THE ROLE OF TLR-2 IN THE PRODUCTION OF TNF-α AND IL-10 BY MACROPHAGE INFECTED WITH A RECOMBINANT BCG (rBCG) EXPRESSING THE MSP-1C OF *Plasmodium falciparum*

SASIREKHA D/O RAJENDRAN

SCHOOL OF HEALTH SCIENCES
UNIVERSITI SAINS MALAYSIA
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%	Percent
&	And
<	Less than
>	More than
±	Plus/minus
°C	Degree Celsius
µ	Micro
µl	Microliter
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cells
BCG	Bacillus Calmette-Guérin
rBCG	Recombinant BCG
CDC	Centers for Disease Control and Prevention
DC	Dendritic cells
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
PBS	Phosphate buffered saline
g	gram

HRP	Horseradish peroxidase
IFN	Interferons
IL	Interleukin
Ig	Immunoglobulin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
MOI	Multiplicity of infection
MHC	Major histocompatibility complex
ml	Milliliter
L	Liter
MSP-1	Merozoite Surface Protein-1
MSP-1C	C-terminus Merozoite Surface Protein-1
NK	Natural killer cells
Th	T helper
TLR	Toll-like receptor
NO	Nitric oxide
OD	Optical density
PBS	Phosphate buffered saline
PfEMP-1	*P.falciparum* erythrocyte membrane protein 1
pRBC	parasitized red blood cells
rpm	Rotation per minute

SD	Standard deviation
SE	Standard error
Sec	Seconds
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UK	United Kingdom
USA	United States of America
WHO	World Health Organization
κ	Kappa
α	Alpha
γ	Gamma
β	Beta

ABSTRAK

Mycobacterium bovis BCG merupakan vaksin yang digunakan pada masa ini untuk mencegah penyakit tuberkulosis. Kehadiran glikolipid seperti lipoarabinomannan (LAM) dan lipomannan (LM) pada dinding sel mikobakteria ini merangsang BCG sebagai vektor vaksin rekombinan untuk patogen lain, termasuk parasit malaria. Faedah mengunakan BCG sebagai vaksin rekombinan termasuklah keupayaanya untuk dicernakan oleh sel persembahan antigen (APC) seperti makrofaj bagi merangsang gerak balas pro-inflammatori yang merupakan mekanisme pertahanan semula jadi yang penting terhadap parasit malaria. Interaksi antara BCG dan makrofaj melibatkan beberapa TLR seperti TLR-2. Oleh itu, kajian ini dijalankan untuk menentukan peranan TLR-2 dalam penghasilan sitokin inflamatori seperti TNF-α dan IL-10 oleh sel makrofaj (J774A.1) yang dijangkiti klon BCG dan rBCG yang mengekspreskan antigen MSP-1C *Plasmodium falciparum*. Rembesan TNF-α dan IL-10 sitokin oleh makrofaj yang dijangkiti dalam ketidakhadiran atau kehadiran TLR-2 telah ditentukan dalam supernatan makrofaj melalui analisis ELISA. Hasil kajian kami menunjukkan bahawa, paras TNF-α and IL-10 sitokin yang lebih tinggi telah dikesan dalam supernatan makrofaj yang dijangkiti oleh BCG dan rBCG sama ada dalam ketidakhadiran atau kehadiran inhibitor TLR-2. Walau bagaimanapun, paras TNF-α dan IL-10 sitokin yang dihasilkan oleh makrofaj yang dijangkiti tanpa kehadiran TLR-2 adalah jauh lebih tinggi berbanding dengan makrofaj yang dirangsang dengan TLR-2. Kesimpulanya, kehadiran inhibitor TLR-2 tidak menghalang penghasilan TNF-α dan IL-10 oleh makrofaj. Oleh itu, keputusan ujian ini mencadangkan bahawa TLR-2 tidak memainkan peranan yang penting dalam merangsang penghasilan TNF-α dan IL-10 oleh makrofaj yang dijangkiti BCG atau rBCG.

ABSTRACT

An attenuated strain of *Mycobacterium bovis* BCG is the only available vaccine used for tuberculosis so far. The presence of glycolipids such as lipoarabinomannan (LAM) and lipomannan (LM) in its cell wall has encouraged the use of BCG as a recombinant vaccine vector for other pathogens, including malaria parasites. The benefits of using BCG as a recombinant vaccine vector include its ability to be ingested by professional antigen presenting cells (APCs) such as macrophage to induce pro-inflammatory responses, an important innate host defense mechanism against malaria infection. The interaction between BCG and macrophage involves several toll like receptors (TLRs) such as TLR-2. Therefore, this study was conducted to determine the role of TLR-2 in the production of inflammatory cytokines such as TNF-α and IL-10 by murine macrophage cell line, J774A.1 infected with BCG and recombinant BCG (rBCG) clones expressing the MSP-1C of *Plasmodium falciparum*. The secretion of TNF-α and IL-10 cytokines by the infected macrophages in the absence or presence of TLR-2 was determined in the supernatant of the infected macrophages by enzyme-linked immunosorbent assay (ELISA). Our result showed that, higher levels of TNF-α and IL-10 were detected in the supernatant of BCG and rBCG infected macrophages either in the absence or presence of TLR-2 inhibitor. However, the levels of TNF-α and IL-10 cytokine production by infected macrophages in the absence of TLR-2 was significantly higher than macrophages stimulated with TLR-2. In conclusion, blocking of TLR-2 does not reduce the TNF-α and IL-10 production by the macrophages. Therefore, this result suggested that TLR-2 does not play important role in stimulating the production of TNF-α and IL-10 by macrophages infected with either BCG or rBCG clone.

CHAPTER 1

INTRODUCTION

Malaria is the world's most widespread infection. According to the World Malaria Report 2011, malaria is prevalent in 106 countries of the tropical and semitropical world, with 35 countries in central Africa. Until today, malaria still one of the most important disease in Malaysia especially in the rural area of the coastal region. This is because Malaysia is located within the equatorial zone with high temperatures and humidity, factor that are usually important for the transmission of malaria. *Plasmodium knowlesi* followed by *P. falciparum* causes the most serious form of disease in Malaysia (William *et al.*, 2013). Various intervention and eradication programs have been implemented by the World Health Organization (WHO) and non-governmental organizations (NGOs), but the prevalence of malaria is increasing, especially in young children. This problem might be due to various possible contributing factors such as genetic diversity, the emergence of multidrug resistance strains (Babiker, 1997), socioeconomic status of people in the malaria hotspot regions and environmental conditions, including climate change (Divya *et al.*, 2014). Given the urgent need to develop an effective malaria vaccine to relieve the growing malaria burden, it is important that full consideration be directed to the data emerging from the studies of innate immunity to infection with blood stage *Plasmodium* parasites.

The innate immune cells such as dendritic cells (DC), macrophages and natural killer (NK) cells play a fundamental role in shaping the adaptive immune response to blood stage malaria. Innate immune responses are also likely to be necessary downstream for the

generation of memory T-cell as well as B-cell responses leading to immunity against the clinical symptoms associated with malaria. The information derived from studies on innate immunity to blood stage malaria may also provide information for the development of novel immunotherapies to alleviate the tremendous burden of malaria. Harnessing the information on innate immune responses to blood stage malaria, for the development of an effective vaccine will require additional studies in several critical areas. One of these areas includes the identification of host receptors and parasite ligands important in initiating these responses (Urban, 2005). However knowledge regarding the mechanism by which malaria parasite was eliminated by the host immune system is still not fully understand.

Development of recombinant DNA using live vectors from viruses and bacteria to deliver foreign antigens to the immune system has become a popular technique nowadays for developing new generation vaccines (Britton, 2003). *Mycobacterium bovis* bacille Calmette-Guérin (BCG) is the most extensively used vector for developing recombinant vaccines for preventing malaria disease. Using this strategy, rBCG clone which expressing C-terminus of the merozoite surface protein-1 (MSP-1C) of *P. falciparum* was cloned in a previous research (Nurul *et al.*, 2010). Moreover, previous studies proved that the rBCG clone capable of stimulating pro-inflammatory cytokine production such as TNF-α in mouse macrophage cell line (Rapeah, 2010). However, the mechanism by which the presence of MSP-1C in BCG renders rBCG that capable to induce more inflammatory responses than parental BCG and LPS is unknown. Therefore, we speculated that the presence of MSP-1C antigen in the rBCG clone increased the role of toll like receptor such as TLR-2 as a pathogen recognition receptor in macrophage, thus enhancing cellular

activation of the cell. Engagement of TLR-2 in enhancing pathogen recognition and production of immuno-regulatory cytokine in mouse macrophage infected with rBCG were higher than in macrophage infected with BCG. Therefore in this study, we aim to investigate the involvement of TLR-2 in enhancing pro-inflammatory cytokine production such as TNF-α and IL-10 in mouse macrophage infected with the rBCG clone. An understanding the role of TLRs pathway is essential to improve the binding and the presentation of the vaccine to the immune system.

Objectives of study

To investigate the role of TLR-2 in the production of TNF-α and IL-10 by macrophages infected with *Mycobacterium bovis* bacille Calmette-Guérin (BCG) and recombinant BCG (rBCG) clone expressing the C-terminus of the merozoite surface protein-1 (MSP-1C) of *Plasmodium falciparum*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria is a life-threatening disease caused by *Plasmodium* parasites that are spread to the people through the bites of infected Anopheles mosquitoes. *P. falciparum* is endemic in most parts of sub-Saharan Africa where it causes the greatest devastation being primarily responsible for the huge toll of young lives lost due to malaria (Snow *et al.*, 2005). Malaria incidence is determined by a variety of factors, particularly the abundance of anopheline mosquito species, human behavior, and the presence of malaria parasites (Martens, 1995). Indirectly, climate change could also have an effect by influencing environmental factors such as rainfall and temperature, even when there's plenty of rainfall to produce breeding pools for the Anopheles mosquitoes that spread malaria, hot temperatures can hamper mosquito development (Impoinvil, 2007).

Half the world's population is at risk for malaria. children under five are at particular risk. who represent 77% of all malaria deaths. Children are at risk because they lack of developed immune systems to protect against the disease (WHO, 2013). Pregnant women living in endemic regions also affected by malaria (Dellicour, 2010). Pregnancy reduces immunity to malaria, increasing the risk of infection which lead to severe illness, and death for the woman. Moreover, other adverse outcomes include spontaneous abortion, stillbirth, low birth weight, and neonatal death (WHO, 2012). Although it is a preventable and treatable infection (CDC, 2014), many challenges continue to complicate malaria control

efforts in hard-hit areas, including poverty, poor sanitation, weak health systems, limited disease surveillance capabilities, drug and insecticide resistance, natural disasters, armed conflict and migration (Senior, 2008).

2.2 Prevalence of world malaria

According to the latest estimates, malaria mortality rates were reduced by about 42% globally and by 49% in the African region between 2000 and 2012. During the same period, malaria incidence rates declined by 25% around the world, and by 31% in the African region. However, this progress is no cause for complacency. The absolute numbers of malaria cases and deaths are not going down as fast as they could. The disease still took an estimated 627 000 lives in 2012, mostly those of children under five years of age in Africa (WHO, 2013). There are 43 countries with ongoing malaria transmission, of this Africa accounts for the majority of estimated malaria cases (80%) and deaths (90%), but only about 13% of the world's population (Figure 2.2).

There are 10 countries with ongoing malaria transmission in South East Asia (Figure 2.3) which accounts for 27 million (13% of estimated cases worldwide), the second highest number after Africa. India, Indonesia, and Myanmar comprise most of the region's estimated malaria cases (97%). However, Bangladesh, Bhutan, the Democratic Republic of Korea, Nepal, and Sri Lanka have made notable achievements in reduced the malaria incidence by 75% between the years 2000 and 2012. In the Eastern Mediterranean, Pakistan, South Sudan, and Yemen made up 88% of the region's estimated malaria cases in 2012 (WHO, 2013).

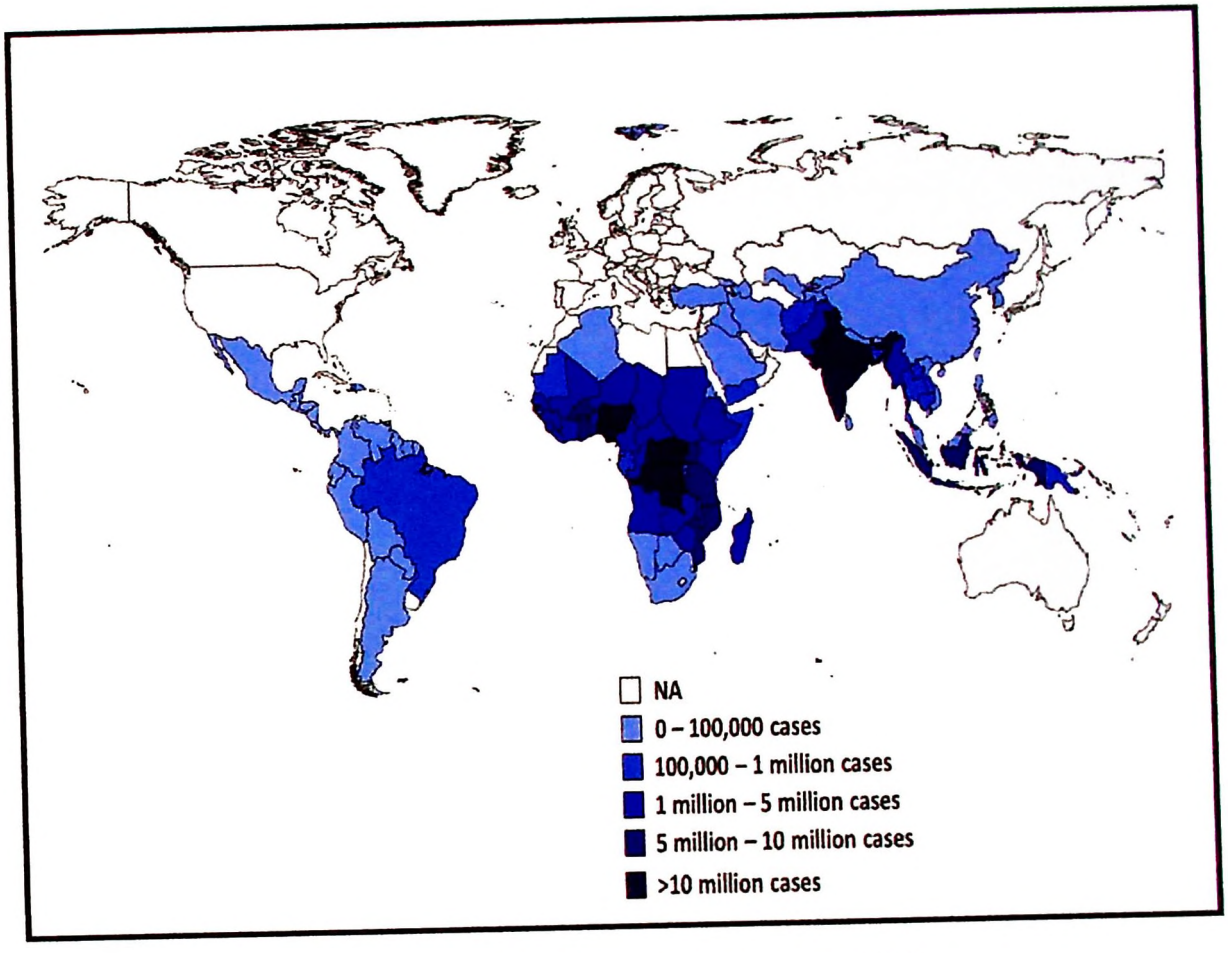

Figure 2.2: Estimated malaria cases 2012 (World Malaria Report, 2013)

2.3 Malaria in Malaysia

Malaria has been reported in Malaysia even before 1900's. In 1990, there were 50,500 cases in Malaysia. A decade later, in the year 2000, the number of reported cases has reduced to 12,705 cases. In 2012, there were 4,725 cases which is a 63% reduction compared to year 2000. There has also been a reduction in the number of malaria deaths from 43 in 1990 to 35 in 2000 and to 16 deaths in 2012. In Peninsular Malaysia: Selangor, Pahang, Kelantan and Perak reported more than 100 cases throughout the year 2012. *P. knowlesi* followed by *P. falciparum* causes the most serious form of disease in Malaysia (William *et al.*, 2013).

However in order to prevent malaria, The Malaria Eradication Programme which was introduced by Malaysia in 1960 had reached the significant milestone of having less than 1 case per 1,000 populations. The National Malaria Elimination Strategic Plan also introduced in the same year with the target of "malaria free" status by 2020. Although several control activities have reduced the incidence of malaria in Malaysia, it is still a major public health problem in the less developed parts of the country. In addition, the greatest challenge for Malaysia in controlling malaria is the frequent travel by people to malaria endemic countries, the presence of illegal immigrant and also the influx of workers from malaria endemic countries in search of work have kept the number of imported malaria cases on the constant high (Vector Borne Disease Sector, 2013).

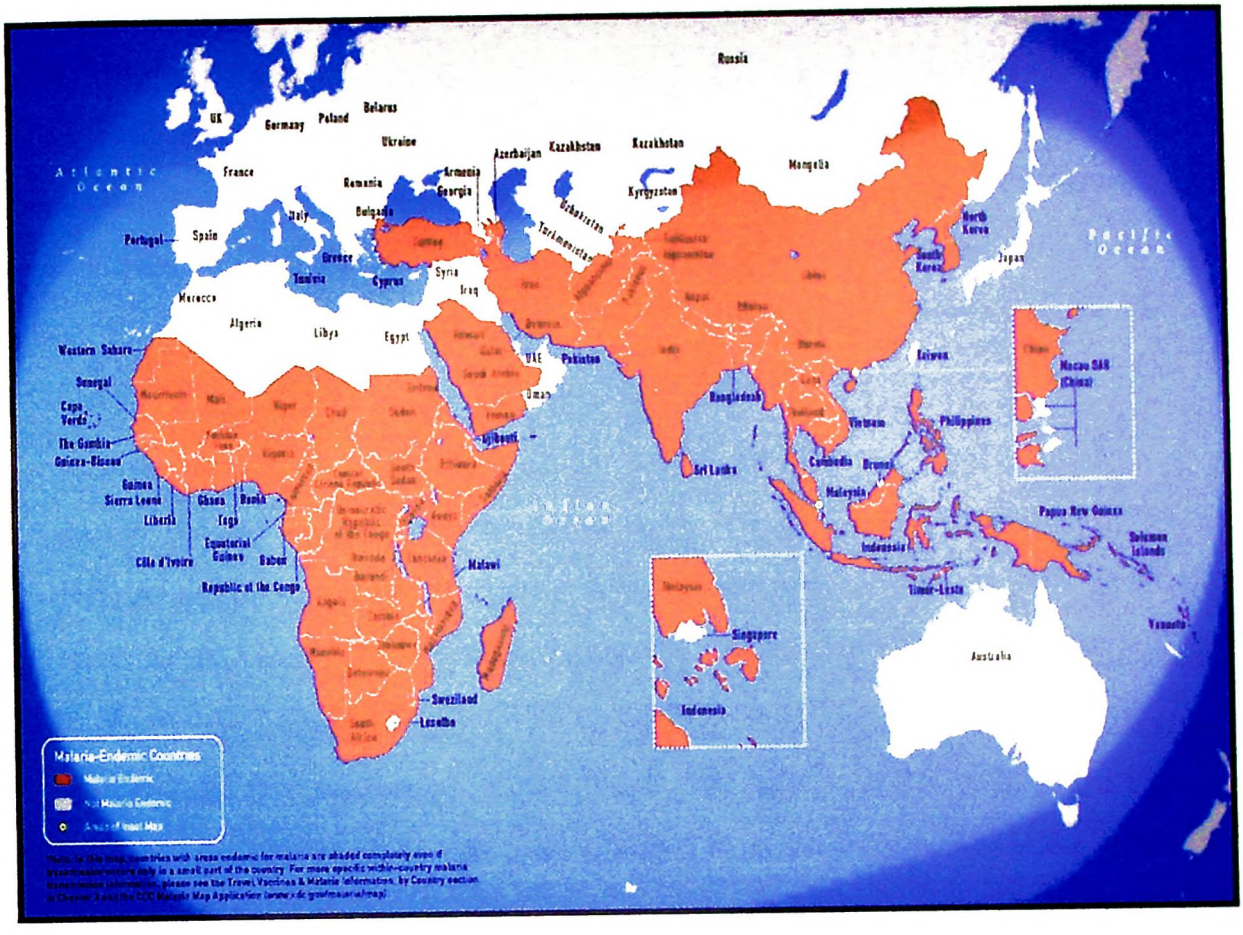

Figure 2.3: Asia prevalence of malaria (http://www.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-infectious-diseases-related-to-travel/malaria.htm).

2.4 Malaria parasite

Four *Plasmodium* species namely, *P. falciparum, P. vivax, P. malariae* and *P. ovale* are responsible for human malaria. Of these, *P. falciparum* and *P. vivax* are the most common and *P. falciparum* is the most virulent. *P. vivax* causes an acute, febrile illness but is rarely fatal. Due to its complete dependence on interaction with the duffy antigen receptor for chemokines (DARC) for invasion of human erythrocytes (Miller, 1976), *P. vivax* is not commonly found in Sub Saharan Africa where greater than 90% of individuals are duffy negative. The vast majority of people living at any risk of *P. falciparum* transmission worldwide is in South East Asia (62.3%), followed by Africa (27.7%), the Middle East (6.1%), and the Americas (3.8%) (Snow *et al.*, 2008).

2.4.1 Life cycle of *P. falciparum*

Malaria parasite has a complex life cycle (Figure 2.4). Infection in human begins when sporozoites, the infective stages, are injected by a mosquito and are carried around the body until they invade liver hepatocytes where they undergo a phase of asexual multiplication (exoerythrocytic schizogony) resulting in the production of many uninucleate merozoites. These merozoites flood out into the blood and invade red blood cells where they initiate a second phase of asexual multiplication (erythrocytic schizogony) resulting in the production of about 8-16 merozoites which invade new red blood cells. This process is repeated almost indefinitely and is responsible for the disease, malaria. As the infection progresses, some young merozoites develop into male and female gametocytes that circulate in the peripheral blood until they are taken up by a female anopheles mosquito when it feeds. Within the mosquito the gametocytes mature into male and female gametes,

fertilization occurs and a motile zygote (ookinete) is formed within the lumen of the mosquito gut, the beginning of a process known as sporogony. The ookinete penetrates the gut wall and becomes a conspicuous oocyst within which another phase of multiplication occurs resulting in the formation of sporozoites that migrate to the salivary glands of a mosquito and are injected when the mosquito feeds on a new host (Cox, 2010).

Each of the lifecycle stages of *P. falciparum* has been targeted by potential vaccine candidates. Vaccines may be directed against three antigenically distinct stages. These include pre-erythrocytic antigens comprising the sporozoite and liver-stage antigens, the asexual blood-stage antigens formed within the erythrocytes, as well as the mosquito stages of the parasite lifecycle. Various types of vaccine constructs including subunit, multi-subunit and whole-cell strategies present the parasite epitopes to the host immune system, with the aim of priming it for subsequent attack. So far, none of the potential vaccines have been successful enough for clinical use (WHO, 2010).

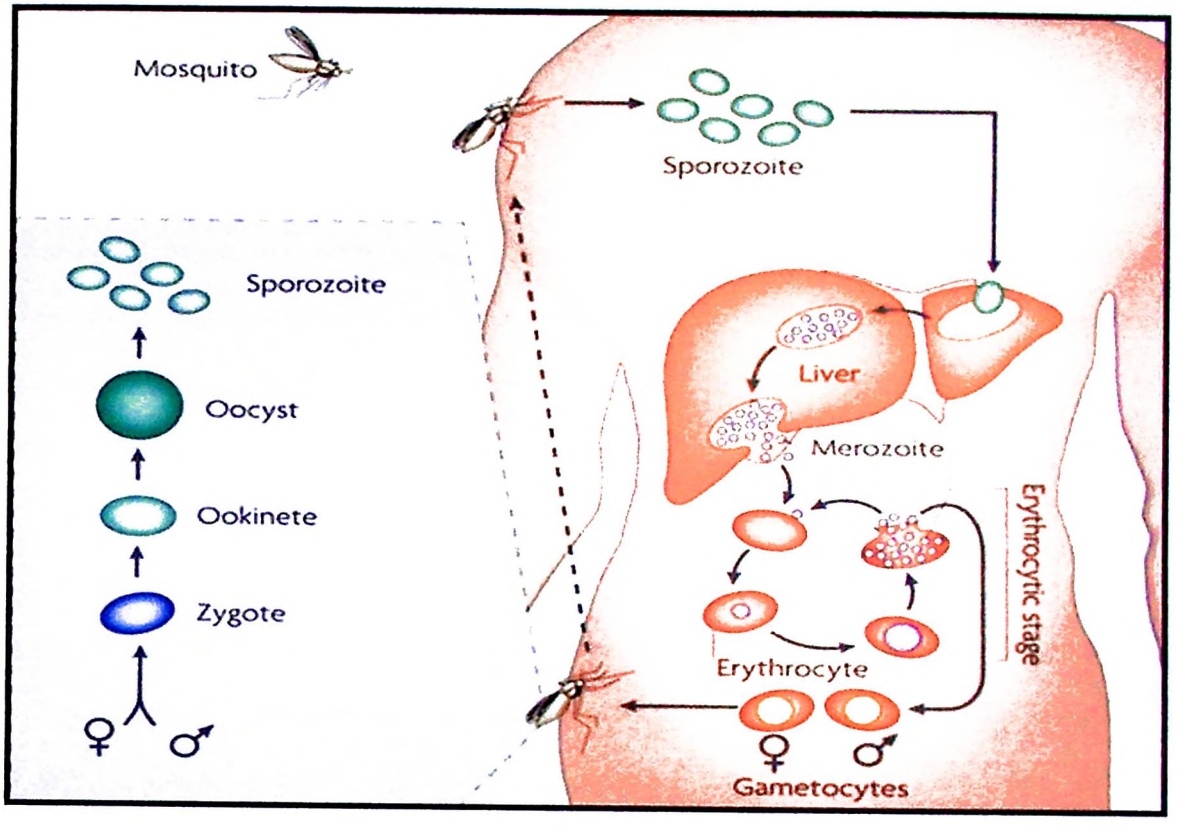

Figure 2.4: The life cycle of *Plasmodium falciparum* (Dondorp, 2010).

2.5 Pathogenesis of malaria

Every year, there are over 500 million clinical cases due to malaria infection. One percent of symptomatic infections may become complicated and develop into severe malaria. Severe malaria may manifest as anemia, hypoglycemia, metabolic acidosis, repeated seizures, coma or multiple organ failure and is estimated to cause over one million deaths annually (Snow, 2005). Cerebral malaria is the most severe neurological manifestation of severe malaria. Parasite sequestration in cerebral microvasculature is thought to be a central factor in pathogenesis and the resulting pathophysiological changes in tissue around the sequestered parasites (MacPherson, 1985). Sequestration results from adherence of parasitized red blood cells (pRBCs) to the endothelial lining (cytoadherence) using parasite derived proteins exposed on the erythrocyte surface (Newbold, 1999). A group of parasite antigens including erythrocyte membrane protein-1 (PfEMP-1) mediate binding to host receptors of which, intercellular adhesion molecule-1 (ICAM-1) is the most important and whose expression is upregulated in areas adjacent to sequester parasites. The sequestered parasite mass is further increased when adherent erythrocytes agglutinate with other pRBCs, form rosettes with non-parasitized erythrocytes or use platelet-mediated clumping to bind to each other. Sequestration impairs perfusion and may aggravate coma through hypoxia.

Cytokines and chemokines play a complex role in pathogenesis and have both protective and harmful effects. Tumor necrosis factor (TNF) is the most extensively studied cytokine in cerebral malaria, upregulates ICAM-1 expression on the cerebral vascular endothelium increasing the cytoadhesion of pRBCs. Near areas of sequestration, there is increased local

synthesis. The timing of this is important since early in the disease, TNF may be protective but prolonged high levels contribute to complications (Hunt, 2003). TNF is also involved in regulating synaptic transmission (strength, scaling and long-term potentiation) (Clark, 2009). Thus, cytokine mediated synaptic changes may contribute to the syndrome of cerebral malaria.

Other than cerebral malaria, severe malarial anemia (SMA) is the primary clinical manifestation and which vast majority of the morbidity and mortality occurs in immune-naïve African children less than five years of age. The pathophysiological processes that contribute to SMA involve direct and indirect destruction of parasitized and non-parasitized red blood cells (RBCs), inefficient or suppression of erythropoiesis, and dyserythropoiesis. One important cause of impaired erythroid responses in children with SMA is dysregulation in the innate immune response (Perkins, 2011). The World Health Organization (WHO) defines SMA as Hb concentrations <5.0 g/dL (or a hematocrit <15.0%) in the presence of any density parasitemia (WHO, 2000). The etiology of SMA can include a number of distinct, as well as overlapping features, including lysis of infected and uninfected RBCs (Dondorp, 1999), splenic sequestration of RBCs (Buffet, 2009), dyserythropoiesis and bone marrow suppression and chronic transmission of malaria in holoendemic regions. However, it is important to stress that high levels of parasitemia, particularly in non-immune individuals, can certainly result in massive lysis and clearance of RBCs, resulting in profound anemia (Molyneux, 1989).

Although the precise mechanisms responsible for reduced reticulocyte responses in children with SMA have been somewhat elusive, it is clear that one important cause of reduced erythropoiesis in children with SMA is due to an imbalance in inflammatory mediators. In an attempt to control the parasitemia, the host releases an array of pro and anti-inflammatory cytokines, chemokines, growth factors, and effector molecules as part of the innate immune response. Depending on the magnitude and timing of inflammatory mediator release, the immune response to malaria can result in either successful control of the parasitemia or alternatively, an inappropriate balance in the inflammatory milieu that can induce damage to the host, including suppression of the erythropoietic response (Helleberg *et al.*, 2005).

2.6 Immunity against malaria

2.6.1 Innate Immunity

The development of effective adaptive immunity to malaria occurs through repetitive infections during childhood (Baird, 1995). In naive individuals, on malaria infection, the host's ability to control rapidly growing parasites relies on the defensive actions of the innate immune system. Otherwise, the parasite could grow exponentially and cause death. Vaccine related research has tended to focus on the identification of target antigen of protective immunity and has inevitably concentrated on adaptive rather than innate immune responses. The innate response to malaria has, until recently, received relatively little attention. However, studies in both mice and humans have repeatedly shown that pro-inflammatory cytokine are essential mediators of protective immunity to erythrocytic malaria and these cytokine can derive from either the innate or adaptive arm of the immune

response (Artavanis, 2002). Innate immunity is important in the early control of malaria infection because it restricts parasite replication and impedes the progression of severe and fatal disease. Innate immune response components include antigen-presenting cells (APCs) such as monocytes, macrophages and dendritic cells, other effector cells such as natural killer (NK) cells, as well as secreted components like the complement system, cytokine and acute phase proteins, which provide the host with immediate defense against invading pathogens (Stevenson, 2004).

2.6.1.1 Macrophage

Macrophages are the major type of phagocytic cell involved in innate immune protection against malaria. Macrophages have several functions including the removal of cell debris, killing pathogenic microorganisms, and the processing and presentation of antigens ingested by lymphocytes. Therefore, the activation of macrophages is a key event for effective innate and adaptive immunity (Lee, 2007). In addition, infection of the macrophages by *Mycobacterium tuberculosis* leads to the activation of multiple microbial mechanisms, including the production of pro-inflammatory cytokine, which limit the growth of ingested organisms and ligation of pattern recognition receptor allows them to resist the cytopathic effects of intracellular pathogens and can induce production of oxygen radicals and nitric oxide (NO) (Giacomini *et al.*, 2001). Phagocytosis of parasites by macrophages has important roles in innate immunity during malaria parasite infection by facilitating the killing of pathogens and priming the adaptive immune response (Rapeah, 2010).

2.6.1.2 Toll Like Receptors (TLRs)

Toll like receptors are a family of innate immune receptors known as pattern recognition receptors (PRRs) whose critical role involves the recognition of pathogen associated molecular patterns (PAMPs) of invading pathogens (Akira, 2006). TLRs are broadly distributed on the cells of the immune system such as macrophage, dendritic cells (DC), neutrophils, B cells, as well as mucosal epithelial and endothelial cells (Iwasaki, 2004). To date, 10 TLRs in human and 13 TLRs in mice have been described. TLRs play an important role in innate recognition of *Mycobacteria* which its cell wall associated lipoprotein induce production of IL-12, a strong pro-inflammatory cytokine.

TLRs are transmembrane glycoproteins that contain a ligand binding domain in the extracellular N-terminus, and a signaling domain in the intracellular C-terminus. On ligand recognition, TLRs bind to specific adaptor proteins through their cytoplasmic toll (TIR) domain and activate various mitogen-activated protein kinase (MAPK) and NF-kB signaling pathways (Gowda, 2007). This signalling activation leads to the induction of a wide range of immunological responses, including the production of cytokines, chemokines, cell adhesion molecules, and co-stimulatory molecules (Zhu, 2011). The first demonstration of parasite antigens detected by TLRs was by Campos *et al.* (2001), who showed that glycosylphosphatidylinositol (GPI) anchors from *Trypanosoma cruzi* activate TLR-2. The activation of macrophages by malarial GPIs involves engagement of TLR-2 resulting in the intracellular signalling and production of cytokine such as IL-12, TNF-α and NO (Campos, 2001). Figure 2.7 shows how GPI induced TLR meidated cell signaling pathway.

2.6.1.2 (i) Toll-like receptor 2 (TLR-2)

TLR-2 recognizes a wide range of PAMPs derived from various pathogens, ranging from bacteria, fungi, parasites and viruses (Akira *et al.*, 2006). These ligands include di and tri-acyl lipopeptides, peptidoglycan, lipoteichoic acid, porin, lipoarabinomannan, zymosan *Trypanosoma* GPI-mucin and hemagglutinin protein (Wieland *et al.*, 2004 , Campos *et al.*, 2001). It has been suggested that TLR-2 recognizes a wide spectrum of microbial components because it forms heterodimers with other TLRs such as TLR-1 and TLR-6, both of which are structurally related to TLR-2. TLR-2 also forms heterodimers with non-TLR molecules such as CD36, CD14 and dectin-1 (Takeuchi *et al.*, 2001). Studies showed that, *P. falciparum*, that cause malaria,can trigger TNF-α and IL-6 production in murine macrophages by means of TLR2/TLR1 or TLR2/TLR6 activation with GPI. This GPI is only recognized in merozoites, the erythrocyte infective form of the protozoan (Baeza *et al.*, 2010).

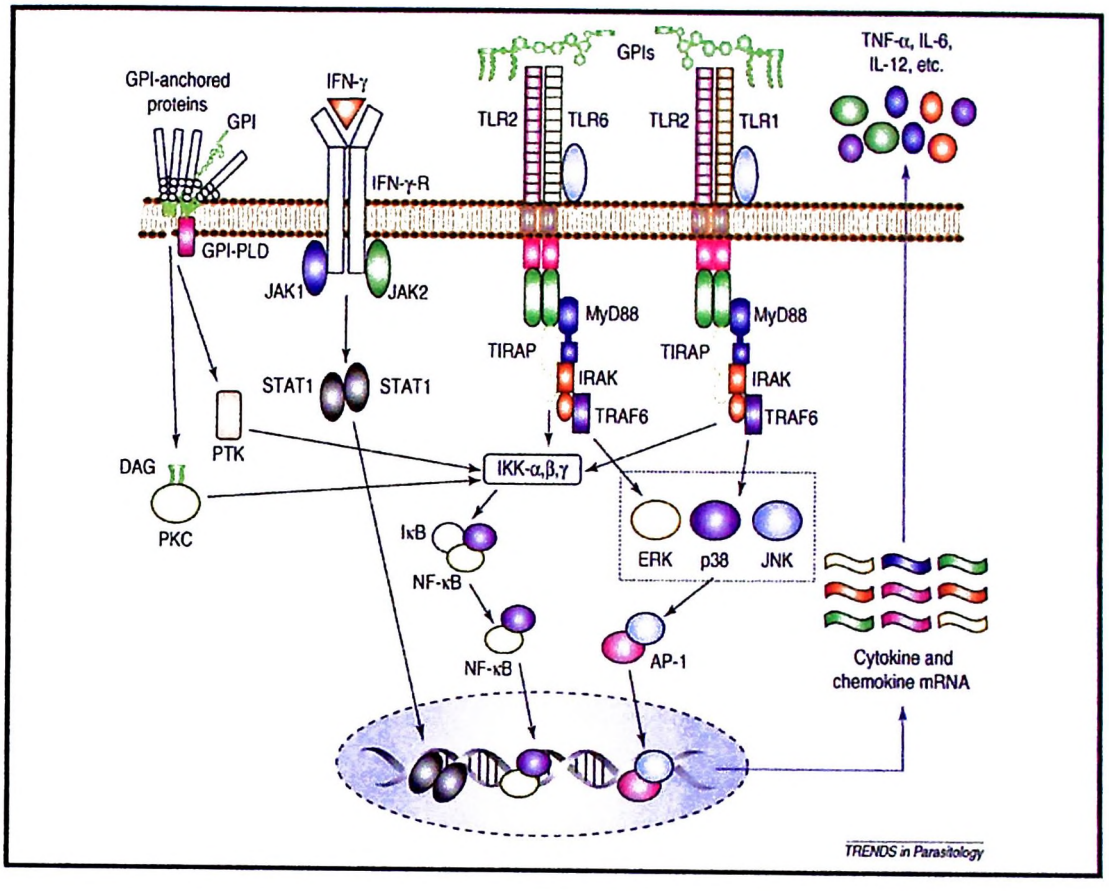

Figure 2.7: Representation of GPI-induced TLR mediated cell signaling pathway (Gowda, 2007).

2.7 Inflammatory cytokines

Cytokine are low molecular weight regulatory proteins or glycoproteins secreted by various immune effector cells in the body in response to a number of stimuli. Some cytokines that clearly promote inflammation are called pro-inflammatory cytokines, whereas other cytokines that suppress the activity of pro-inflammatory cytokine are called anti-inflammatory cytokine (Hillenbrand *et al.*, 2010). A successful type 1 response to malaria requires a well-timed and proportional release of pro-inflammatory cytokine such as IL-12, IL-1β, IFN-γ and TNF-α to minimize infection (Perkins *et al.*, 2011). Cytokine such as IL-4, IL-10, IL-13, and TGF-β suppress the production of IL-1, TNF, chemokines such as IL-8, and vascular adhesion molecules. The pro-inflammatory response which is initiated by *M. tuberculosis* is antagonized by anti-inflammatory mechanisms. However, an uncontrolled pro-inflammatory response may lead to excessive tissue damage, while a predominance of anti-inflammatory effects may favor outgrowth of *M. tuberculosis* (Crevel *et al.*, 2002). Therefore, a "balance" between the effects of pro-inflammatory and anti-inflammatory cytokine is thought to determine the outcome of the disease (Dodoo *et al.*, 2002).

2.7.1 Tumour necrosis factor alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine principally produced by activated immune cells, such as macrophages, CD4+ lymphocytes, neutrophils and mast cells. TNF-α is produced mainly as a soluble 17-kDa secreted protein and also in transmembrane form at the surface of macrophages. TNF-α may have both beneficial and detrimental functions. It can activate host defense and promote resistance to infectious diseases, and it can also be involved in toxicity and inflammatory processes. TNF-α is produced and released by immune host cells following exposure to various malarial antigens at different steps of the life cycle of *Plasmodium* species (Gimenez, 2003). TNF-α does not kill parasites directly but exerts protection through the induction of mediators such as nitric oxide (Yazdani *et al.*, 2006). The studies show that TLR expression and responses are enhanced in individuals carrying asymptomatic *P. falciparum* infection. The expression levels of the TLR-2 gene, the number of TLR-2 expressing monocytes, and TLR-2 signaling per cell induced by the TLR-2 ligand Pam3Cys were all increased in *P. falciparum* infected subjects. The latter was reflected by increased MAPK activation that resulted in higher production of TNF-α and IL-10 (Hartgers *et al.*, 2008).

2.7.2 Interleukin 10 (IL-10)

Recent experimental studies suggest that anti-inflammatory cytokine down-regulate the potentially pathogenic pro-inflammatory cytokine responses in malaria to mediate protective immunity (Sanni *et al.*, 2004). In fact, in mild malaria, inflammatory responses are down regulated by anti-inflammatory cytokine such as IL-4, IL-10, and TGF. The Th1 cytokine necessary for controlling early parasitemia, need to be counter balanced later in

the infection by Th2 cytokine, which enhance antibody production (Tsakonas *et al.*, 2003). It has been reported that the suppression of IFN-γ and TNF-α secretion by IL-10 synthesis reduces the pathological effect of macrophages during cerebral malaria (Jason *et al.*, 2001). There is an evidence and named that the recognition of antigens from infectious agents via TLR-2, and potentially TLR-4 and CD14, lead to IL-10 secretion and down-regulation of pro-inflammatory responses. The mechanism by which IL-10 dampens the production of TNF occurs *via* the inhibition of NF-κB and the establishment of a negative-feedback loop that inhibits TLR mediated cellular activation. Increased production of IL-10 further inhibits production of reactive nitrogen species and oxygen radicals, which play a role in controlling the infection. For many pathogens that fall within these two extremes, particularly those that establish persistence, there is a critical balance between where IL-10 is beneficial either for the host or for the pathogen. Thus, the timing as well as the relative amounts of pro-inflammatory and anti-inflammatory cytokine production is critical for safe resolution of infection.

2.8 Malaria antigen

2.8.1 C-terminus of the merozoite surface Protein-1 (MSP-1C)

MSP-1C or also known as MSP-1_{19} is a 19 kDa blood-stage antigen produced by proteolysis of a high molecular weight precursor, 195 kDa MSP-1 protein. During merozoite invasion of red blood cells, the protein is processed by proteases and released from the parasite surface except for a 19 kDa C-terminal region of MSP-1 which remain on the surface of the invading merozoites. This proteins on the surface of *P. falciparum* merozoites are good targets for vaccine development against malaria because they are

accessible to antibodies in the plasma. Antibodies directed against merozoite proteins function by blocking RBC invasion, initiate parasite clearance by opsonisation making the merozoite susceptible to phagocytic cells or complement mediated damage (Wieland *et al.*, 2004 and Krug *et al.*, 2004). This protein is responsible for protective immunity against malaria infection (O'Donnell, 2001) and is one of the most promising malaria vaccine candidates (Bisseye, 2011). Therefore, previous study was conducted to clone expressed the MSP-1_{19} in *Mycobacterium bovis bacille* Calmette-Guerin (BCG). The expressions of the recombinant proteins were detected by specific monoclonal antibodies (mAbs) namely, 12.10 and 1E1 against MSP-1_{19} (Nurul, 2010). Moreover, another study showed that the rBCG clone capable of stimulating phagocytic activity and pro-inflammatory cytokine production as well as apoptosis activity in mouse and human macrophages much higher than BCG and LPS (Rapeah *et al.*, 2010).

2.9 Malaria vaccine

Malaria parasite resistance to artemisinin which the core compound in the world's most effective antimalarial medicines and mosquito resistance to insecticides remain major concerns (WHO, 2013). Therefore, the development of a malaria vaccine carries huge expectations. However, vaccine research over the past three decades has been characterized by lack of adequate knowledge about the immune mechanisms underlying protection. Of the 6000 to 8000 malaria proteins so far identified, the few that have been the among blood-stage molecules, MSP1 alone or combined with MSP2, has been included in several human trials. However, the inhibition of merozoite invasion obtained with monoclonal antibodies has not been induced to date by immunization (Lawrence, 2000).

RTS, S is the vaccine that appears to be the most promising but may still have problems of interpretation, since certain subjective cutoffs have been used to compute a quantitative efficacy (Alonso, 2004). Mice immunized with GPI glycan were protected against severe disease conditions such as blood acidosis, pulmonary oedema, and vascular occlusion by macrophages and cerebral deaths, without any effect on the parasite growth or burden (Schofield, 2002). With the possibility that the parasite GPI and merozoite surface protein may act as the ligands for the 'pattern recognition receptor TLRs', and find a significant place in a malaria vaccine.

2.9.1 Development of recombinant BCG vaccine against malaria

The *M. bovis* bacillus Calmette-Guérin (BCG) is an only licensed vaccine that has been used for a century to helped controlling tuberculosis (TB). BCG is attractive as a vaccine vector because of its extensive safety record in humans, heat stability, low production cost, induction of long-lasting type 1 helper T cell (Th1) immunity, CD8+ T cell triggering, adjuvant activity, usability in newborns and its mucosal immune induction by oral administration. Taking the current situation of serious epidemics of emerging and reemerging diseases mainly in developing African and Asian countries into account, a new global vaccine should be affordable in such areas. Therefore, the low price and heat stability of BCG based vaccines would be desirable.

The most characteristic response to BCG is the induction of innate immunity by cell wall components through toll-like receptors (TLRs) 2 and 4 on dendritic cells and macrophages. After phagocytosis, BCG is degraded by lysosomal enzymes, and the processed antigen can