	Hour
kDa	Kilodalton
L	Litre
M	Molar
µ	Micro-
µg	Microgram
µL	Microlitre
Min	Minutes
mL	Millilitre
mM	Millimolar
µg/mL	Microgram per millilitre
%	Percentage
Rpm	Revolutions per minute
Sec	Second
V	Voltage
X	Times
&	And
≈	Approximately

LIST OF ABBREVIATIONS

CTL	Cytotoxic T-lymphocytes
ddH20	double distilled water
e.g.	For example (Latin: exempli gratia)
IFN-γ	Interferon gamma
Ig	Immunoglobulin`
IL	Interleukin
NK cell	Natural killer cell
°C	Degree Celsius
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
RBC	Red blood cell
RT	Room temperature
TGF	transforming growth factor
Th1	T helper 1
TNF- **α**	Tumour necrosis factor α
v/v	Volume to volume
WHO	World Health Organization
γδ	Gamma/delta

PENGESANAN ANTARA POLIMORFISME GEN IFN- γ +874T/A DAN JANGKITAN *Plasmodium falciparum* DALAM KALANGAN PENDUDUK SUDAN

ABSTRAK

Malaria merupakan penyakit bawaan parasite yang utama di dunia dan bertanggung jawab terhadap 214 juta jangkitan setiap tahun, dan menyebabkan lebih daripada 438,000 kematian. Di Sudan, *Plasmodium falciparum* merupakan parasit utama yang menyebabkan malaria. Polimorfisme gen sitokin boleh merubah pembangunan protein ini dan oleh itu memberi kesan kepada gerak balas imun terhadap jangkitan malaria. Interferon- (IFN-), sitokin pro-inflamasi telah terbukti mempunyai kesan antiparasit dan imunopatogenik semasa jangkitan malaria. Polimorfisme nukleotida tunggal (SNP), + 874T /A, dalam intron pertama gen IFN-telah menunjukkan hubungan dengan kerentanan manusia terhadap banyak penyakit termasuk malaria di sesetengah populasi. Oleh itu, tujuan kajian ini adalah untuk mengkaji sama ada variasi genetik pada lokus *IFNG* (IFN- +874T/A gene) berkait rapat dengan jangkitan malaria *Plasmodium falciparum* dalam kalangan komuniti Sudan. Tiga puluh empat sampel darah kering populasi Sudan dari Kem IDP Ardamata di Al-Geneina Town, Sudan disahkan terlebih dahulu sama ada positif malaria dan berasal daripada jangkitan *Plasmodium falciparum* atau tidak menggunakan kaedah PCR bersarang. PCR kemudian digunakan untuk mengetahui hubungan antara jangkitan *P. falciparum* dan polimorfisme gen IFN- + 874T/A. Keputusan ujian PCR bersarang menunjukkan bahawa daripada 34 sampel yang dianalisis, 17 sampel adalah positif terhadap jangkitan *P. falciparum* manakala 17 sampel lagi adalah negatif. Walau bagaimanapun, tidak ada hubungan yang signifikan antara gen IFN- + 874T/A dengan jangkitan *P. falciparum*. Sebagai kesimpulan, data ini menawarkan titik permulaan untuk analisis

fungsional dan genetik wilayah genom IFN-γ dalam jangkitan malaria dalam kalangan penduduk Sudan, yang dapat merangsang kepada pembangunan vaksin malaria yang berpotensi pada masa hadapan.

DETECTION OF IFN-γ +874T/A GENE POLYMORPHISM AND *Plasmodium falciparum* INFECTION IN ARDAMATA IDP CAMP, AL-GENEINA CITY SUDAN

ABSTRACT

Malaria is the primary parasite disease and is responsible for around 214 million infections annually, resulting in more than 438,000 deaths. In Sudan, *Plasmodium falciparum* is typically accountable for severe malaria. Cytokine gene polymorphisms can alter the development of these proteins and therefore affect the immune response to disease. Interferon- (IFN-), a pro-inflammatory cytokine, has been shown to have antiparasitic and immunopathogenic effects during malaria infection. A single nucleotide polymorphism (SNP), +874T/A, in the first intron of the IFN- gene, has presented associations with human susceptibility to many diseases, including malaria in certain populations. Therefore, the purpose of this study was to investigate whether genetic variation at the *IFNG* locus (IFN-+874T/A gene) affects susceptibility to *P. falciparum* malaria infection in the Sudanese population. Thirty-four (34) dried blood samples of Sudanese communities from the Ardamata IDP Camp in Al-Geneina Town, Sudan were first confirmed with nested PCR for malaria positive confirmation and *Plasmodium falciparum* identification. PCR was then used to test the relationship between *P. falciparum* infection and IFN- +874T/A gene polymorphism of the samples. The result of the nested PCR showed that out of 34 samples, 17 samples were malaria positive and *P. falciparum* positive, and 17 samples were negative. However, our result suggests that the IFN- +874T/A mutation has no association with *P. falciparum* infection. In conclusion, this data offers a starting point for functional and genetic analysis of the IFN-γ genomic region in malaria infection affecting Sudanese

populations, which will promote the potential production of successful malaria vaccines.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria remains one of the significant public health problems due to its prominent high mortality and morbidity rate (Kosaisavee et al., 2011). Currently, it was estimated that about 216 million cases were recorded with 445 000 death worldwide in 2016. Unsurprisingly, the African region was representing the highest malaria cases, with 90% episodes (WHO, 2017). In Sudan, the disease transmission rate was estimated to approximately 90% (Hamid et al., 2016; Niaz et al., 2018). Five different species of protozoan parasites named *Plasmodium* have been known to cause malaria in humans. *P. falciparum* cases were more frequent in the African region while *P. vivax* shows high frequencies in the Americas, South-East Asia, and Eastern Mediterranean Regions (Trampuz et al., 2003; Kosaisavee et al., 2011). The high transmission rate is mainly attributed to the spread of Anopheles mosquitos, particularly, *Anopheles arabiansis* (Niaz et al., 2018).

Malaria has been associated with several clinical manifestations ranging from asymptomatic, mild (uncomplicated), and severe (complicated) illness, which often leads to death (Hamid et al., 2016). These conditions might be attributed to the genetic polymorphism, which can also be related to its ability to adapt to various geographical locations as well as different hosts (Mahgoub et al., 2012; Diering, 2018). Accordingly, WHO has taken into consideration the most vulnerable individuals as a prerequisite for prevention and treatment (WHO, 2017). These include infants and children with

the age of less than five years, pregnant women, and patients with HIV/AIDS (WHO, 2017).

The control of malaria infection is currently managed by chemoprophylaxis, which mounts the blood stage of malaria infection, thereby, preventing the malaria illness (WHO, 2017). However, with the appearance of drug resistance by *P. falciparum* for certain drugs have raised the requirement for the development of an effective vaccine for durable protection against malaria infection. Therefore, a quite appreciated attempt on this track has already been made to come out with a long-lasting, highly immunized as well as highly effective vaccine (Patel et al., 2017), but unfortunately, genetic polymorphisms within most vaccine candidate genes were the major obstacles toward achieving this goal (Gosling & von Seidlein, 2016; Campbell et al., 2017). Until now, there is still no approved vaccine available in the market, even though several potential vaccine candidates targeted against sporozoite, merozoite, and gametocyte stages of *P. falciparum* are now under various stages of clinical evaluation (Patel et al., 2017).

Interferon gamma (IFN-γ) is a key immunological mediator that has a protective role in host defense against malaria but also contributes to underlying disease pathology. The target cells of IFN-γ during *P. falciparu*m infection are monocytes/macrophages, neutrophils, Th2 cells and parasite-infected hepatocytes. IFN-γ also serves as a macrophage activating factor involved in the innate immune response to malaria. Activated macrophages release tumor necrosis factor α (TNF-), transforming growth factor β (TGF-β), interleukins (IL)-1 and IL-6, and reactive oxygen and nitrogen radicals. Additionally, IFN-γ activates iNOS and induces the L-

arginine dependent NO pathway via signal transducers associated with transcription. This NO pathway leads to the subsequent elimination of the infected hepatocytes (Elghazali et al., 1997). IFN-γ induces cellular activation by binding to a receptor complex - the interferon-gamma receptor (IFN-γR). IFN-γR is ubiquitously expressed on all monocytes, macrophages, T cells, B cells, NK cells, neutrophils, fibroblasts, and endothelial cells (Kwak, 2012). The receptor is a heterodimer of two subunits, α, and β, that are integral membrane proteins. Subunit α (IFN-γR1) is essential for IFN-γ binding, receptor trafficking, and signal transduction (Bach et al., 2003). At the same time, the β subunit (IFNγR2) functions as a transmembrane accessory factor. The heterodimeric IFNγR interacts with STAT1, GAF, JAK1, and/or JAK2 in the downstream IFN-γ signal transduction pathway (Samanez-Larkin et al., 2009). Several polymorphisms (SNPs and microsatellite repeat) from the promoter, introns, and UTR region of the IFN- gene have been reported (Koch et al., 2006). Some of these polymorphisms have been associated with diseases other than malaria (Wang et al., 2012). A previous study also reported that several polymorphisms in the promoter region of IFN R1 affect gene regulation which will also influence the susceptibility to severe malaria (Koch et al., 2012).

1.2 Significant of the study

Malaria is one of the leading causes of death in Sudan. Although intensive control measures in recent years have resulted in a substantial reduction in disease burden, due to the violent conflict in Darfur, limited control options and resource availability maintain the high risk of malaria in displaced camps, making the disease responsible for most deaths. Therefore, more effective malaria control, like vaccination, is probably the best option. However, due to the complexity of malaria infection, until

now there is no disease vaccine available. Host genetic factors have been shown to influence intensity of malaria infection and clinical malaria, a very important factor in vaccine development. Several candidate genes were associated with severe malaria resistance, including IFN- , a Th1 cytokine known to help eliminate malaria parasites by enhancing macrophage phagocytic activity, generating intermediate reactive oxygen and L-arginine-derived nitric oxide, and stimulating T-cell proliferation. Several studies have reported associations between Interferon gamma (IFN-γ) (or also known as *IFN* G in some literatures) polymorphisms and susceptibility to disease. The first intron of the IFN-γ gene contains a highly polymorphic CA-repeat microsatellite, whose 12 CA-repeatallele is associated with high levels of IFN-γ production and has been associated with resistance or susceptibility to various diseases including malaria in other populations. However, very limited data can be found on the association of IFN-γ gene polymorphisms with *P. falciparum* infection in Sudanese population. Therefore, understanding the IFN-γ gene polymorphisms in Sudanese population is likely to be important for the future development of worldwide therapies and vaccine-based interventions since IFN-γ stimulates Th1-type responses which are of parasitological importance in malaria elimination.

1.3 Objectives of the study

1.3.1 General objective

To detect the IFN- **γ** gene polymorphism and *Plasmodium falciparum* infection in malaria patients of Ardamata IDP Camp, Al-Geneina City, Sudan

1.3.2 Specific objectives

1. To determine malaria positive infection and *P. falciparum* identification in blood samples of malaria patients from Ardamata IDP Camp, Al-Geneina City, Sudan using nested polymerase chain reaction (nested PCR)
2. To determine the association between IFN- gene polymorphism at single nucleotide +874T/A with *P. falciparum* infection in malaria patients of Ardamata IDP Camp, Al-Geneina City, Sudan

1.4 Hypothesis

1. Nested PCR confirmed that all the blood samples isolated from malaria patients of Ardamata IDP Camp, Al-Geneina City, Sudan are positive malaria and the infection is caused by *P. falciparum.*

2. There is a positive association between IFN- gene polymorphism at single nucleotide +874T/A with *P. falciparum* infection in blood samples of malaria patients from Ardamata IDP Camp, Al-Geneina City, Sudan

1.5 Flow chart of the study

The activities of the study are summarized in Figure 1.1.

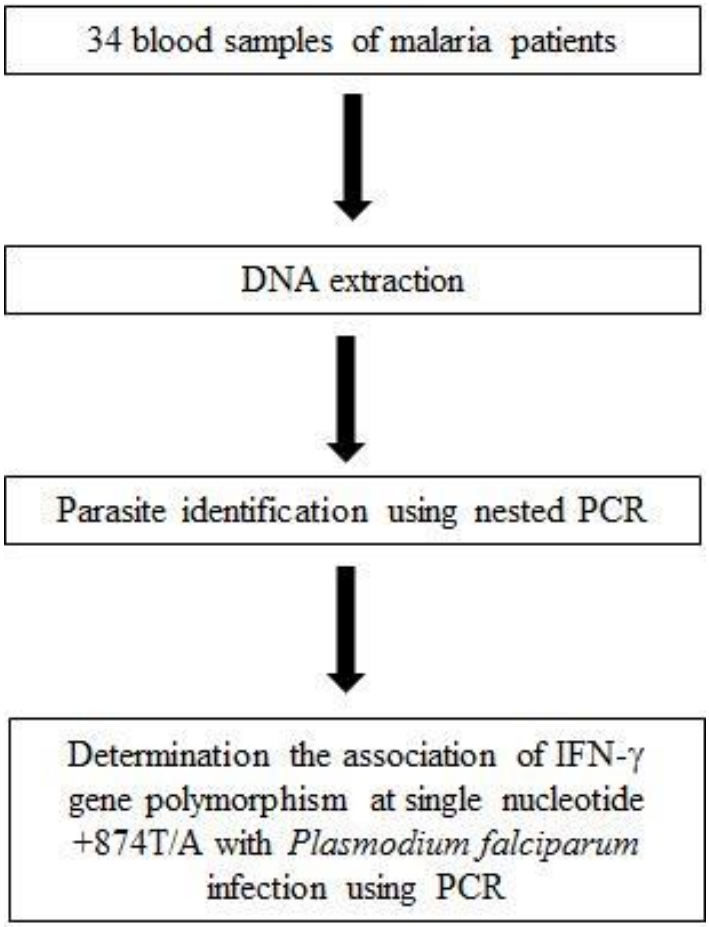

Figure 1.1 Flow chart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria

Malaria is one of the world's most serious infectious diseases. World Health Organization (WHO) reported 225 million malaria cases worldwide, with 781,000 deaths due to annual *Plasmodium* infection. Four species of Plasmodium are responsible for almost all human infections (*P. falciparum, P. vivax , P. malariae* and *P. ovale*) (Cavasini et al., 2000). Malaria parasites share the characteristic febrile episodes with their susceptibility to frequent intermittent paroxysms of chills, rigors, and sweating. They are also associated with many signs of other infectious diseases, including body aches, headache and nausea, general fatigue, and prostration. Untreated malaria infections are characterized by spleen enlargement. Serious, life-threatening conditions usually occur in *P. falciparum* malaria. These cause inflammation of vital organs during cerebral malaria including the lungs, kidneys, liver, and, most notably, the brain. Serious anemia can develop. These are the factors associated with the majority of acute malaria mortality. Chronic malaria infection can lead to nephrotic syndrome and this, too, can ultimately become fatal (Carter & Mendis, 2002).

Repeated malaria attacks over several years due to any species of the parasites seriously damage body and mind. Cachexia and the splenic enlargement becomes a constant feature. The general definition of the disorder is lethargic and with sunken and sallow eyes, spindly limbs, and hard swollen belly. In this situation, the individual affected succumbs to illnesses or other difficulties that would scarcely endanger a normal healthy person. Under the burden of chronic malaria, both efficiency and lifespan are reduced considerably. However, an individual's malaria experience at a

given time is strongly influenced by the form and degree of antimalarial immunity he or she may have acquired (Deribew et al., 2017).

2.1.1 Global malaria

Malaria is endemic in regions of 107 countries, mainly in sub-Saharan Africa (Figure 2.1). The spatial limits of distribution and seasonal activity are sensitive to climatic factors, as well as local disease control capability. In endemic areas where transmission occurs in regular, long-term seasons, fatality rates are highest among children who have not yet developed immunity to the disease. In epidemic areas where malaria transmission occurs in short seasons or in the form of epidemics, serious deaths are likely to occur in all age categories. After the Global Malaria Eradication Program launched by the World Health Organization (WHO) in the 1950s, 79 countries eliminated malaria. Most of this progress has been made in the extratropics (Eurasia, North America, North Africa, and Australia) where malaria transmission has been highly seasonal due to temperate climate conditions and mainly due to *P. vivax*. In the early 1970s, WHO abandoned the idea of eliminating malaria in the tropics, particularly in Africa, due to deficiencies in local public health services. It was then replaced by a control policy using new control measures such as vector control through insecticide spraying, use of bed nets, systematic early detection and treatment of cases. Between 2000 and 2010, the incidence of malaria decreased by 17% worldwide and by 33% in African regions. There were 655,000 reported malaria deaths in 2010, of which 86% were children under the age of five. In 2010, the majority of malaria deaths occurred in Africa (91% of the global burden). They were due to *P. falciparum* (98% of infections) which causes the most serious clinical form of the disease (Caminade et al., 2014).

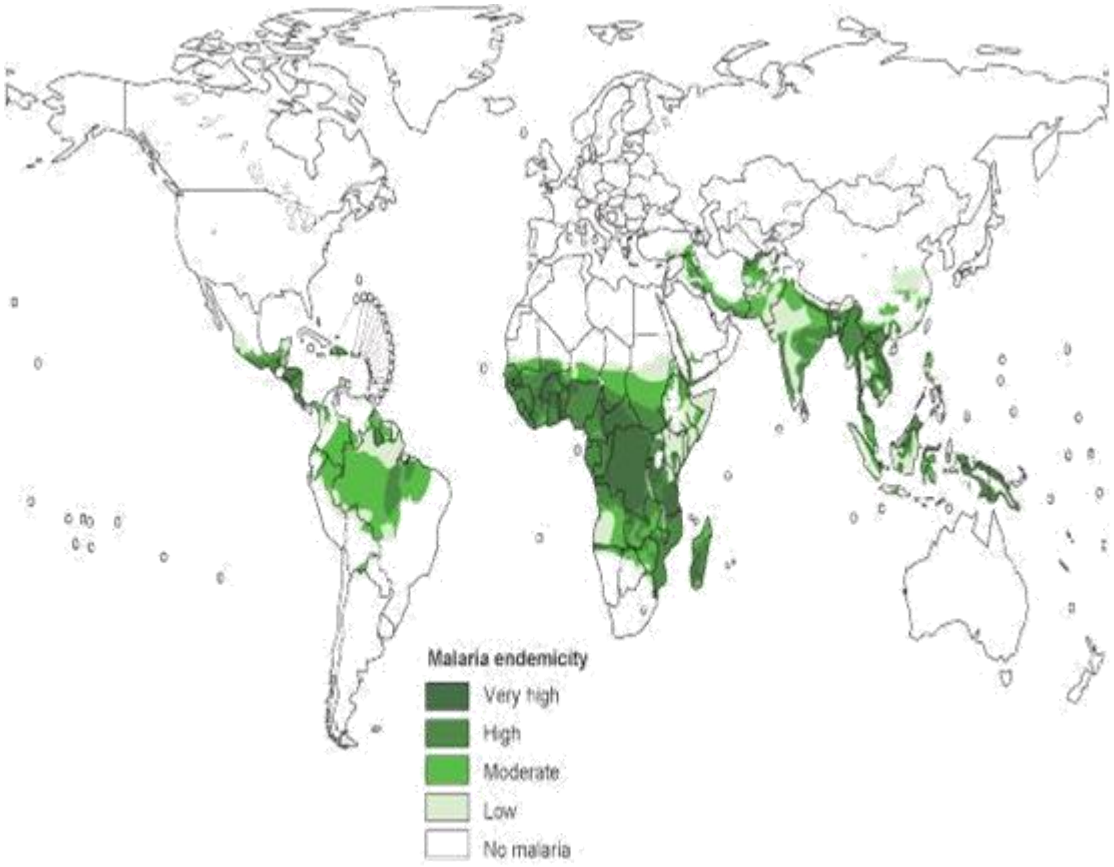

Figure 2.1: Global distribution of malaria transmission risk (World Malaria report, 2005).

Measuring malaria mortality is a public health problem that was faced with different solutions. Outside Africa, these figures typically depend on government reported deaths, modified in different ways to compensate for insufficient coverage. Neeraj Dhingra and colleagues use the findings of verbal autopsies to predict malaria mortality in India. Non-medical field staff performed verbal autopsies for 122,291 group deaths in 6,671 Indian sites between 2001 and 2003. Fieldworkers used verbal autopsies to determine fever cause and severity. Two qualified physicians coded the findings for death, and disputes were secretly adjudicated if there was no consensus. After adjudication, investigators reported that 3·6% of deaths between 1 month and 70 years of age were malaria-related. Deaths initially attributed to malaria by one of the two physicians (3·8%) were used as a possible upper bound and those subsequently rated by both physicians were used as lower bound (1·7%) (Hay et al., 2011).

2.1.2 Malaria in Sudan

Malaria remains a major health problem in Sudan despite the efforts of the National Malaria Control Program (NMCP), with significant morbidity and mortality due to the emergence of resistance of *P. falciparum* parasite to chloroquine, especially in children under five. At about 44,000 deaths in 2002, it caused 9 million incidents, accounting for 20-40% of the overall outpatient attendance and about 40% of admissions. In addition, 2,877,000 disability-adjusted life-years (DALYs, which is a disease burden measure) lost in Sudan in 2002 due to malaria mortality (WHO, 2005; Abdalla et al., 2007). Males face the highest pressure and mortality with more DALYs loses in females. The scope of malaria clinical presentation ranges from asymptomatic parasitemia, febrile fever, to serious potentially lethal disease in multiple transmission areas. It is due to factors such as parasite organisms and immune status of patients.

Severe malaria in Sudan is usually due to *P. falciparum*, however cases have also been described due to *Plasmodium vivax* (Mahgoub et al,, 2002).

2.2 Malaria parasite

Malaria is a mosquito-borne disease that affects several vertebrate species, including rodents, reptiles, birds, and primates. The infection is transmitted by the bite of an infected female Anopheles mosquito carrying a protozoan parasite of the genus Plasmodium. There are over 175 species of plasmodia currently recognized. Many of these are known to cause malaria in non-human vertebrates. However, human malaria can solely be caused by four Plasmodium species**:** *P. vivax*, *P. ovale*, *P. malariae*, and *P. falciparum*. Among these, *P. falciparum* is the most virulent and is responsible for almost all cases of severe disease and deaths. *P. falciparum* is the most common parasite in tropical Africa and exists in all malaria endemic regions worldwide while other species are variably distributed in different geographical locations. More than four hundred species of Anopheles were identified, but only seventy are known to transmit malaria to various vertebrates naturally. Human malaria parasites are transmitted by a few Anopheles species. *A. gambiae*, *A. arabiensis*, and *A. funestus* are the most prevalent in Africa. In contrast, other species such as *A. albimanus*, *A. culicifacies*, *A. dirus* and *A. anthropophagus* are found in other parts of the world (Hay &Snow, 2006).

2.2.1 Life cycle of Plasmodium falciparum

P. falciparum has a remarkably complex life cycle (Figure 2.2). The infection begins when sporozoites are released into the bloodstream by a female *Anopheles* mosquito. The inoculated sporozoites circulate before they invade the liver parenchymal cells,

where they undergo an asexual replicative phase called exo-erythrocytic schizogony. This liver stage of parasite lasts for approximately a week before releasing merozoites, which invade red blood cells (RBCs) (Siciliano & Alano, 2015).

Erythrocyte invasion is crucial for parasite survival. It provides a rich source of nutrients in a niche (the parasitophorous) that is primarily protected from host immune defense. The erythrocytic schizogony involves rapid successive mitotic divisions and generates thousands of merozoites that are released to re-invade new erythrocytes. This stage is responsible for the acute clinical episodes of malaria (Wurtz et al., 2011). After a period of 7 to 15 days of asexual replicative cycles in RBCs, a small proportion of the merozoites transform into male and female gametocytes, the micro- and macro-gametocytes. The half-life of the mature gametocyte in the blood is about 2.4 days during which it could be taken up by a feeding *Anopheles* mosquito. A few minutes after the mosquito's ingestion, the micro-gametocytes exflagellate in the insect midgut, each forming eight micro-gametes that move to fertilize the female macro-gametes. Eighteen to 24 hours after fertilization, the zygote elongates into an ookinete, which traverses the membranes and the epithelial cells of the mid-gut and transforms into an oocyst. The oocyst enlarges progressively as the nucleus repeatedly divides to form sporozoites. Mature sporozoites exit the oocyst to the coelomic cavity and migrate to salivary glands awaiting transfer to a new victim (Talman et al., 2010).Infectious mosquitoes inject sporozoites into the bloodstream of the human host, which then circulates through the blood to the liver. An exo-erythrocytic stage in the liver leads to merozoites escaping into the bloodstream. The merozoites invade the red blood cells and undergo an asexual replicative cycle (erythrocytic schizogony) to release several merozoites that can reinvade new red blood cells. Occasionally,

erythrocytic trophozoites turn into gametocytes that are released into the bloodstream (Talman et al., 2010).

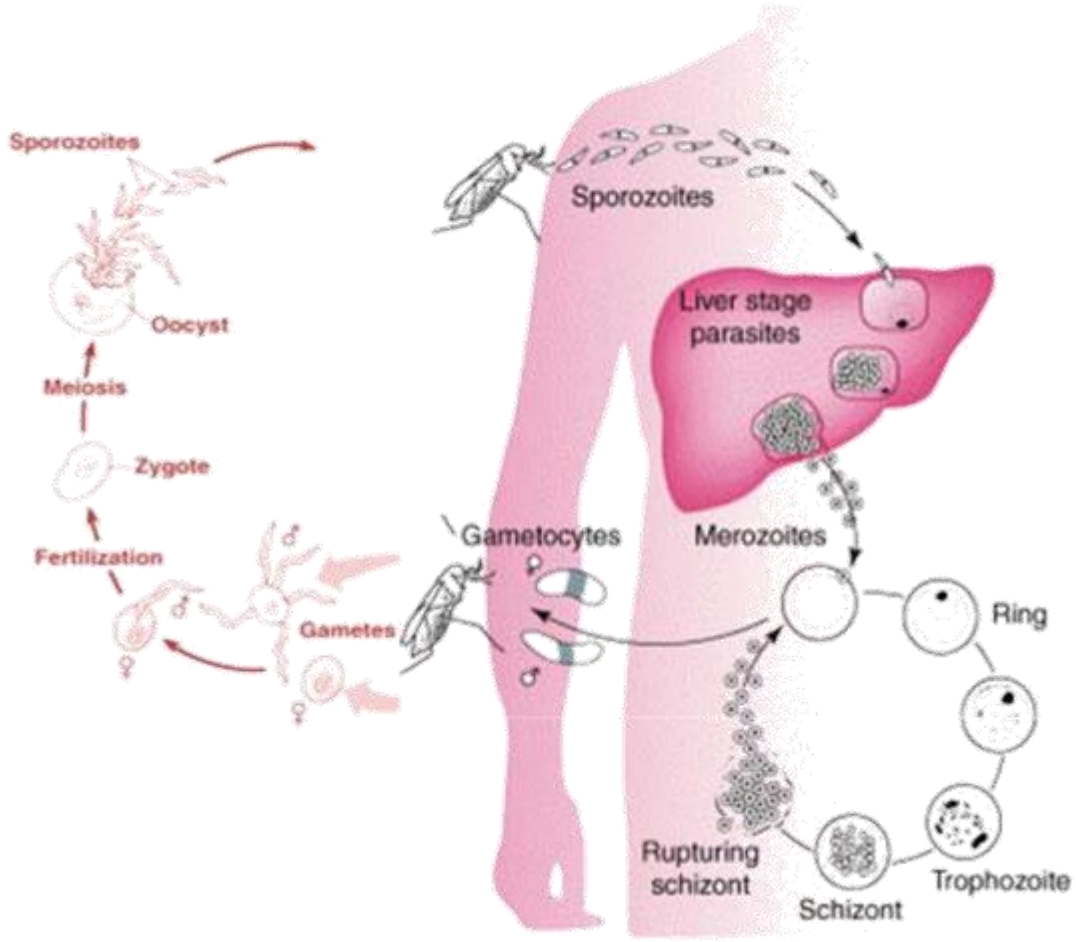

Figure 2.2: The life cycle of *Plasmodium falciparum*

(Modified from the Center of Biologics Evaluation and Research, US, Food and Drug Administration: www.fda.gov/Cber/blood/malaria071206sk.htm)

2.3 Immunity to malaria

The immune system plays an important role in the host defense against *P. falciparum* infection and in reducing the severity of the disease once it occurs. While most people living in endemic areas of malaria are disproportionately exposed to infectious bites from the *Anopheles* mosquitoes, a substantial proportion of the infected individuals either cure the infection spontaneously or remain asymptomatic, i.e. harboring any parasitemia without serious clinical disease. A small number of infected people, mainly young children, experience clinical malaria symptoms, and only fewer than 5% of such clinical cases result in severe life-threatening complications (Rowe et al., 1995). The age-dependent development of immunity to malaria significantly affects malaria's clinical pattern. Specific immune system components including antibody-dependent and cell-mediated mechanisms operate at different stages of the life cycle of the parasite (Figure 2.2) (Branch et al., 2001). Protective immunity against malaria, however, takes years of exposure to infection before it improves dramatically, and even with frequent infections, this immunity is rarely sterile and is unique to the organisms, variants, strains, and level. This inefficient, sluggish development of anti-malaria resistance is due to the virulence and heterogeneity of *P. falciparum* (Lee et al., 2008).

2.3.1 Innate immunity to malaria

Early interactions between sporozoites and host cells do not produce a significant systemic immune response and disease. The phases that elicit fast immuno-inflammatory responses are just merozoites. While not well known, the defense against malaria included many humoral components against innate immunity

comprising the complement proteins, C-reactive proteins, and mannose-binding lectins. Nonetheless, the major inborn effector mechanisms against malaria parasites are regulated by cellular components, including monocyte-macrophages, natural killer cells, naturally occurring T-cells, and cells (Boldt et al., 2009). One of the first instances of innate resistance to *P. falciparum* infection is the activation of monocyte-macrophages. Infected red blood cells (iRBCs) are actively involved in phagocytosis and clearance of circulating monocytes and tissue-resident macrophages in the liver, spleen, and other tissues (Turrini et al., 2003).

Malaria antigens, such as hemozoin pigment and membrane-anchored glycosylphosphatidylinositol, macrophages phagocytosis or surface receptor action, activate these cells to produce a series of pro-inflammatory mediators and cytokines. Inflammatory agents produced may have direct anti-parasite activity or may contribute to the activation of other immune mechanisms. However, these cells are also involved in severe malaria (Ferro et al., 2008). Natural killer T cells (NKTs) are a specialized subset of lymphocytes that share the properties of both T cells and NK cells. They differ from conventional T-cells in that; they express a distinct NK.1.1 marker and possess a unique single invariant Vш14 + antigen receptor that recognizes lipids and glycolipids presented by CD1d molecules rather than peptide-MHC complexes (Kinam Park, 2014). Experimental models have shown the role of NKTs in providing protection against murine malaria (Morris Jones et al., 1996). NKT cells were linked to malaria-associated splenomegaly and to the expansion of the splenic B cell pool forming a parasite-specific antibody (Tsunoda et al., 2009).

Gamma/delta (/) T cells are a minor subset of T lymphocytes characterized by an antigen receptor formed by and chains rather than the common 5-007 and β chains that make up the antigen receptors in conventional T cells (Walker et al., 2016). Peripheral / T cells are strongly regulated during malaria and respond to malaria antigens through proliferation and lymphokine production. Activated γ/δ T-cells but not T-cells from malaria-naïve donors have been shown to inhibit parasite replication in erythrocytes *in vitro*, indicating an essential innate role protection against malaria (Morris Jones et al., 2018). Natural killer (NK) cells interact with iRBCs and release pro-inflammatory cytokines, particularly IFN- γ. In non-immune donors, NK cells may kill plasmodia-infected erythrocytes in the absence of opsonizing antibodies through direct cell-cell interaction. Early production of IFN-γ NK cells stimulated by IL-12 during malaria infection is associated with a better prognosis of the disease (Sam & Stevenson 2015).

In addition, NK interaction with iRBCs induces the production of chemoattractant IL-8, suggesting the role of NK cells in the recruitment and activation of other leukocytes during malaria infection. Production of chemoattractant IL-8, suggesting the role of NK cells in the recruitment and activation of other leukocytes during malaria infection (Gardiner, 2014). Natural killer T cells (NKTs) are a specialized subset of lymphocytes that share the properties of both T cells and NK cells. They differ from conventional T-cells in that; they express a distinct NK.1.1 marker and possess a unique single invariant V5-00714 + antigen receptor that recognizes lipids and glycolipids presented by CD1d molecules rather than peptide-MHC complexes (Lantz & Bendelac, 2012).

2.3.2 Adaptive immunity to malaria

2.3.2(a) Anti-sporozoite immunity

Malaria protection may be induced in animal models and humans following immunization with irradiated sporozoites. However, it confers complete resistance to subsequent sporozoite challenges; such protection is stage-specific and does not withstand problems with Plasmodium blood-stage. Anti-sporozoic antibodies are the main agents that promote phagocytosis and inhibit the invasion of hepatocytes (Mccutchan et al., 2014). These antibodies are primarily directed against the most abundant sporozoic surface proteins, circumsporozoic proteins (CSPs), thrombospondin-associated adhesive proteins (TRAPs), and several other sporozoic and liver-stage antigens. Most adults in malarious areas have been found to have a high titer of *P. falciparum* CSP antibodies and an immune sera from sporozoite-immunized human volunteers. Naturally, infected individuals have been found to block the invasion of hepatocytes by *P. falciparum* sporozoite (Berzins et al., 1986).

2.3.2(b) Immunity to liver stages of malaria parasite

Several cellular mechanisms are implicated in generating protective immunity against Plasmodium's liver stages (Connelly et al., 2004). T cells were shown to be involved in the generation of protective immunity in rodents immunized with irradiated sporozoites). The passive transfer of CD8+ T cells and CD4+ T cells clones derived from sporozoite immunized mice was found to confer protection Cytotoxic T lymphocyte response to CS proteins was observed in field studies and believed to protect against pre-erythrocytic stages of malaria (Hill et al., 2002).

2.3.2(C) Immunity to blood stages of malaria parasite

The invasion of red cells is a crucial step in malaria infection and is the cause of clinical disease. Hence erythrocytic stages of *P. falciparum* are likely to be the most critical targets for protective immune responses. Protective immunity against erythrocytic stages involves antibody-dependent and cell-mediated immunity (Sanni et al., 2002).

2.3.2(d) Antibody mediated immunity to blood stages of *P. falciparum*

Passive transfer of anti-*Plasmodium* specific antibodies to non-immune mice and treatment of *P. falciparum*-infected patients with immunoglobulins isolated from normal immune individuals leads to a decrease in parasitemia and clinical symptoms (Nnedu et al., 2011). Moreover, studies in gene-targeted and B-cell-deficient mice have shown that B-cells are essential for the resolution of blood-stage murine malaria infections. Thus, protection from disease was thought to be primarily an antibody-mediated response (Langhorne, 2012). In people living in endemic malaria areas, exposure to repeated infections with different variants of *P. falciparum* induces the production of polyclonal antibodies, predominantly IgM, IgG isotypes, and acquired malaria protective immunity correlates with age and the level of antibodies to asexual blood-stage parasite antigens. Consequently, a clinical episode in a child living in an endemic malaria zone represents an infection with a parasite that expresses VSA that is not recognized by the existing antibody repertoire (Maddison, 2016).

3.3.2(e) Cell-mediated immunity to blood stages of *P. falciparum*

Brown and co-workers made the first observations showing the importance of T lymphocytes in recovery from malaria infections in their thymectomized rat experiment (Brown et al., 2011). Later, it was shown that B-cell-deficient mice can be

immunized by natural Plasmodium infection and that the adoption of $CD4^+$ T-cell clones could provide protection against malaria (Brown et al., 2002). However, as erythrocytes do not express MHC molecules, it seems unlikely that T cells could have direct cytotoxic effects on infected erythrocytes. Alternatively, it was suggested that T cells respond to malaria antigens by secreting cytokines. Consistent with this view, it has been shown that stimulation of T cells by malaria antigens presented by antigen-presenting cells (APCs) results in the release of various cytokines. Gene-knocked-out mice experiments have shown that immune responses to the blood stages of Plasmodium are mostly mediated by T-lymphocytes (Plebanski et al., 2002). Both the Th1 and Th2 types of $CD4^+$T cells were involved in the protection of malaria and pathogenesis in mice. It has been shown that, during the acute phase of the murine infection with *Plasmodium chabaudi chabaudi* AS, $CD4^+$ T cells of the type Th1 produced by IL-2 and IFN- are essential, whereas the type Th2 of these cells is required during the later stages to provide assistance to B-cells through IL-4 secretion (Taylor-Robinson & Phillips, 2013). The secretion of IFN- by T lymphocytes is believed to induce cytophilic IgG blood-stage-specific antibodies and to aid in antibody-dependent cellular inhibitor mechanisms (Taylor-Phillips, 1994).

2.4 Immuno-pathogenesis of severe malaria

Malaria is a very complicated disease. Several factors determine the clinical outcome of *P. falciparum* infection, including the intensity of parasite transmission, parasite virulence, multiple host genetic, nutritional, and immunological characteristics (Rowe et al., 1995; Idro et al., 2010). However, mechanisms leading to serious life-threatening complications of malaria are not fully understood (Rowe et al., 1995; Lundblom et al.,

2013). The mechanisms underlying the pathogenesis of severe malaria remain controversial and several assumptions have been made to explain the cause of this deadly syndrome (Conroy et al., 2010; Lundblom et al., 2013). The mechanical (sequestration) hypothesis assumes that *P. falciparum*-parasitized erythrocytes bind to post capillary veneers, hindering blood flow, thereby reducing tissue perfusion and reducing waste disposal leading to brain pathology (Obradovic, 2005; Kunwittaya et al., 2014). Histological examination of brain sections of fatal cases of malaria revealed sequestration of iRBCs in multiple organs and tissues (MacKenzie et al., 2008). However, due to lack of sequestration in some patients with cerebral symptoms and the fact that most patients with malaria recover fully without evidence of ischaemic damage, the mechanical hypothesis cannot explain the cause of CM properly. Alternatively, it has been suggested that malaria toxins bind and stimulate monocytes to secrete pro-inflammatory cytokines (Stevenson & Ghadirian, 1989; Carter et al., 2005). Although cytokines themselves are not harmful, they may induce the production of nitric oxide (NO), which spreads through the blood-brain barrier and interferes with the synaptic function as a general anesthetic, leading to a reduced state of consciousness (Craig et al., 2012). Several malaria antigens, such as membrane-anchored glycosylphosphatidylinositol (GPI) and the by-product of parasite metabolism, hemozoin, may cause the release of cytokines from various host immune cells (Gallego-Delgado et al., 2015). Released cytokines up-regulate the expression of different endothelial adhesion receptors, such as ICAM-1, PECAM-1 / CD31, and thus enhance iRBC cyto-adhesion and sequestration (Bagot et al., 2002; Craig et al., 2012). Sequestered parasites in the brain vasculature may release more toxins locally and induce inflammatory mediators that disrupt brain function and metabolism leading to severe malaria.

2.5 Role of cytokines in immuno-pathogenesis of malaria

Cytokines have been shown to be critical molecular markers of cell-mediated immune response and are known to be key players in malaria control. Studies have shown that cytokines are associated differently with symptoms of disease, parasitemia, disease severity, and complications (Hechavarría et al., 2013). Effective proinflammatory cytokine response is needed to address parasitemia and control malaria infection (Freed-Pastor & Prives, 2012). In addition, enhanced IFN- levels have been seen as a surrogate marker for protection of childhood malaria (Fiore & et al., 2008). Increased TNF-5-007 stimulated phagocytosis and improved clearance of parasitized erythrocytes (Autino et al., 2012).

On the other hand, proinflammatory cytokines appear to contribute to adverse disease outcomes as the prolonged and robust response of cytokines such as TNF-5-007, IFN- , IL-8, and IL-18 has been associated with severe disease syndromes in both human and experimental models (Hansen & Schofield, 2010; Malaguarnera et al., 2010). Severe malaria has been shown to be induced by IL-18 through IFN- production of Th1 cells and NK cells, primarily in the presence of IL-12 (Plant et al., 2001). Reduced plasma levels of TGF-β have been seen in Gabonese children with severe malaria (Malaguarnera et al., 2010; Perkins et al., 2011; Sawian et al., 2013). In contrast, higher TGF-β was observed under critical conditions of malaria in Burkina Faso (Berg et al., 2014;). A recent study in Fulani and non-Fulani ethnic groups in Mali showed that IL-1β was found at a high level while IL-10, a cytokine that limits the production of proinflammatory cytokines and chemokines seen during *P. falciparum* infection, was found to be low in Fulani compared to non-Fulani groups (Tatematsu et al., 2018). In the same area and from the same research group, Fulani

has been shown to have higher levels of several inflammatory cytokines compared to the non-Fulani ethnic group, in particular IFN-(McCall et al., 2010).

Figure 2.3 shows the possible regulation of adaptive immunity to blood-stage malaria by cytokines produced by cells of the innate immune response. Maturation of DCs has associated with the upregulation of expression of MHC class II molecules, CD40, CD80, CD86 and adhesion molecules, and the production of cytokines including interleukin-12 (IL-12). IL-12 activates natural killer (NK) cells to produce IFN-γ and induces the differentiation of T helper 1 (TH1) cells. The production of cytokines, particularly IFN-γ, by NK cells, results in DC maturation. It enhances parasite-derived maturation stimuli, facilitating the clonal expansion of antigen-specific naive CD4+ T cells. IL-2 produced by antigen-specific TH1 cells further activates NK cells to produce IFN-γ, which induces DC maturation and activates macrophages, further amplifying the adaptive immune response. Cytokines such as IL-10 and transforming growth factor-β (TGF-β) negatively regulate both innate and adaptive responses. NO, nitric oxide; TCR, T-cell receptor; TNF, tumour-necrosis factor (Chen et al., 2014).

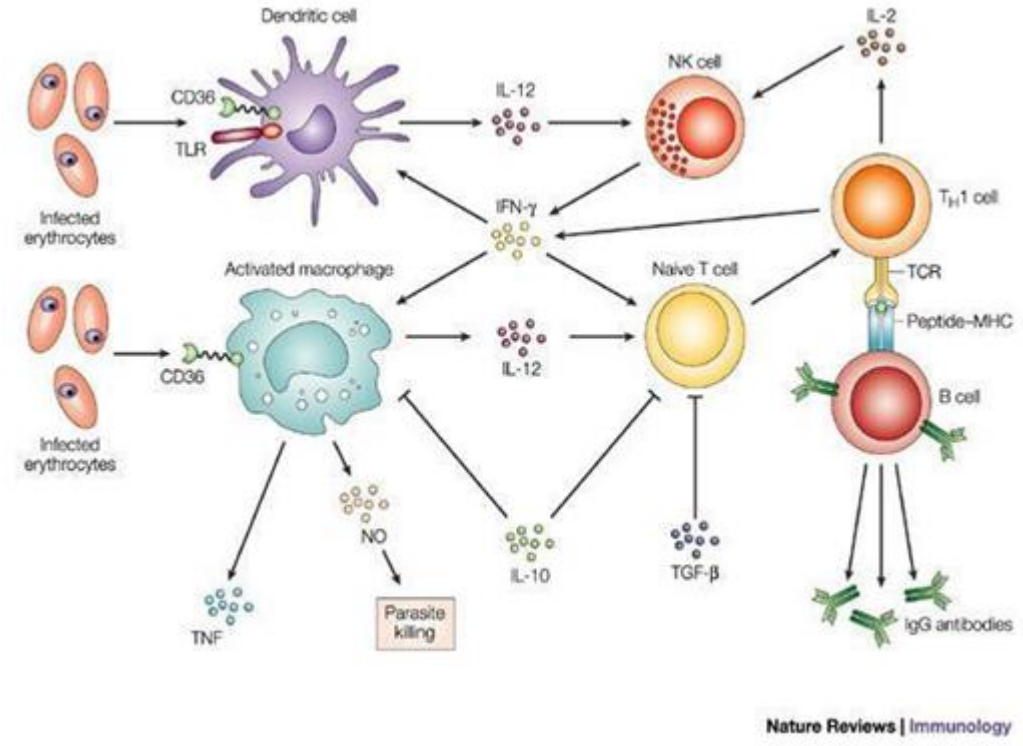

Figure 2.3: Linking innate and adaptive immunity to blood-stage malaria (McKenna et al., 2005)

2.6 The role of IFN-γ in malaria disease

IFN-γ (or sometimes also indicate as IFNG) secretion by innate and adaptive immune cells is essential for the control of various diseases including malaria. It is an essential cytokine in controlling *Plasmodium* infection in the liver and blood stages of the parasite life cycle. It can also exacerbate the severity of the malarial disease, depending on the temporal and spatial production of IFN-γ (King & Lamb, 2015; Rowe & Wright, 2020).

Plasmodium infection induces IFN-γ production from various innate and adaptive immune cell subsets at different stages of the life cycle (Figure 2.4). Various immune cell subsets produce IFN-γ in response to Plasmodium infection. NK, γδ, and NKT cells are primarily responsible for the early production of IFN-γ in response to the parasite's liver and blood stages and play a role in the first control of parasite growth. IFN-γ-producing CD8+ T cells have also been shown to limit intrahepatic parasite growth through an IFN-γ-inducible, nitric oxide-dependent mechanism (Sanches-Vaz et al., 2019). Once an adaptive immune response is initiated, IFN-γ produced by CD4+ T cells optimally activates CD8+ T cells, B cells, and macrophages. IFN-γ influences isotype switching in B cells leading to the production of cytophilic antibodies capable of binding free parasites and blocking red blood cell invasion (Nasr et al., 2014). Besides, this cytokine is also mediating parasite clearance through opsonization (Jagannathan et al., 2014) and binding the surface of infected red blood cells to promote antibody-dependent phagocytosis (King & Lamb, 2015). Production of IFN-γ from CD4+ T cells also optimally activates macrophages to phagocytose infected red blood cells and free parasites (Harawa et al., 2019). All of these mechanisms are important for optimal control of parasite growth during Plasmodium