EXPRESSION OF TOLL-LIKE RECEPTOR (TLR) 2 ON MACROPHAGE INFECTED WITH rBCG CLONE EXPRESSING MSP-1C OF *Plasmodium falciparum* AND ITS EFFECT ON IL-12 AND IL-1β PRODUCTION

by

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ABSTRAK

Reseptor "Toll like" (TLR) merupakan salah satu "pattern recognition receptor" (PRRs) yang terlibat dalam imuniti semulajadi bagi melawan patogen semasa fagositosis. TLR mengawal transkripsi tindak balas inflamatori oleh sel yang dijangkiti. Antara TLR yang dikenalpasti, TLR2 merupakan salah satu reseptor yang terlibat dalam pengesanan mikobakterium dan parasit malaria oleh makrofaj. Kajian ini telah dilaksanakan untuk mengesan pengekspresian protein TLR2 oleh makrofaj tikus yang dijangkiti oleh BCG dan rekombinan BCG (rBCG) yang mengekspres protein MSP-1C *Plasmodium falciparum* dengan mengunakan pemblotan Western. Peranan TLR terhadap prenghasilan sitokin inflamasi seperti IL-12 and IL-1β juga turut dikaji dalam supernatan sel yang dijangkiti dengan kehadiran atau tanpa kehadiran inhibitor TLR2 dan diransang dengan LPS+IFN-γ mengunakan kaedah ELISA. Keputusan menunjukan kedua-dua sel yang dijangkiti dengan BCG dan rBCG mengekspreskan protein TLR2. Walaubagaimanapun, pengekspresan protein TLR2 oleh makrofaj yang dijangkiti rBCG lebih tinggi secara signifikan berbanding makrofaj yang dijangkiti BCG. Untuk penghasilan sitokin, kehadiran inhibitor TLR2 tidak memberi kesan kepada penghasilan IL-12, tetapi berjaya mengurangkan penghasilan IL-1β oleh makrofaj yang dijangkiti kedua-dua BCG dan rBCG. Sebagai kesimpulan, keputusan kajian ini menunjukan makrofaj mengekspres reseptor TLR2 semasa dijangkiti oleh BCG dan rBCG. Namun, reseptor TLR2 ini hanya penting untuk penghasilan sitokin IL-1β tetapi tidak untuk penghasilan sitokin IL-12 oleh makrofaj tersebut.

ABSTRACT

Toll like receptor (TLR) is a pattern recognition receptor (PRRs) that involve in innate immune response against invading pathogen during phagocytosis. TLR controls the transcription of inflammatory response by the infected cells. Among the TLRs identified, TLR2 is one of the receptor that involved in the recognition of mycobacterium and malaria parasites by macrophages. This study was conducted to determine the expression of the TLR2 protein in a mouse macrophage cell line infected with BCG and recombinant BCG (rBCG) expressing the C-terminus of merozoite surface protein (MSP-1C) of *Plasmodium falciparum* by Western blot. The role of the TLR in the production of pro-inflammatory cytokine production such as IL-12 and IL-1β was also evaluated in the supernatant of infected cells in the presence or absence of TLR2 inhibitor and LPS+IFN-γ stimulation using ELISA. The result demonstrated that both BCG- and rBCG-infected macrophages express significant TLR2 protein. However, the expression of TLR2 by the rBCG-infected macrophages was significantly higher than those expressed by BCG-infected macrophages. For the cytokine production, the presence of TLR2 inhibitor does not affect the production of IL-12, but capable to reduce the production of IL-1β by the macrophage infected with both BCG and rBCG. In conclusion, our results indicate that the macrophage expresses TLR2 receptor during BCG and rBCG infection. However, this TLR2 receptor only important for the production of IL-1β cytokine but not the production of IL-12 cytokine by the macrophages.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Malaria is a major health concern in many countries. Approximately 500 million people were affected and 2 to 3 million deaths cause by malaria reported each year (Gowda, 2007). About 85% of the deaths are among children of five years old (Kokwaro, 2009). This disease causes a socioeconomic burden mostly to people in endemic areas. Most of the high risk population lives in remote area with less access to health care center and they cannot afford the treatment once they get infected. It was reported that African lost approximately US$12 billion every year due to this disease.

The emergence of anti-malarial drugs resistance parasite becomes a great challenge to manage the disease. The most commonly used anti-malarial drugs are chloroquine and sulphadoxine/pyrimethamine (Olliaro *et al.*, 2001). The development of parasite resistance to chloroquine is widespread and parasite resistance to sulphadoxine/pyrimethamine is well established in South-East Asia and parts of Latin American. Emergence of anti-malarial drugs parasite is due to the inappropriate used of the drugs. Treatment of malaria disease by using the anti-malarial drugs with similar basic chemical compound can lead to the development of the resistance (Kokwaro, 2009). The consequence of this situation is deleterious. A more expensive drug is needed to treat the disease (Olliaro *et al.*, 2001).

People in non-endemic region is also at risk due to increasing globalization and population migration (Gowda, 2007).

Innate immunity against malaria parasite is required to control the early phase of parasite replication and allowing adaptive immunity to develop (Stevenson & Riley, 2004). Stimulation of innate immune response involves recognition of pathogen-associated molecular pattern (PAMP) of the *Plasmodium* parasite by the pattern recognition receptor such as toll like receptor (TLR) (Gowda, 2007). Ten human TLR had been recognized known as TLR1 to TLR10. In malaria infection, TLR2 and TLR4 involve in the recognition of glycosylphosphotidylinositol whereas TLR9 involve in the recognition of parasite DNA complex with hemozin (Hartgers *et al.*, 2008). The recognition of pathogen by TLR leads to activation of intracellular signaling pathway. Pathways involve in the malaria infection are ERK, p38, JNK and NF-κB pathway. These entire pathways are important for pro-inflammatory cytokine production such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-12 (Gowda, 2007). TNF-α and IL-1β can induce production of nitric oxide. The nitric oxide has parasiticidal properties against the malaria parasite (Dhaniah *et al.*, 2014).

As of now, there is no licensed vaccine against malaria. A safe and effective vaccine against malaria is important to reduce mortality and morbidity cause by the parasite. Most of the current vaccine development will target a single stage of *Plasmodium* parasite mostly blood stage (Hill, 2011). A number of research groups are focusing on the development of the vaccine that targeting the invasive stage of the malaria parasite (Chitnis & Blackman, 2000). Erythrocytic invasive stage is responsible for clinical manifestations of this disease.

The release of malaria parasite and erythrocyte material induce pathophysiology process, lead to onset of the symptom (Bartoloni & Zammarchi, 2012). The most prominent candidate antigen for vaccine development is 19 kDa C-terminus of *P. falciparum* merozoite surface protein-1(MSP-1C). This protein produces from proteolytic cleavage of MSP-1 protein precursors. MSP-1C remains on the surface of merozoite and carried into the new uninfected erythrocytes. Previous study had shown antibodies against MSP-1C can provide protective immunity against malaria infection (Nurul & Norazmi, 2011).

Previously, we had successfully constructed a recombinant BCG (rBCG) expressing the MSP-1C of *P. falciparum* by using assembly PCR. *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) had been chosen as a recombinant vector due to their stability to deliver foreign antigen into the host immune system. Despite of the various controversies of the BCG, it is proven to be safe and had been given to 2.5 million people worldwide. It has a great adjuvant activity, able to elicit both humoral and cell-mediated immune response and inexpensive. Due to this entire attribute, many efforts had been taken to develop BCG as a vaccine vehicle that able to simultaneously express recombinant antigen (Ohara & Yamada, 2001). In this study, we want to determine the involvement of TLR in enhancing pathogen recognition, activation of intracellular signaling and production of cytokines in mouse macrophage infected with the rBCG vaccine. The study is important in order to get knowledge on the role of TLR and their pathway in the binding and presenting the vaccine.

1.2 Objective of study

1. To determine the expression of TLR2 protein in macrophage infected with BCG and rBCG clones expressing the MSP-1C of *Plasmodium falciparum* by using Western blot.
2. To determine the involvement of TLR2 in the production IL-12 and IL-1β by macrophages infected with the BCG and rBCG clones expressing the MSP-1C of *Plasmodium falciparum* in the presence of LPS and IFN-γ stimulation by using ELISA.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria disease

Malaria is one of the main causes of mortality worldwide. It is caused by infection of *Plasmodium* parasites. There are more than 100 species of *Plasmodium* but only four species can cause malaria in human which are *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi* (CDC, 2012). In addition, there is one species that naturally infect macaques, which is also able to cause malaria in human.

Different *Plasmodium* species are distributed to a different geographical area. *Plasmodium falciparum* is found worldwide and the most malignant form. It contributes the most for death, cause by the malaria in Africa (CDC, 2012). A second species of *Plasmodium* parasite is *P. vivax* which more dominant in Asia, Latin America and some parts of Africa (CDC, 2012). It has a wider distribution compared to other species (WHO, 2013). The factors that contribute to wider distribution of *P. vivax* are it can develop in *Anopheles* mosquito vector at lower temperature and can survive at extreme temperature and climate (WHO, 2013). In addition, *P. vivax* has a dormant liver stage called hypnozoites.

Other than *P. vivax*, other species that ability to develop into the hypnozoite is *P. ovale*. However, this species can infect Duffy-negative individual while *P. vivax* cannot. *P.*

ovale is widely distributed in Sub-Saharan Africa while *P. malariae* can be found worldwide (CDC, 2012).

The fifth species of *Plasmodium* that can cause infection in human is *P. knowlesi*. In 1967, Chin *et al.* had showed that *P. knowlesi* causes infection in human. The natural reservoir for this parasite is the long-tailed and pig-tailed macaques (White, 2007).

2.2 Malarial epidemiology

2.2.1 Worldwide

Malarial disease cause by *Plasmodium* parasite is a major cause of disease and death in tropical countries. It causes more than 300 million cases and between one to three million deaths per year (Stevenson & Riley, 2004). As reported by WHO (2014), there are 97 countries and territories had ongoing malaria transmission with most of the deaths and cases reported in Sub-Saharan African. It was estimated about 198 million cases of malaria and 584000 deaths had occurred in 2013. Most of the deaths reported were among the African children. Increased prevention and control measures had dramatically reduced the malaria burden in many places. The mortality rate had reduced by 47% since 2000.

The emergence of antimalarial resistance parasite had become the largest obstacle to control malaria. Drug resistance develops due to inadequate doses of antimalarial treatment, failure to complete the treatment and uncontrolled used of poor quality drugs (Crawley, 1999). Another challenge was changing in land use such as deforestation, irrigation and road construction that create site suitable for vector breading. Insecticide was used as a vector control. However, the increasing prevalence of mosquito resistance to chemical becomes a great challenge to control the vector (Kokwaro, 2009). In non-endemic region, transmission of malaria disease occurs due to entering of foreigner to their country.

2.2.2 Malaysia

In 2012, there were 4725 reported malaria cases in Malaysia. It was found that 50.2% of the human malaria infection is caused by *P. vivax*. The *P. falciparum* that can cause a severe malaria infection also present in Malaysia. Among the human malaria infection, 30.7% of the infection cause by *P. falciparum*.

Most of the cases in Malaysia distributed in less develop state as shown in Figure 2.1. Sabah (74.0 %) had the highest reported cases. In Peninsular Malaysia, more than 100 cases were reported in Selangor, Pahang, Kelantan and Perak. About 2.5 % of the cases occur among the children of 5 years old age. There was a decline in the number of malaria cases in Malaysia from 12705 cases in 2000 to 4725 cases in 2012. The mortality rate due to malaria infection also declines from 35 deaths in 2000 to 16 deaths in 2012. The decline in malaria infection and death was due to varying eradication program done by the government. However, there was a new challenge arise in eliminating malaria in Malaysia.

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The entering of foreigner and tourist had become a source of malaria for Malaysian (Ministry of Health, 2013).

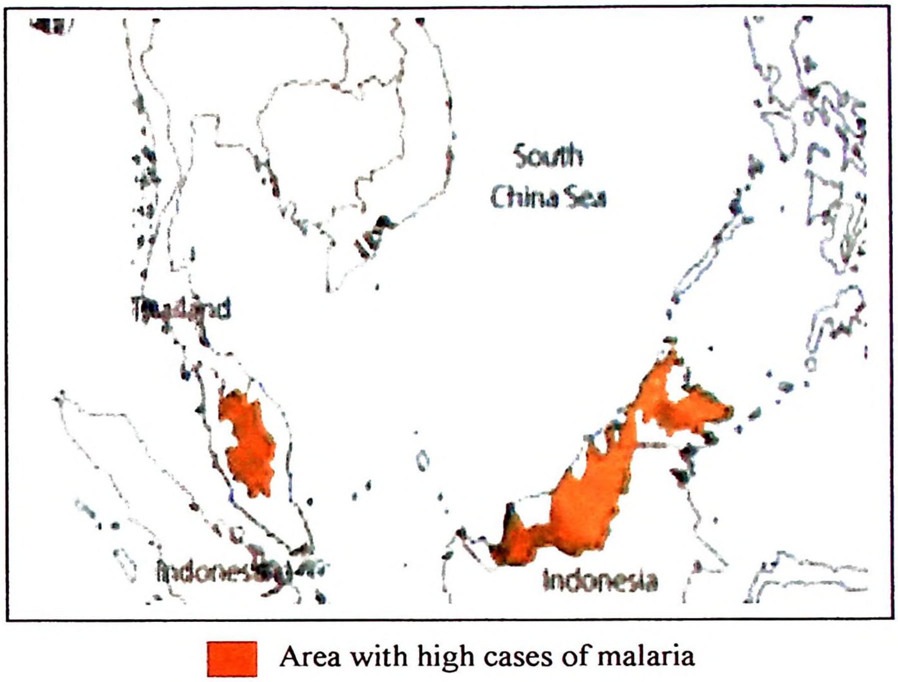

Figure 2.1 Distribution of malarial cases in Malaysia (Adapted from World Malaria Report, 2013).

2.3 Life cycle of *Plasmodium* parasite

The life cycle of *Plasmodium* parasite is complex. Malaria disease is a vector borne where it is transmitted by the mosquitoes. The species that responsible for transmission of this disease is *Anopheles* mosquitoes. Female *Anopheles* mosquitos inject the sporozoite into the suitable host during the blood meals. A complete cycle for development of sporozoite in the mosquito is about 10 to 18 days and infected mosquito does not suffer noticeably (CDC, 2014).

The sporozoites inject into human during a blood meal. The sporozoite then enters liver, where it develops into schizont that contains thousands of merozites. For *P. vivax* and *P.ovale*, they have a dormant stage called hypnozoite. This dormant stage will remain in the liver for weeks or years before develop into schizont. *P. falciparum* and *P. malariae* have no dormant stage (Fujioka & Aikawa, 2002). Mature schizont will release merozoites into the bloodstream that able to invade red cells. Invasion of merozoite into red blood cell is essential steps and must occur rapidly because extracellular merozoite is short live (Fujioka & Aikawa, 2002) and the antigen on their surface is particularly susceptible to immune attack (Cowman & Crabb, 2006). The entire process of invasion takes 30 seconds as shown in Figure 2.2 (Fujioka & Aikawa, 2002) which involve initial long distance recognition of surface receptor followed by reorientation. Then, a tight junction formed between the parasite and host membrane. The tight junction then moves from apical to the posterior end. As the junction moving, the ligands that assist in parasite invasion will be removed by the serine protease. Lastly, the parasite creates a parasitophosphorus vacuole to coat itself from host cytoplasm and create an environment suitable for its development (Cowman & Crabb, 2006).

The eryrtocytic stage is responsible for most of the clinical sign and symptom of the malaria. A rapid parasite growth with massive destruction of erythrocytes leads to a variety of pathologic conditions (Gowda, 2007). Another factor that contributes to pathologic condition during erytrocytic stage is the release of parasite and erythrocyte material with the activation of cytokine cascade. Liver stage is asymptomatic because only a few numbers of liver cells involve (Bartoloni & Zommarchi, 2012). Therefore, individual survival depends on their ability to control the replication of blood-stage parasite (Stevenson & Riley, 2004).

In the red blood cells, the merozoite develops through ring, trophozoite, and schizont stage. Red blood cells contain mature schizont will burst and release merozoite that able to invade new red blood cells. A small proportion of merozoite will develop into sexual forms which are microgametocyte (female) and microgametocyte (male). Mature gametocytes taken into midgut of the mosquito. In the mosquito, gametocyte mature and fuse to form zygotes. Non motile zygote then transforms into motile ookinetes within 18 to 24 hours (Fujioka & Aikiwa, 2002). The ookinetes travel and penetrate the midgut wall of the mosquito where they develop into oocyst. Then sporozites are released from a mature ookinates after ten to 24 days of infection (Fujioka & Aikiwa, 2002) .The new life cycle begins after sporozite is injected into the susceptible human during a blood meal.

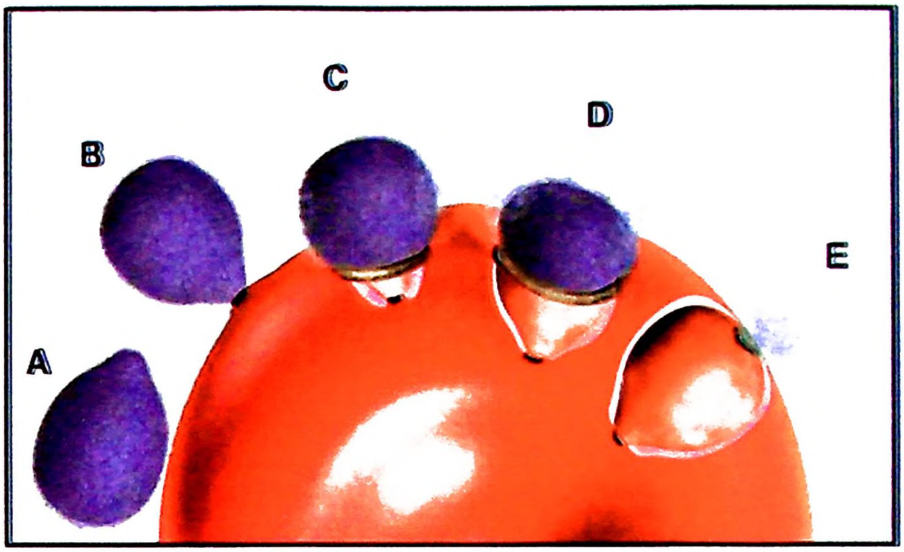

Figure 2.2 Invasion of merozoite into red blood cell. Invasion of *Plasmodium* parasite involve long distance recognition of surface receptor (A) followed by a reorientation process where the low-affinity contact are maintained (B). Then a tight junction is formed involving high-affinity ligand receptor interaction and then the parasite moves from the apical to the posterior pole (C and D). Upon reaching the posterior end, the adhesive proteins are proteolytically removed (E) (Adapted from Cowman & Crabb, 2006).

2.4 Pathogenesis of malaria

Infection with *P. falciparum* may lead to more severe complication and eventually death. *P. falciparum* can lead to severe complication such as cerebral malaria, severe anemia, renal failure and pulmonary affection (Heddini, 2002). The feature that contributes to severity of *P. falciparum* is the ability of the parasite to adhere to the endothelium (Mackintosh *et al.*, 2004). Infected red blood cells adhere to vascular endothelium of various organs including the brain, placenta and heart. Adherence of infected erythrocytes to endothelial in large numbers cause specific organ dysfunction by various mechanisms. The proposed mechanism includes poor local perfusion, local released of cytokines and nitric oxide and local metabolic derangements (Heddini, 2002). Potential receptors for infected red blood cell binding including thrombospondin, CD36, intracellular adhesion molecule 1(ICAM-1), vascular cell adhesion molecule, E-selctin, chondroitin sulphate A (CSA), CD31 and hyaluronic acid (HA) (Mackintosh *et al.*, 2004).

Infected red blood cell also able to adhere to non-infected red blood cell in a phenomenon called resetting. The mechanism of resetting that lead to pathologic changes including facilitating invasion into new erythrocyte, a shield from the host immune response, and reduce blood flow to facilitate contact with endothelial receptor. Infected red blood cell may also able to adhere to other infected red blood cell forming a large clump with platelets or autoagglutinates (Mackintosh *et al.*, 2004).

2.5 Immunity against malaria

Immunity to malaria is complex. It is both species and stage specific. Both innate and adaptive immunity can reduce levels of parasitemia, prevent pathologic damage and reduce the number of circulating infected cells. Innate immunity limits the maximum parasite density, but adaptive immunity is required for complete parasite elimination (Stevenson & Riley, 2004). Innate immune response to malarial infection is important since it functions to limit the initial stage of parasite replication, controlling the first waves of parasitemia and allowing the host to develop adaptive immune response (Stevenson & Riley, 2004). It is the first line of defense mechanism that protects the host. An effective adaptive immunity only develops through repetitive infection. For a naïve individual who did not develop effective immunity, innate immunity is important to control rapidly growing parasites (Gowda, 2007).

The activation of innate immune response involved recognition of pathogen associated molecular pattern (PAMPs) of the parasite by host pattern recognition receptors (PRRs). During malarial infection, the schizont burst releases a large amount of merozoite. Free merozoites in the bloodstream will be targeted by host immunity. Other than the merozoite, the parasite waste product such as hemozoin and parasite membrane fragment may also become the target of the immune system. Glycosylphosphatidylinositol (GPI) is one of the antigen targeted by the immune system. *P. falciparum* GPIs have been identified as an important parasite factor that activates the host innate immune system. The PRRs that responsible to recognize this protein is toll like receptor (TLR). Recent study had shown GPIs were recognized by TLR2 and to a lesser extend TLR4 (Gowda, 2007).

Activation of TLR leads to production of various cytokines. During malarial infection, the host response by produces pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-12, and interferon (IFN)-γ to control parasite growth(Gowda, 2007). However, uncontrolled production of these cytokines may lead to the pathogenesis such as severe malarial anemia and cerebral malaria. Hence, the regulation of immune response against malaria is required. The production of immunoregulatory cytokines is needed to downregulate the immune system when the parasitemia level starts to reduce. Important immunoregulatory cytokines are IL-10 and transforming-growth factor (TGF)-β. IL-10 can inhibit IL-12 production by dendritic cell and macrophage. Inhibitions of IL-12 cause downregulation of IFN-γ that is produced by NK cells and T cells. TGF-β regulates the innate immune response by directly inhibit IFN-γ production by dendritic cells (Stevenson & Riley, 2004).

2.5.1 Toll like receptor (TLR)

The recognition of pathogen by innate immunity depends on a limited number of germline-encoding receptor that recognized pathogen associated molecular protein (PAMPs). Toll-like receptor (TLR) is important in recognition of pathogen and initiation of inflammatory and immune response. Activation of TLR results in induction of antimicrobial genes and cytokine production. It also plays an important role in inducing adaptive immune response by increasing the activation of co-stimulatory molecule and antigen-presenting capacity (Janeway & Medzhitov, 2002). TLR is a transmembrane type 1 glycoprotein that consists of three domains, which are extracellular domains, transmembarene domain and cytoplasmic toll/IL-1 receptor (TIR). The extracellular leucine-rich domain is important for recognition of the pathogen associated molecular protein (PAMPs). Transmembrane and cytoplasmic domain is important to initiate intracellular signaling activity (Basto & Leitao, 2014). Until recently, 10 members of TLR in human had been recognized. All of these TLR have difference ligand specificities, expression pattern and target gene that induce due to their activation (Janeway & Medzhitov, 2002). TLR1, TLR2, TLR4, TLR5 and TLR11 are expressed on the cell surface while TLR3, TLR7, TLR8 and TLR9 are found in endosomal and lysosomal membranes (Gowda, 2007). Once TLR has encountered specific ligand, it will activate intracellular signaling pathway that causes induction of inflammatory cytokines such as type 1 IFN. The specificity of the signaling pathway is controlled by the adapter protein.

According to Gowda (2007), GPI of *Plasmodium* parasite is mainly recognized by TLR2 and a lesser extent by TLR4. Hartgers *et al.* (2008) had conducted a study to analyze TLR responsiveness of school children in an area where malaria is endemic. The result showed that the expression level of the TLR2 gene and the number of TLR2 express by the monocyte increased in the infected individual. TLR2 able to recognize a wide range of ligands and is expressed on antigen presenting cell such as macrophage, monocyte and dendritic cell. It recruits adapter protein of myeloid-differentiation primary-response protein 88 (MyD88) and TIR domain-containing adaptor protein (TIRAP) and initiate a signaling pathway that leads to activation of NF- κB and MAP kinase (MAPK) pathway (Basto & Leitao, 2014). Figure 2.3 shows a signaling pathway of TLR when activated.

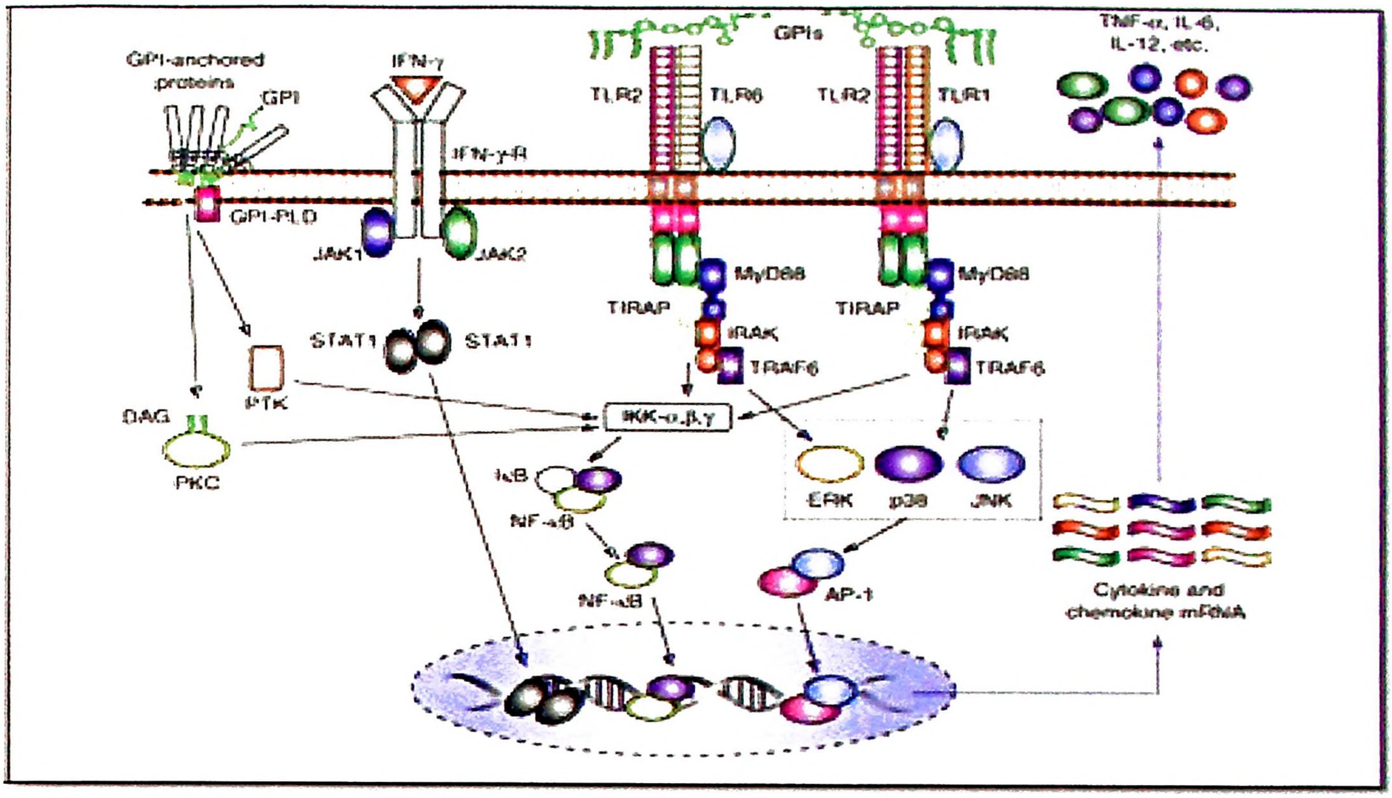

Figure 2.3 The signals induce by TLR after recognizing GPI of the malaria parasite. Malaria GPI is mainly recognized by TLR2/1 hetrodimer and to lesser extends through TLR4. Stimulation of TLR lead to recruitment of adaptor protein MyD88 and TIRAP followed by the activation of various signaling molecule that activate MAP kinase and NF-κβ pathway and initiation of cytokines gene transcription (Adapted from Gowda, 2007).

The MAPK pathway including extracellular signal-regulated kinase (ERK), C-Jun N-terminal kinase (JNK) and p38 with NF-κB are also involved in the expression of pro-inflammatory cytokines and mediators including TNF-α, IL-6, IL-12, and nitric oxide (NO). The activation of this signaling pathway reduces markedly in the MyD88$^{-/-}$ macrophage compared to wild type macrophage (Gowda, 2007). MyD88 is an adaptor protein that associated with TLR after it is activated. The MyD88 will recruit members of interleukin-1 receptor associated kinase (IRAK) family. Due to the stimulation, IRAK phosphorylated and dissociated from MyD88. Then IRAK will activates tumor necrosis factor receptor-associated factor 6 (TRAF6). Activation of TRAF6 leads to activation of the transforming growth factor-β activated protein kinase 1 (TAK1). This sequence then continues to activate the expression of IKK by TAK1 and lastly leads to NF-κB activation. NF-κB is a dimeric transcription factor. In inactive condition, NF-κB binds to inhibition protein known as NF-κB (IκB) inhibitor in the cytoplasm. During activation of the TLR, IκB is phosphorylated by serine residue for ubiquitination and degradation by proteasome. Then free NF-κB migrates to the nucleus and regulates cytokine gene transcription (Kawai & Akira, 2006). According to Gowda (2007), NF-κB pathway regulates the production of TNF-α, IL-6, IL-12 and NO by macrophages.

TRAF6 stimulation also leads to activation of ERK, JNK and p38 pathway. This MAPK pathway leads to activation of AP-1 transcription factor. AP-1 then migrates to the nucleus to initiate gene transcription that related to cytokine production (Kawai & Akira, 2006). The ERK pathway is important in positive regulation of IL-6 and IL-12. For p38 pathway, it has a little role in production of TNF-α and nitric oxide, but the pathway is important for the production of IL-6 and IL-12. Two difference isoforms of JNK are express by monocyte and macrophage which are JNK1 and JNK2. Both JNK isoforms play a crucial role in production of TNF-α, IL-6 and NO. JNK2 is important for the production of IL-12 (Gowda, 2007).

Activation of macrophage can occur due to an encounter with a pathogen, their protein or activation by other cytokines. A previous study showed that the production of pro-inflammatory response by macrophage was increased in LPS+IFN-γ activation. This indicates the role of LPS and IFN-γ in the activation of macrophage (Dhaniah *et al*, 2014). IFN-γ can be produced by natural killer cell after stimulated by IL-12 (Stevenson & Riley, 2004).

Other than MAPK and NF-κB pathways, there is another pathway that also involved in the production of pro-inflammatory cytokine such as IL-12. Earlier studied had suggested that the GPI of malaria parasite might bind to a cell membrane of host cell and activates the protein kinase C (PKC) and protein tyrosine kinase (PTK) pathways. Both of these pathways can activate MAPK and NF-κB expression that leads to the expression of IL-12 by macrophage (Gowda, 2007).

2.5.2 Pro-inflammatory cytokines

2.5.2.1 Interleukin-12

Interleukin (IL)-12 cytokine is secreted by various immune cells such as dendritic cells, monocytes and B-cells when stimulated with bacterial cell wall components, intracellular pathogens and CD40 ligation (Perkins *et al*., 2011). Production of IL-12 can be enhanced by other cytokines and chemokine such as granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN-γ, while other cytokine such as IL-4, IL-10, IL-11, IL-13, and monocyte chemotactic protein (MCP)-1 can reduce production of IL-12. During malarial infection, IL-12 cytokine stimulates IFN-γ production by natural killer (NK) cells, promotes cell mediated-immune response and modulates humoral immune response by inducing antibody-isotype switching (Gowda, 2007).

Among the cytokine and chemokine produces against the *Plasmodium* parasite, IFN-γ plays a crucial role because it induces the production of nitric oxide (NO) that can kill the parasite. Exogenous IFN-γ has a short half-life and does not consistently able to give protection against sporozite (Sedegah *et al*., 1994). Production of IFN-γ can be induced by IL-12 and hence the level of IFN-γ can be maintained. Early and effective immune response against the *Plasmodium* parasite by IFN-γ in the IL-12 and IL-18 dependent is important to control the level of parasitemia (Wroczynska *et al*., 2005). The transcription of the IFN-γ is enhanced by the presence of IL-18 and IL-12 (Torre *et al*., 2002). IL-12 is also required for the production of the protective immunoglobulin G2a (IgG2a) antibody and enhances erythropoiesis to reduce anemia related symptom (Angulo & Fresno, 2002).

2.5.2.2 Interleukin-1β

Interleukin (IL)-1β is a cytokine released as part of the innate immune response to *Plasmodium falciparum*. IL-1β cooperates with IL-1α and TNF-α to stimulate the production of NO and IFN-γ in murine models. However, unregulated production of IL-1β can leads to pathologic condition such as anemia (Perkins, 2011).

2.6 Development of malaria vaccine

A major concern in the elimination of malaria disease worldwide is arising of antimalarial resistance species. According to the WHO (2014), resistance of antimalarial medicine is a recurring problem. Parasite resistance has been detected in 5 countries of the Greater Mekong subregion including Cambodia, Laos, Myanmar, Thailand and Viet Nam. Prevention of malaria transmission is done by vector control using insecticide. This intervention is less efficient due to the emergence of mosquito resistance pyrethroids in many countries. Hence, the prophylactic treatment by vaccination has become an ultimate eradicative strategy.

There is still no licensed vaccine against *P. falciparum*, a major cause of infectious mortality. In order to eliminate malaria, effective vaccine is required. Decades ago, an attenuated malaria parasite had been shown able to induce immunity to infection, but its progression towards effective vaccine is slow (Vaughan & Kappe, 2012). Most current vaccines that are being developed only target at a single stage of the parasite's life cycle.

Vaccine against the early pre-erythrocyte stage is suggested to be the effective malaria vaccine candidates (Hill, 2011).

The erythrocyte stage of the parasite is responsible for malaria pathogenesis. Vaccination against a blood stage of Plasmodium parasite can prevent the invasion of red blood cell and malaria complication such as malaria anemia, renal failure and severe malaria in pregnancy. The merozoite surface is an obvious target for the development of a malaria vaccine. A number of potential vaccine antigen candidates that have been identified including merozoite surface protein (MSP)-1, MSP-2, MSP-3, MSP-4, MSP-5, MSP-8, RAP1/2, AMA-1 and EBA 175 which involved in the process of merozoite invasion into red blood cell (Muzumdar *et al.*, 2010).

2.6.1 Malaria antigen

Invasion of merozoite into red blood cell required the interaction of specific receptors on the eryrocyte membrane with ligands on the surface of the merozoite (Fujioka & Aikawa, 2002). The surface of merozoite is largely comprised of GPI anchor proteins which are potentially erythrocyte ligands. Currently, there are nine GPI anchored proteins identified. There are also other proteins that important in merozoite adherence including cysteine-rich domains (Cowman & Crabb, 2006).

2.6.2 C-terminal 19 kDa domain (MSP-1C) protein

Merozoite surface protein (MSP) -1 is a dominant antigen and the most abundant protein on the surface of merozoite (Cowman & Crabb, 2006). It is the most extensively studied protein of *P. falciparum* (Muzumdar *et al.*, 2010). MSP-1 could be involved in the initial recognition of the red blood cell in a sialic acid-dependent pathway (Fujioka & Aikawa, 2002). According to O'Donnell and Blackman (2005), MSP-1 protein can induce invasion-inhibitory or protective antibody response in animal and human.

MSP-1 is synthesized as a ~200 kDa precursor which will then be processed in two steps. In the first stage, a complex of four fragments that is present on the merozoite surface will be produced (Muzumdar *et al.*, 2010). The complex exists as a non-covalent associated fragment and mediates the primary low affinity interactions between merozoite and red blood cell (O'Donnell & Blackman, 2005). The secondary processing occurs at invasion stage, where the complex is shielded from the surface except for the C-terminal 19-kDa domain (MSP-1C) that remains attached to the GPI moiety (Muzumdar *et al.*, 2010). A study conducted by Blackman *et al.* (1990) by using monoclonal antibodies to trace MSP1 fragments during erythrocyte invasion had shown that only MSP-1C is detected at the parasite surface of newly invade red cells. The C-terminal 19-KDa fragment is well conserved among *P. falciparum* isolates and contains epidermal growth factor (EGF) that responsible for merozoite invasion (Muzumdar *et al.*, 2010). The inhibition of MSP-1 processing by specific antibodies may inhibit merozoite invasion (Muzumdar*et al.*, 2010). A finding from a study conducted by Blackman *et al.* (1990) had suggested that an antibody against MSP-1C may interrupt malaria asexual blood stage.

A previous study showed that the mutated version of MSP-1C cloned into a BCG japan strain can induce inhibitory antibody but not blocking antibody. Inhibitory antibodies cause decrease invasion and replication of the parasite while blocking antibody interfere with the binding of inhibitory antibody (Nurul & Norazmi, 2010).

2.7 *Mycobacterium bovis* bacille Calmette- Guerin (BCG)

Mycobacterium bovis bacille Calmette-Guerin (BCG) is a live attenuated bovine tubercle bacillus that is used as a vaccine against tuberculosis (*Stover et al.*, 1991). The League of Nations recommended BCG as the official vaccine against human tuberculosis in 1928. Until today, BCG remains the official and commercially available vaccine against tuberculosis (Bastos *et al.*, 2009). The vaccine is the most widely used around the world and shows low incidence of serious complication (Stover *et al.*, 1991). It is not influenced by maternal antibody, so vaccination can be done at any rate and any time after birth. A single inoculum given can sensitize tuberculoprotein for a longer period of time. BCG is also inexpensive to produce (Stover *et al.*, 1991). Because of these attributes, a major effort has been made to develop the vaccine as a vector to express another antigen (Ohara & Yamada, 2001). Immunizations with BCG give protection during childhood but the efficacy is insufficient to prevent pulmonary tuberculosis in adults. The development of rBCG can provide protection against *M. tuberculosis* more efficiently compared to the wild type strain BCG (Bastos *et al.*, 2009). Development of BCG as a vector expressing multiple antigens is based on its adjuvant properties and ability to replicate inside the antigen presenting cells (APC) such as macrophage and dendritic cell (Bastos *et al.*, 2009). BCG