APOPTOSIS ACTIVITY AND MITOGEN ACTIVATED PROTEIN KINASE EXPRESSION OF THE MOUSE MACROPHAGE CELL LINE J774A.1 INFECTED WITH A RECOMBINANT BCG EXPRESSING THE C-TERMINUS OF MEROZOITE SURFACE PROTEIN-1 OF *Plasmodium falciparum*

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by

ANIS FADHILAH BINTI ZULKIPLI

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LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
AMA1	Apical membrane antigen 1
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATCC	American Type Culture Collection
AV	Annexin-V
B.C.E.	Before Christian era
BCG	Bacille Calmette-Guerin
Bcl-2	B-cell lymphoma 2
CFU	Colony forming unit
CO_2	Carbon dioxide gas
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer and xylene
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
GPI	Glycosylphosphatidylinositol
HCl	Hydrochloric acid
HRP	Horseradish peroxidase

IDV	Integrated density values
Ig	Immunoglobulin
IL	Interleukin
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KCl	Pottasium chloride
KH_2PO_4	Potasium dihydrogen phosphate
kDa	Kilo Dalton
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MIF	Migration inhibitory factor
MOI	Multiplicity of infection
MRI	Measurement of mean relative intensity
MSP-1	Merozoite surface protein-1
$MSP\text{-}1_{19}$	19 kDa C-terminus of merozoite surface protein-1
MSP-1C	C-terminus of the merozoite surface protein-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrogen phosphate
NO	Nitric oxide
OADC	Oleic acid, albumin fraction V, dextrose and catalase
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis

PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PBS-T20	Phosphate-buffered saline-Tween-20
PCR	Polymerase chain reaction
pH	Potential hydrogen
PI	Propidium iodide
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
rBCG	Recombinant BCG
RIPA	Radioimmunoprecipitation assay
RM ANOVA	Repeated measures analysis of variance
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SPSS	Statistical Package of Social Sciences
TEMED	N,N,N',N'-Tetramethylethylenediamine
TB	Tuberculosis
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween-20
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-α
TNFR	Tumor necrosis receptor
TNF-R1	Tumor necrosis receptor 1
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
USA	United States of America

LIST OF SYMBOLS

α	Alpha
β	Beta
cm	Centimeter
° C	Degree Celcius
g	Gram
h	Hour
L	Liter
M	Molar
µ	Micro
nm	Nanometer
%	Percentage
x g	Times gravity
v	Voltage
w/v	Weight/Volume
X	Times
±	Plus-minus
<	Less than

AKTIVITI APOPTOSIS DAN PENGEKSPRESAN PROTEIN PENGAKTIFAN MITOGEN KE ATAS SEL MAKROFAJ MENCIT J774A.1 YANG DIJANGKITI KLON BCG REKOMBINAN YANG MENGEKSPRESKAN PROTEIN PERMUKAAN TERMINUS C MEROZOITE-1 DARIPADA *Plasmodium falciparum*

ABSTRAK

Apoptosis makrofaj merupakan mekanisme yang berkesan dalam mengawal jangkitan intrasel semasa gerak balas imun semulajadi terhadap pelbagai patogen termasuk parasit malaria. Kajian ini dijalankan untuk menentukan aktiviti apoptosis dan pengekspresan protein pengaktifan mitogen (MAPK) dalam sel makrofaj mencit J774A.1 yang dijangkiti klon BCG dan BCG rekombinan (rBCG) yang mengekspreskan terminus C protein permukaan merozoite-1 (MSP-1C) daripada *Plasmodium falciparum* selama 48 jam. Pewarnaan nukleus menggunakan Hoest 33342 menunjukkan bahawa klon rBCG berupaya meningkatkan kondensasi nuklear dan peringkat morfologi apoptosis dalam sel makrofaj yang dijangkiti berbanding BCG dan LPS. Analisis flow sitometri menggunakan pewarnaan Annexin-V dan PI membuktikan bahawa klon rBCG meningkatkan peratusan aktiviti apoptotik awal di dalam sel makrofaj mencit yang dijangkiti berbanding sel yang dijangkiti oleh klon BCG dan dirangsang dengan LPS. Gerak balas apoptosis yang ditunjukkan ini seiring dengan pengurangan pengekpresan protein anti-apoptotik Bcl-2 dan peningkatan pengekspresan protein p53. Ujian permeteran warna menunjukkan klon BCG berupaya mengekspreskan aktiviti kaspase-1, -3, -8 dan -9 manakala klon rBCG hanya mengaktifkan pengekspresan kaspase-1 and -9 di dalam sel makrofaj

yang dijangkiti, mencadangkan penglibatan laluan apoptosis mitokondria (intrinsik). Tambahan pula, jangkitan klon BCG dan rBCG berupaya merangsang pengekspresan protein ekstrasel ERK2 yang lebih tinggi dalam sel yang dijangkiti tetapi mengurangkan secara signifikan pengekspresan protein p38 dan JNK, yang menunjukkan penglibatan sesetengah MAPK dalam aktiviti apoptosis makrofaj yang dijangkiti. Sebagai kesimpulan, kedua-dua klon BCG dan rBCG berupaya meningkatkan aktiviti apoptosis di dalam sel makrofaj mencit, J774A.1. Mekanisme ini adalah penting untuk menyingkirkan patogen seperti parasit malaria semasa aktiviti fagositosis makrofaj. Walaubagaimanapun, klon rBCG menunjukkan aktiviti apoptosis yang lebih tinggi berbanding klon BCG.

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ABSTRACT

Macrophage apoptosis exerts an efficient mechanism in controlling intracellular infection during innate immune response against various pathogens including malaria parasites. This study was carried out to determine the apoptosis activity and mitogen activated protein kinase (MAPK) expression in mouse macrophage cell line J774A.1 infected with a *Mycobacterium bovis* bacille Calmette-Guerin (BCG) clone and a recombinant BCG (rBCG) clone expressing the C-terminus of merozoite surface protein-1 (MSP-1C) of *Plasmodium falciparum* for 48 hours. The nuclear staining with Hoechst 33342 showed that the rBCG clone was capable of increasing the nuclear condensation and morphological stages of apoptosis in the infected cells compared to the BCG-infected cells and the LPS-stimulated cells. The flow cytometric analysis using Annexin-V and PI staining confirmed that the rBCG clone significantly increased the percentage of early apoptotic activity in the infected macrophage higher than the one stimulated by the parent BCG clone and LPS. This apoptotic response corresponded with the reduction of the anti-apoptotic Bcl-2 protein expression and higher p53 expression. The colorimetric assay demonstrated that the BCG clone is capable of stimulating higher production of caspase-1, -3, -8 and -9 while the rBCG clone only stimulated the expression of caspase-1 and -9 in

the infected macrophages, suggesting the involvement of mitochondrial-mediated (intrinsic) pathway of apoptosis. In addition, an infection with BCG and rBCG clones stimulated higher extracellular signal-regulated kinase 2 (ERK2) protein expression in the infected cells but significantly reduced the expression of p38 and c-jun N-terminal kinase (JNK) proteins, suggesting the involvement of certain MAPK in the apoptosis activity of the infected macrophages. In conclusion, both the BCG and rBCG clones are capable of inducing macrophage apoptosis activity in the mouse macrophage cell line J774A.1. This mechanism is important for the elimination of pathogens such as malaria parasite during the phagocytosis activity of macrophage. However, the rBCG clone showed higher apoptosis activity than those produced by the parent BCG clone.

CHAPTER ONE

INTRODUCTION

1.1 Study background

Malaria remains the public health concerns owing to the high rate of mortality and morbidity (Wiwanitkit, 2011). It annually affects millions of people throughout the world, especially older people and pregnant ladies. Mostly, children under the age five years are vulnerable to life-threatening anaemia and cerebral malaria (World Health Organization, 2015). The obligate intracellular parasite, *Plasmodium* is the causative agent of malaria disease. The infection is transmitted to humans through the saliva of the female Anopheles mosquitoes (Sinden *et al.,* 2002). *Plasmodium falciparum* causes the most serious pathologies of malaria disease in human due to its capability to multiply rapidly in the red blood cells. Infections with this parasite can be lethal in the absence of quick detection of the disease (Sinden *et al.,* 2002*;* Snow *et al.,* 2005; Ministry of Health Malaysia, 2014; World Health Organization, 2015).

The development of a safe and effective vaccine that elicits lasting immune responses against malaria has been a major agenda for controlling the disease due to the spread of drug-resistant parasites and insecticide-resistant mosquitoes in many parts of the world (Brogdon *et al,* 1998; Phillips, 2001; Cravo *et al.,* 2015). The clinical symptoms and pathologies associated with malaria occur during the blood stage infection. At this stage, the parasites express various antigens such as AMA1, EBA175 and MSP1. Among these, the 19 kDa C-terminus of the merozoite surface protein-1 (MSP-1_{19}) or also known as MSP-1C has been extensively studied as a

blood-stage malaria vaccine candidate. A previous study showed that antibodies produced against the MSP-1C have been reported to be associated with protection from symptomatic malaria disease (Wan Omar *et al.*, 2007).

Mycobacterium bovis bacilli Calmette-Guerin (BCG) is the only vaccine used for tuberculosis. It represents one of the most promising live vectors for the delivery of foreign antigen to the human immune system, including malaria parasites (Bloom, 1989). Previously, our group has constructed a recombinant BCG clone that expresses the MSP-1C of *Plasmodium falciparum* (Nurul *et al*., 2010). Our constructed vaccine represents a promising candidate to prevent malaria infection by inducing appropriate humoral and cellular immune responses in mice. The vaccine candidate is also capable of stimulating the production of pro-inflammatory cytokines as tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and nitric oxide (NO) and the expression of toll-like receptors in mouse macrophage cell line J774A.1 better than the parent BCG clone. Indeed, the previous finding had demonstrated that the phagocytic activity of macrophage infected with the rBCG clone was increased, resulting in a significant reduction of macrophage viability as well as the viability of the rBCG clone itself (Rapeah *et al.,* 2010).

1.2 Problem statement

The rBCG clone expressing MSP-1C antigen was capable to stimulate higher production of cytokines, nitric oxide and iNOS in mouse macrophage but reduced the viability of the cells (Rapeah *et al*., 2010). We assumed that this phenomenon involved macrophage apoptosis activity due to the fact that the pro-inflammatory

response of macrophage induced by BCG was associated with apoptosis activity (Keane *et al.,* 2000; Riendeau & Kornfeld, 2003; Vanzembergh *et al.,* 2011) and MAPK expression (Cheung *et al.,* 2009). However, information on the ability of the rBCG clone to induce apoptosis activity in macrophage during innate immune response is still lacking and requires more study.

1.3 Rationale of the study

Macrophage apoptosis and MAPK expression is an essential mechanism which exerts protective effects against mycobacteria during innate immune response (Schorey *et al.,* 2003; Raja, 2004; Briken *et al.,* 2008). A previous study has shown that the rBCG clone expressing the MSP-1C of *Plasmodium falciparum* was capable of stimulating the production of a strong pro-inflammatory response such as TNF-α, IL-1β and NO in mouse macrophage cell line J774A.1. We hypothesized that this phenomenon involves macrophage apoptosis mechanism and MAPK activation. To prove this hypothesis, this study was carried out to evaluate the ability of the vaccine candidate to stimulate macrophage apoptosis as well as the signaling pathway involved in this event. The information about these mechanisms will assist in the development of an efficient vaccine against malaria parasite (Jo et *al*., 2007).

1.4 Objectives of the study

1.4.1 General objective

The general objective of this study was to investigate the apoptosis activity and MAPK expression in mouse macrophage cell line J774A.1 infected with parent BCG clone and rBCG clone expressing the MSP-1C of *Plasmodium falciparum*.

1.4.2 Specific objectives

1. To determine the viability of J774A.1 mouse macrophage cells infected with BCG and rBCG clones.
2. To determine the apoptosis activity in mouse macrophage cell line J774A.1 infected with BCG and rBCG clones.
3. To determine the expression of p53, Bcl-2 and Bax in mouse macrophage cell line J774A.1 infected with BCG and rBCG clones.
4. To determine the caspase activity in mouse macrophage cell line J774A.1 infected with BCG and rBCG clones.
5. To determine the expression of MAPK protein in mouse macrophage cell line J774A.1 infected with BCG and rBCG clones.

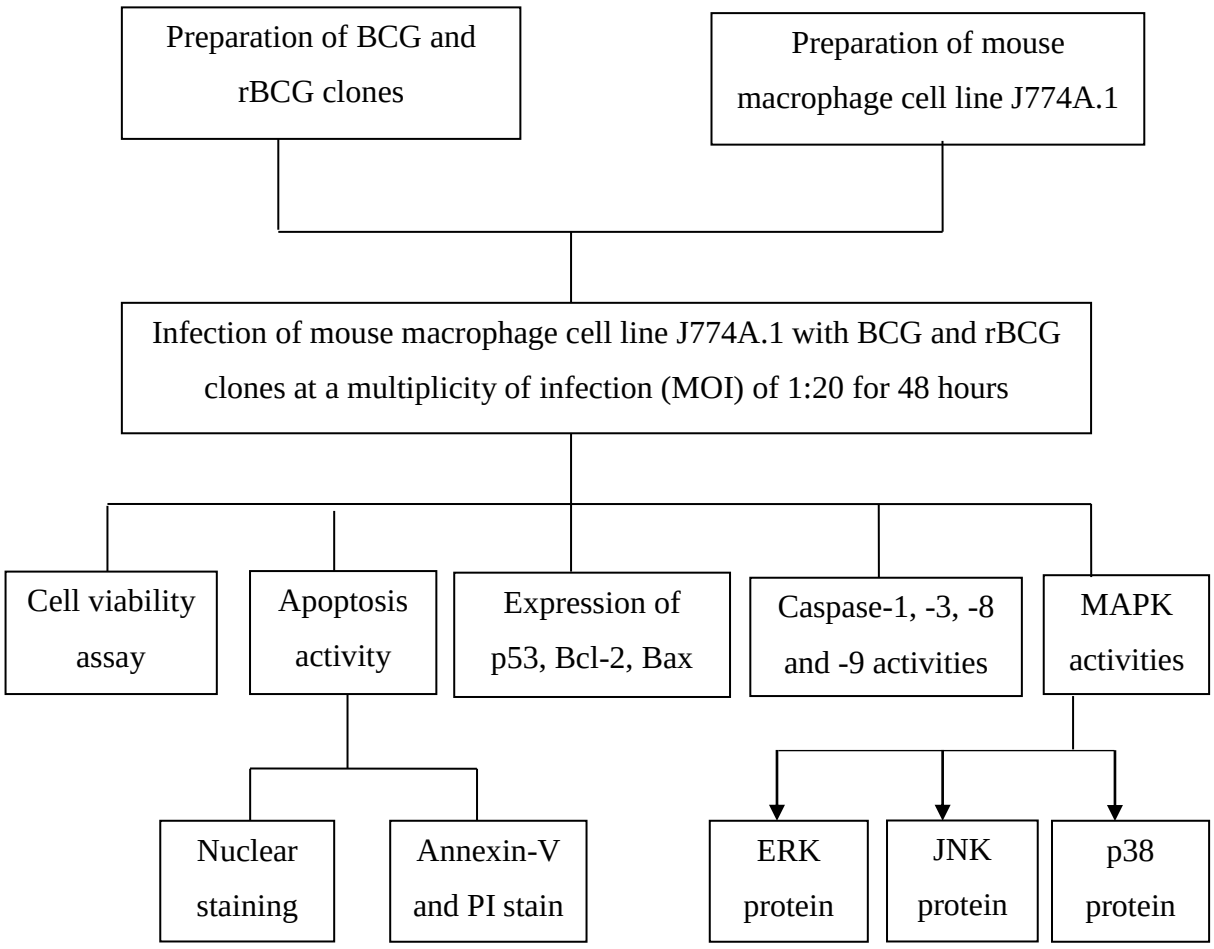

Figure 1.1: Flow chart of the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of malaria

Malaria is an ancient disease which was mostly described in a Chinese document, clay tablets from Mesopotamia, Egyptian papyri and Hindu texts before the Christian era (B.C.E.) (Bruce-Chwatt, 1988). The clinical descriptions of malarial fever were first demonstrated by Hippocrates in about 400 B.C.E. In 1880, Charles Louis Alphonse Laveran detected live microscopic bodies in blood of malaria patient and suggested it as the malaria parasite. All claims of various parasites causing malaria were verified after the development of the staining method using Eosin and Methylene Blue by Dimitri Romanowsky in 1891 (Cox, 2010). In 1896, MacCallum from Johns Hopkins Medical School found the sexual cycle of the malaria parasite (MacCallum, 1987). In 1902, Ronald Ross found that infected mosquitoes were accountable for transmission of malaria in 1897 (Jarcho, 1984; Hoffman *et al.*, 1999). Between 1898 and 1900, an Italian group revealed the sporogony of *Plasmodium falciparum* in Anopheles mosquitoes (Dobson *et al.*, 1999). In 1912, the asexual cycle of *Plasmodium falciparum in vitro* was first cultivated by C. C. Bass and Foster M. Johns in human blood, but limited to a few initial maturation cycles (Kreier, 1980). Later in 1976, continuous culture of *Plasmodium falciparum* in a medium of red blood cells was successfully done by William Trager and it is a starting point of discovery of vaccine for malaria (Trager & Jensen, 1976). The first synthetic Spf66 vaccine for *Plasmodium falciparum* infection was developed by a Colombian Biochemist, Dr. Manuel Elkin Patarroyo in 1987 (Graves *et al.*, 2008).

2.2 Malaria burden

2.2.1 Worldwide

Malaria infection is a serious worldwide health issue which contributes to millions of deaths. According to the latest estimates in 2015, about 214 million cases of malaria and 438 000 malarial deaths were reported (World Health Organization, 2015). Globally, approximately 306 000 children before the age of five years are vulnerable to get malaria and its developing disease (Heelan *et al.*, 2002; World Health Organization, 2015). African Region is the most affected region with malaria burden, where an estimated 292 000 cases of malaria deaths were occurred in African children. The majority of malaria deaths in the African continent were caused by *Plasmodium falciparum* (World Health Organization, 2015; CDC, 2016). Figure 2.1 shows areas where malaria transmission occurs.

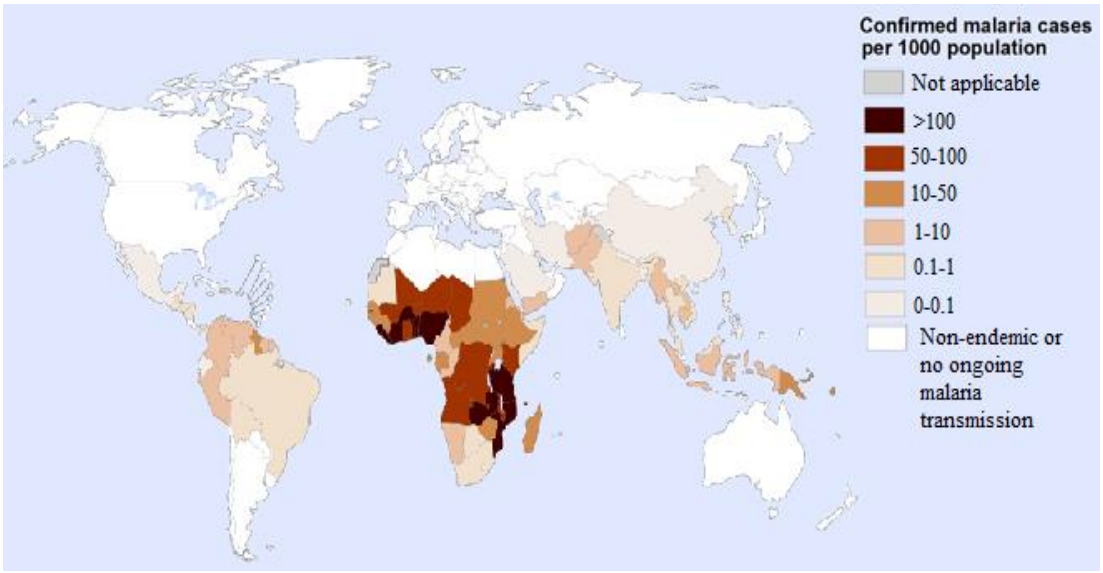

Figure 2.1: Global distribution of malaria transmission risk for 2015. The figure is adapted from World Health Organization (2015).

2.2.2 Malaysia

In Malaysia, approximately 3923 new cases of malaria infections and nine cases of malaria deaths were reported in 2014. These infections comprise of human malaria infections and zoonotic malaria infections. Among the human malaria infections, *Plasmodium vivax* accounted most of the malaria infections (8%) followed by *Plasmodium falciparum* (7%) (World Health Organization, 2015). As shown in Figure 2.2, malaria infections were usually found in the states of Sabah and Sarawak. There were a few cases of malaria infections reported in Peninsular Malaysia (Kheong *et al.,* 2010; Rundi *et al.,* 2011; Ministry of Health Malaysia, 2013; World Health Organization, 2015). *Plasmodium knowlesi*, a simian malaria parasite that infects human was detected in the forested regions of Malaysia especially in Sarawak (Singh *et al.*, 2010). These species accounted 38% of the reported cases of malaria in 2014 (World Health Organization, 2015). It was reported that 61.9% of malaria cases were between the ages of 20 to 49 years and the most affected group were in the age group of 20 to 29 years (25%). Also, 2.5% of all malaria cases were in children below the age of five. Malaria predominantly affected male (78.2%) compared with female (21.8%). Approximately, 8.5% of the female patients who suffered malaria infection were pregnant women (Ministry of Health Malaysia, 2013).

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Figure 2.2: Distribution of confirmed malaria cases (per 1000 population). The figure is adapted from the World Health Organization (2015).

2.3 Malaria parasites

2.3.1 *Plasmodium* species

Plasmodium is the causative agent of malaria infection, which is transmitted through the saliva of female Anopheles mosquitoes to human (Levinsion, 2008). Five species of *Plasmodium* that infect human are *Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax, Plasmodium knowlesi* and *Plasmodium ovale* (Mendis *et al.,* 2001; Singh *et al.,* 2004; Collins & Jeffery, 2005; Snow *et al.,* 2005; Collins & Jeffery, 2007). The various species of parasites are classified based on their disease's severity (Wipasa *et al.,* 2002) and have different microscopic appearances during the blood stage life cycle. Among the five species, the most life-threatening species is *Plasmodium falciparum* due to its ability to multiply rapidly in the patient's blood which contributes to severe anaemia in the patient. *Plasmodium falciparum* infection may lead to death if untreated (Sinden & Gilles, 2002; Snow *et al.,* 2005).

2.3.2 Life cycle of *Plasmodium*

The mode of infection and developmental stages in the hosts are similar in all human malaria parasites. As illustrated in Figure 2.3, the complex life cycles of *Plasmodium* consist of two major stages; asexual development in the human host and sexual development in the mosquito vector (Gilles, 1997). While infected mosquitoes draw its blood meal, the spindle-shaped sporozoites are injected into the human bloodstream through their salivary glands. Mostly, this liver stage is asymptomatic here the majorities of sporozoites invade hepatocytes of liver and multiply in it (Sherman, 1998; Amino *et al.,* 2006).

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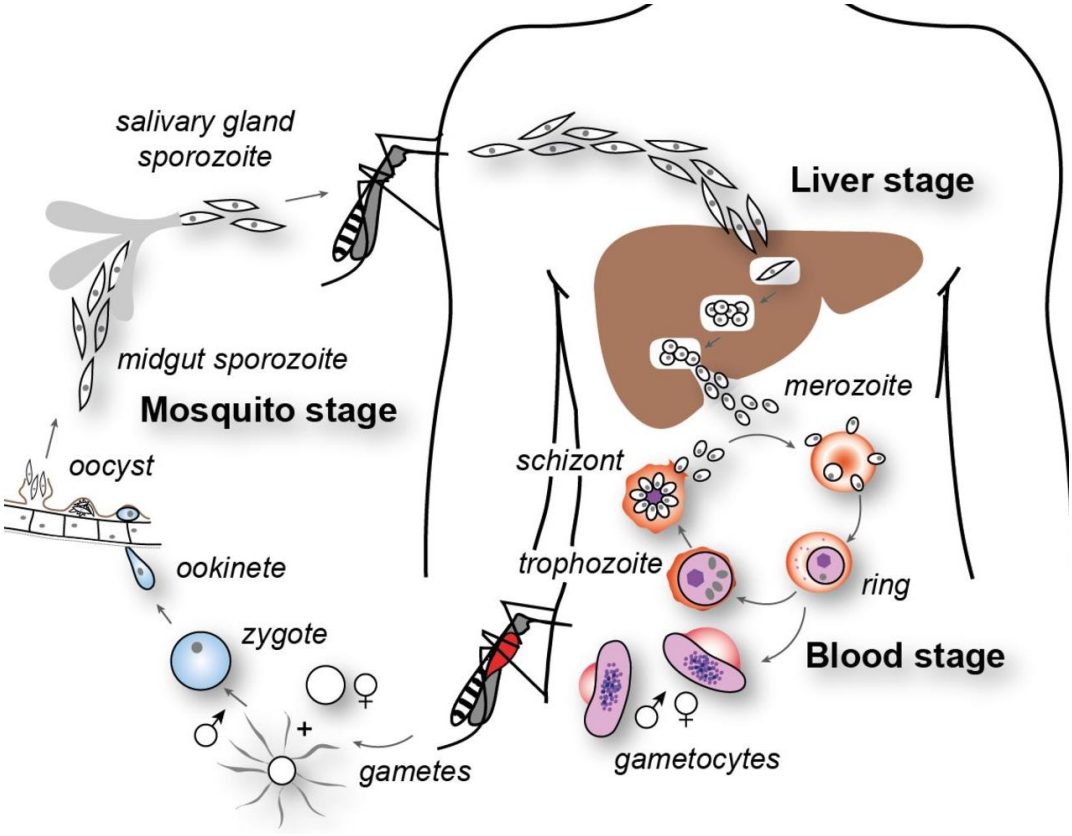

Figure 2.3: Life cycle of *Plasmodium falciparum*. The figure is adapted from Cowman *et al.* (2012).

The maturation and differentiation of sporozoites into merozoites inside hepatocytes take place within one to two weeks. About 30,000 merozoites are free to leave from ruptured liver cells into the blood circulation. Then, the newly formed merozoites initiate asexual multiplication cycle where they invade red blood cells in the bloodstream. During the asexual blood stage, the formed merozoites develop into trophozoites and then, turn into schizonts. Every schizont produces 10,000 to 30,000 merozoites which then infect other erythrocytes to restart a new cycle (Sherman, 1998; Ginsburg *et al.*, 1990; Amino *et al.*, 2006).

The sexual cycle is initiated when some of the released merozoites from infected RBC turned into mature gametocytes. The mature gametocytes circulate in the human bloodstream and can be ingested by the mosquitoes. The maturation process of gametocytes continues in the midgut of mosquito where the union of gametes results in the formation of zygotes. The zygotes become motile forms of ookinete which can pass through the midgut wall of mosquito and then, grows into oocysts. Over time, the oocysts expand and rupture which lead to the formation of sporozoites. The released sporozoites are transmitted to infect new human being through the mosquito salivary gland (Bousema & Drakeley, 2011).

2.4 Clinical symptoms and manifestations

The person infected with malaria parasites remains asymptomatic during the liver stage of parasite life cycle. The infected person will suffer nonspecific symptoms of uncomplicated malaria such as fever, headache and chills when infected erythrocyte ruptured to release merozoites. During the erythrocytic cycle, severe forms of malaria pathologies including cerebral anaemia (coma), hypoglycemia, metabolic acidosis, renal failure and severe anaemia were observed (Miller *et al.*, 1994; Lamikanra *et al.*, 2007; Haldar & Mohandas, 2009). The majority complication of severe malaria cases such as kidney failure and coma contribute to deaths. Thrombocytopenia and hepatosplenomegaly are other complications of severe malaria which occur in infected people especially in the children (Sinden & Gilles, 2002; Vivas *et al.*, 2008).

2.5 Pathogenesis of malaria

Sequestration is a key event of pathogenesis of malaria leading to severe malaria. This event occurred when infected RBC from blood circulation binds to the endothelium of capillaries and venules of the host cell (Fayard *et al.*, 2010). Sequestration can be characterized by the blockage of blood vessel and impaired oxygen supply. This phenomenon can happen in vital organs and leads to organs dysfunction (Turner *et al.*, 1994). The adhesion of infected RBC in endothelium of the brain results in cerebral malaria (Miller *et al.*, 1994; Wassmer *et al.*, 2003). Furthermore, the occurrence of cerebral malaria is mediated by the production of NO and other pro-inflammatory cytokines (Clark *et al.*, 1992). Sequestration events in

intervillous spaces of placenta also cause complications in infants including low birth weight and infant anaemia (Rogerson *et al.,* 2007).

Another pathogenesis of malaria is rosetting, described as when the infected RBC adheres to the uninfected RBC leading to the formation of rosettes. This phenomenon contributes to severe form of microvascular disease (Udomsangpetch *et al.,* 1989; Chen *et al.* 2000). In addition, glycosyl-phosphatidyl inositol-anchored protein of *Plasmodium falciparum* plays a significant role in the pathogenesis of severe malaria through induction of pro-inflammatory response in mammalian hosts (Schofield *et al*., 1993; Arrighi & Faye, 2010).

2.6 Prevention and treatment of malaria

Antimalarial drugs such as chloroquine, mefloquine, quinine and artemisinin have been widely used in the treatment of malaria. Due to the complexity of malaria parasites, these drugs only destroy specific morphological stages of malarial life cycle (Bjorkman *et al.,* 1990; Heelan *et al.,* 2002; Cravo *et al*, 2015). Thus, the combinations of artemisinin derivatives with other drugs (mefloquine, piperaquine and amodiaquine) were used for malaria treatments in order to control malaria symptoms as well as reduce parasite density (Price, 2000). Moreover, the emergence of drug-resistant parasite provides great challenges to the treatment of malaria (Bjorkman *et al.,* 1990; Cravo *et al*, 2015).

Together with treatments of malaria, long term mosquito abatement was implemented for reduction of infected mosquito (vector) population. Several

strategies such as elimination of mosquito breeding sites and the use of insecticides were applied in many parts of the world to prevent malaria parasites transmission from mosquitoes to human (Heelan *et al.,* 2002). In addition, insecticide-treated nets have been used to protect human from the bite of infected mosquito as well as kill sporozoite-infected mosquito. Still, this strategy is partially effective because some of the mosquitoes have developed into insecticide-resistant mosquitoes (Heelan *et al.,* 2002; Bockarie *et al.,* 2006).

2.7 Malaria vaccine development

2.7.1 Different targets of malaria vaccine

The development of a malaria vaccine is a public agenda since the widespread of the drug-resistant malaria parasites and insecticide-resistant mosquitoes gave a difficulty in combating malaria infection (Phillips, 2001). Various malaria vaccine candidates have been developed against the diverse stages of life cycle such as asexual blood stage, pre-erythrocytic stage and transmission-blocking stage (Wipasa *et al.,* 2002; Hill, 2011). Figure 2.4 shows malaria vaccine candidate antigens.

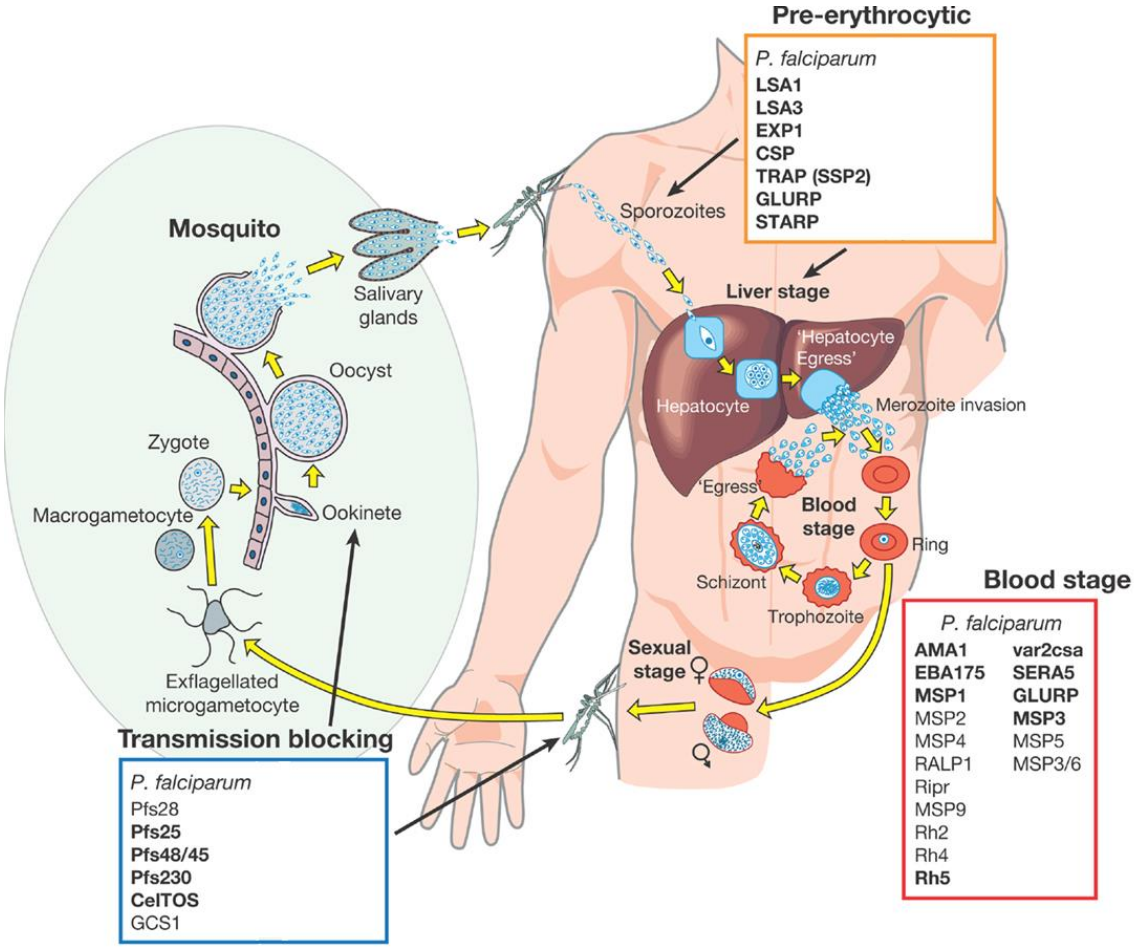

Figure 2.4: Malaria vaccine candidate antigens. All the candidate antigens for *Plasmodium falciparum* are superimposed on the *Plasmodium* life cycle, to indicate the category of malaria vaccine being developed and the life cycle stage targeted. Antigens indicated in bold are those that are currently being evaluated in pre-clinical trials or have entered at least Phase 1 clinical trials according to the World Health Organization malaria vaccine rainbow tables. This figure is adapted from the World Health Organization (2013).

The vast majority of malaria vaccine candidates are blood stage vaccines. These vaccines target merozoites antigen that is involved during the invasion of RBCs (Ellis *et al,* 2010; Birkett *et al.,* 2013; Riley *et al.,* 2013). The purpose of these vaccines is to reduce or prevent severe form of malaria complications that were developed from RBC invasion at the blood stage. Among these vaccines, apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) are leading vaccine candidates that have been extensively studied (Ellis *et al,* 2010).

Pre-erythrocytic vaccines are potential vaccine candidates that target sporozoites of *Plasmodium* during pre-erythrocytic stage. The reduction of sporozoites can indirectly reduce the release of merozoites into the blood circulation, preventing the progression of blood-stage disease (Bejon *et al.,* 2005; Duffy *et al.,* 2012). Among the pre-erythrocytic vaccines, RTS,S vaccine is the first malaria vaccine candidate that enters Phase III trials. Vaccination with RTS,S induce antibodies against the circumsporozoite protein (CSP) of *Plasmodium falciparum.* However, this vaccine was partially effective in treating severe malaria complication (Agnandji *et al.,* 2012; Ajua *et al.,* 2015; Moorthy *et al.,* 2015).

Meanwhile, malaria transmission-blocking vaccines target gametocytes and oocyst at the sexual life cycle. Although these vaccines cannot prevent clinical illness of malaria, but these vaccines prevent human-to-mosquito transmission and greatly assist in the elimination of malaria infections (Bousema & Drakeley, 2011; Birkett *et al.,* 2013; Riley *et al.,* 2013; Nunes *et al.,* 2014).

2.7.2 C-terminus of merozoite surface protein-1

Several parasite proteins have been identified as vaccine candidate antigens that are capable to stimulate host's natural immunity (Doolan *et al.,* 2003; Dutta *et al.,* 2009; Hviid, 2010). Among them, the merozoite surface protein 1 (MSP-1) has been extensively studied as the candidate antigen during the blood stage cycle. This antigen can be found in human malaria parasites (Matsumoto *et al.,* 1998). During the cleavage process of MSP-1, only COOH-terminal 19-kD fragment of MSP-1 ($MSP\text{-}1_{19}$) remains on the surface of merozoite during RBC invasion (Blackman *et al.,* 1990; Blackman *et al.,* 1991). *In vitro* study demonstrated that antibodies produced in response of $MSP\text{-}1_{19}$ inhibited RBC invasion by merozoite (Blackman *et al.,* 1990). In addition, protective immunity induced by MSP-1 was observed in the *Plasmodium yoelii* model (Holder *et al.,* 1981; Burns *et al.,* 1989; Daly *et al.,* 1993). Also, that vaccination with MSP-1 of *Plasmodium falciparum* protects non-human primate from the malaria infection (Herrera *et al.,* 1990).

2.7.3 Recombinant BCG as a potential vaccine candidate

Mycobacterium bovis bacille Calmette-Guerin (BCG) is a non-pathogenic and attenuated strain of *Mycobacterium bovis*. BCG has been widely used as a tuberculosis (TB) vaccine because it preserves the immunogenic characteristics of TB (Britton & Palendira, 2003). Since it offers low toxicity, adjuvant potential and long-lasting immunity, this vaccine has been extensively used as a vaccine vehicle for the presentation of protective antigens in the development of new live recombinant vaccines (Bloom, 1989; Stover *et al.,* 1991). However, mice immunized

with BCG only stimulate nonspecific resistance against malaria infection (Clark *et al.,* 1976; Murphy *et al.,* 1981; Lussow *et al.,* 1990). Thus, the combination of BCG and MSP-1C is a reasonable approach in order to enhance, broaden and extend immune protection against a range of diseases including malaria (Magalhaes *et al.,* 2008; Stover *et al.,* 1994).

Previously, a rBCG expressing the synthetic MSP-1C of *Plasmodium falciparum* was constructed (Nurul *et al*., 2010). The vaccine was capable of stimulating a strong inflammatory action in macrophages by enhancing its phagocytic activity and secretion of pro-inflammatory cytokines (TNF-α, IL-1β, NO). The vaccine candidate was also able to reduce the viability of infected macrophages (Dhaniah *et al*., 2014).

2.8 Immunity against malaria infection

2.8.1 Innate immunity

Innate immunity is an efficient way to control early infection of infectious agents such as mycobacteria. Recognition of mycobacteria through toll-like receptors (TLRs) leads to the initiation of mitogen-activated protein kinase pathways (Medzhitov *et al.,* 2000; Akira *et al.,* 2004; Akira *et al.,* 2006). These mechanisms contribute to pro-inflammatory responses which is important in innate host defense against various infectious agents (Joyoti *et al.,* 2012). According to Artavanis *et al.* (2003), the secretion of pro-inflammatory cytokines in innate immunity was capable to protect mice and humans from erythrocytic malaria. Also, innate immunity plays a crucial part in inhibiting the replication of malaria parasites and delays the severe complications of malaria disease (Rojas *et al.,* 1999; Artavanis *et al.*, 2003).

2.8.2 Macrophage

Antigen-presenting cells such as macrophages, monocytes and dendritic cells are key elements of innate immune response. These cells provide a rapid defense mechanism to protect the host from invading pathogens (Stevenson, 2004). Among these cells, macrophage represents as the main phagocytic cell as the activation of macrophage is crucial in innate immune protection against mycobacteria. Macrophages also have several functions such as killing pathogenic microorganisms, presenting the antigens ingested to T-lymphocytes and removing the cell debris (Fenton *et al.*, 1996; Lee, 2007). In addition, macrophage infected with mycobacteria triggering the cellular events of microbial mechanisms such as the secretion of pro-inflammatory cytokine and ligation of pattern recognition receptor. The secretion pro-inflammatory cytokine is capable to inhibit the progression of pathogen growth. Meanwhile, ligation of pattern recognition receptor avoids the cytopathic effects of invading pathogen (Giacomini *et al.*, 2001; Lee *at al.*, 2009).

2.9 Apoptotic cell death

The programmed cell death, apoptosis is an efficient mechanism during the innate immune response which limits the release of intracellular pathogens. The dissemination of mycobacteria was controlled through sequestration of the pathogens within apoptotic bodies (Bailey *et al.*, 2005; Behar *et al.*, 2011). Apoptosis activity can be recognized by the preservation of plasma membrane, the shrinkage of cells, the presence of apoptotic bodies, the condensation of chromatin and the fragmentation of the nucleus. Apoptosis exerts protective activity against

mycobacteria by preventing the release of intracellular components. Also, apoptosis prevents the dissemination of mycobacteria through sequestering the pathogens within apoptotic bodies leading to a reduction in bacterial viability (Behar *et al.,* 2011). Macrophage acts as a phagocyte which engulfs the apoptotic bodies and removed them in order to avoid inflammation and tissue damage (Fadokl *et al*., 1992; Schwartzman *et al.,* 1993; Falasca *et al.,* 1996; Wickman *et al.,* 2012). As shown in Figure 2.5, there are several mechanisms associated with apoptosis include the accumulation of p53 protein and activation of a caspase-dependent, cytolytic pathway (Mitchell *et al.,* 2002).

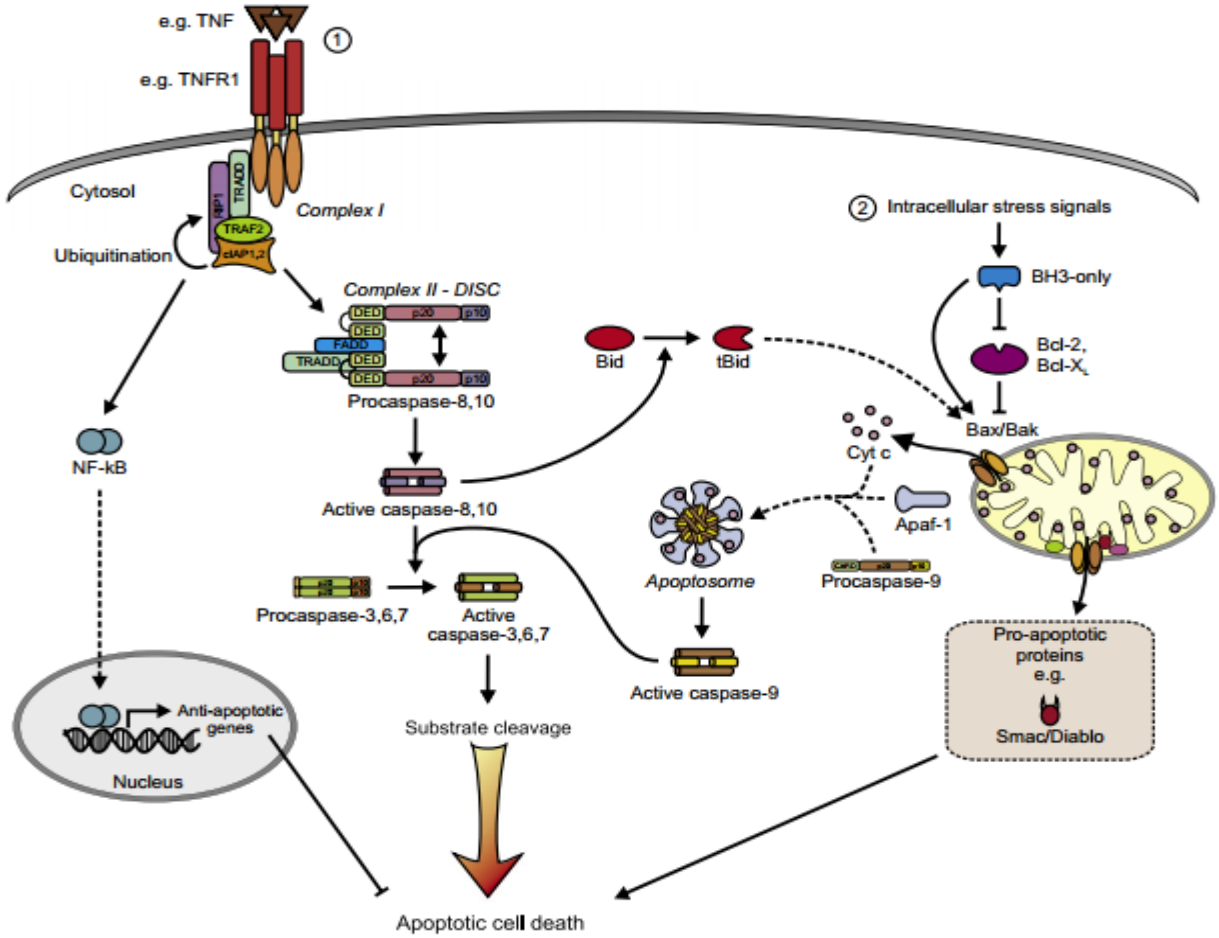

Figure 2.5: Apoptosis signaling pathways. This figure is adapted from Duprez *et al.* (2009).

2.10 Caspase activation

Apoptosis is mediated by caspases enzymes which are known as cysteinyl aspartate-specific proteases. Caspase-dependent apoptosis can be triggered by two different pathways such as death receptor (extrinsic) and mitochondrial (intrinsic) signaling pathways (Figure 2.6). The extrinsic signaling pathway is linked to the ligation and oligomerization of death receptor by their cognate ligands. The binding of death receptor such as tumor necrosis receptor (TNFR) to its cognate ligands (TNF-α) result in the activation of upstream caspase-8 (initiator caspase) (Chen *et al.*, 2002). These events lead to the activation of downstream caspase-3 (effector caspase) triggering apoptosis activity (Lavrik *et al.*, 2012).

As shown in Figure 2.6, the intrinsic pathway is initiated by the permeabilization of mitochondria. The disruption of mitochondria causes the release of apoptotic proteins. Cytochrome c from mitochondria enters the cytosol and results in the formation of apoptosome. These cellular events initiate the activation of caspase-9 (initiator caspase) leading to activation of caspase-3 (effector caspase) for apoptosis process (Li *et al.,* 1997; Green *et al.,* 2004; Gavathiotis *et al.,* 2011; Yivgi-Ohana *et al.,* 2011). The intrinsic pathway involves the release of Bcl-2 family proteins which are crucial regulators of apoptosis. The anti-apoptotic proteins such as Bcl-2, Bcl-w, and Bcl-xl play a role in apoptosis inhibition. Meanwhile, pro-apoptotic proteins such as Bax, Bak and Bcl-xs promote apoptosis activity (Chipuk *et al.,* 2008; Brunelle *et al.,* 2009).