IMMUNOREGULATORY EFFECTS OF RECOMBINANT BCG (rBCG) EXPRESSING C-TERMINUS OF MEROZOITE SURFACE PROTEIN-1 (MSP-1C) OF *Plasmodium falciparum* ON MOUSE MACROPHAGE

by

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LIST OF ABREVIATIONS

ACT	Artemisinin-based combination therapy
AFB	Acid fast bacilli
AMA-1	Apical membrane antigen 1
APC	Antigen presenting cell
BC	Before century
BCG	Bacille Calmette-Guerin
CFU	Colony forming unit
CS	Circumsporozoite
DC	Dentritic cell
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer and xylene
ECL	Enhanced chemiluminescent
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
GAP-12	GATA sequence in the IL-12 promoter (GA-12)-binding protein
GPI	Glycosyl phosphatidyl inositol
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IDV	Integrated density values

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRS	Indoor residual spraying
ITN	Insecticide treated net
kDA	Kilo Dalton
LLIN	Long-Lasting Insecticidal Nets
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
Mø	Macrophage
MHC	Major histocompatibility complex
MOH	Ministry of Health Malaysia
MOI	Multiplicity of infection
MRI	Mean relative intensity
mRNA	Messenger ribonucleic acid
MSP-1	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
NK	Natural killer
NO	Nitric oxide
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PEPSCAN	Peptide scanning
PI	Phagocytic index

pLDH	Parasite lactate dehydrogenase
QBC	Quantitative buffy coat
RBC	Red blood cell
rBCG	Recombinant Bacille Calmette-Guerin
RDT	Rapid diagnostic test
RIPA	Radioimmunoprecipitation assay
RM ANOVA	Repeated measured analysis of variance
rmIFN-γ	Recombinant mouse interferon-gamma
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDS-PAGE	Sodium dodecyl sulphate–Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
SMA	Severe malarial anemia
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UV	Ultra violet
USA	United States of America
WHO	World Health Organization

KESAN IMMUNOREGULATORI KLON BCG REKOMBINAN (rBCG) YANG MENGEKSPRESKAN TERMINUS C PROTEIN PERMUKAAN MEROZOITE-1 (MSP-1C) DARIPADA *Plasmodium falciparum* KE ATAS SEL MAKROFAJ MENCIT

ABSTRAK

Fagositosis oleh makrofaj adalah mekanisme pertahanan pertama terhadap jangkitan parasit malaria dalam sistem imun semula jadi. Setelah diaktifkan, makrofaj akan bertindak sebagai sel radang dan sel anti-mikrob melalui rembesan sitokin pro-inflamatori dan nitrik oksida (NO). Dalam kajian ini, kesan immunoregulatori klon BCG rekombinan (rBCG) yang mengekspreskan terminus C protein permukaan merozoite-1 19-kDa (MSP-1C) daripada *Plasmodium falciparum* ke atas sel makrofaj mencit, J774A.1 telah dikaji. Keupayaan klon rBCG merangsang fagositosis, pertumbuhan makrofaj, pengekspresan reseptor toll-like (TLR), pengeluaran sitokin pro-inflamatori, NO dan sintase nitrik oksida indusibel (iNOS) oleh makrofaj dalam kehadiran atau ketiadaan lipopolisakarida (LPS) atau LPS + interferon (IFN)-γ telah ditentukan. Keputusan menunjukkan bahawa klon rBCG mampu meningkatkan aktiviti fagositik, pengekspresan TLR dan pengeluaran sitokin pro-inflamatori seperti tumor nekrosis factor (TNF)-α dan interleukin (IL)-1β serta NO dan iNOS oleh makrofaj yang telah dijangkiti apabila dibandingkan dengan BCG. Keputusan juga menunjukkan bahawa klon rBCG mengurangkan pengeluaran IL-12 p40 dan pertumbuhan makrofaj yang telah dijangkiti serta pertumbuhan klon rBCG itu sendiri apabila dibandingkan dengan BCG. Kesan immunoregulatori ini adalah penting dalam tindak balas imun semula jadi terhadap parasit malaria. Oleh itu, klon rBCG ini mungkin berpotensi digunakan untuk mengawal jangkitan malaria.

IMMUNOREGULATORY EFFECTS OF RECOMBINANT BCG (rBCG) EXPRESSING C-TERMINUS OF MEROZOITE SURFACE PROTEIN-1 (MSP-1C) OF *Plasmodium falciparum* ON MOUSE MACROPHAGE

ABSTRACT

Macrophage phagocytosis acts as the first line of defence against malaria parasites infection in the innate immune system. Once activated, macrophages act as inflammatory and microbicidal cells through the production of pro-inflammatory cytokines and nitric oxide (NO). In this study, the immunoregulatory effects of a recombinant BCG (rBCG) clone expressing the 19-kDa C-terminus of the merozoite surface protein-1 (MSP-1C) of *Plasmodium falciparum* was examined on the mouse macrophage cell line J774A.1. The ability of the rBCG clone to stimulate phagocytosis, macrophage viability, expression of toll-like receptors (TLRs) and production of pro-inflammatory cytokines, NO and inducible nitric oxide synthase (iNOS) by the macrophages in the presence or absence of lipopolysaccharide (LPS) or LPS + interferon (IFN)-γ was determined. The results demonstrated that the rBCG clone was able to enhance the phagocytic activity, TLRs expression and production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β as well as NO and iNOS of the infected macrophages when compared to parent BCG. The results also demonstrated that the rBCG clone reduced the IL-12 p40 production and the viability of the infected macrophages as well as the growth of the rBCG clones when compared to BCG. These immunoregulatory effects are important in innate immune response against malaria parasite. Thus, this rBCG clone may potentially be used to control malaria infection.

CHAPTER ONE

INTRODUCTION

1.1 History of Malaria

Many ancient histories already described the symptoms of malaria. The word malaria came from an Italian word, “mal’aria” which means “bad or evil air”. In ancient Chinese, before 4700 years ago, it had been described in the Nei Ching (the Canon of Medicine by Emperor Huang Ti), where the symptom apparently refers to repeated fever associated with enlarged spleens and a tendency to epidemic occurrence. Sumerian and Egyptian texts, 3500 to 4000 years ago, also described the fever and spleens enlargement that suggested to malaria symptoms. A Greek physician, Hippocrates who lived in the fifth century B.C., was the first to describe the clinical picture of malaria. While in 25th B.C., Celsus recorded the various types of malaria in the literature and depopulation of rural area. In Sanskrit medical texts, the symptoms of malaria were described and attributed to the bites of certain insects (Gilles, 2002).

A Qinghao plant known as sweet wormwood was first described for its antifever properties during the second century B.C. in ancient Chinese. In 1971, a Chinese scientist had found an active ingredient from the Qinghao known as artimisinin. In the seventeenth century, Indian tribes used the Peruvian bark as the treatment for fevers. It is only in 1820 that Pelletier and Caventou in France had successfully isolated an active compound named quinine from the Peruvian bark. Both artimisinin and quinine are now known as very effective antimalarial drugs (Gilles, 2002).

In 1880 Charles Leveron, a French army surgeon who worked in Algeria, first saw and described malaria parasites in the red blood cells of a patient. However, how this disease was transmitted from a person to a person remains unknown until late 1987. It was Ronald Ross, a British officer in the Indian Medical Service, who first discovered that malaria parasites could be transmitted from an infected patient to mosquitoes. The whole picture of this malaria parasites complex life cycle was then became clear as a result from studies by a group of Italian researchers led by Grassi from 1898 to 1899 (Gilles, 2002).

1.2 Malarial burden

1.2.1 Worldwide

World Health Organization (WHO) estimated that 2.8 billion people (half of the world's population) are living in malaria transmission risk areas, where 1.1 billion people (one fifth of this population) live in areas with a high risk of malaria transmission (WHO, 2012a). It is found that African region has the largest number of people living in areas with high risk of malaria transmission followed by South-East Asia region. However, the largest populations at some risk of malaria transmission are found in South-East Asia and Western Pacific regions. Figure 1.1 shows areas where malaria transmission occurs.

In 2011, it is estimated that there are 219 million cases of malaria worldwide, where 79 % cases were in African region, 15 % cases were in South-East Asia and 5% in Eastern Mediterranean region. Among the malaria cases, 91 % were due to *Plasmodium falciparum* infection. It is estimated that approximately 660 000 malaria deaths occurred worldwide in 2011, of which 90 % deaths were in African region,

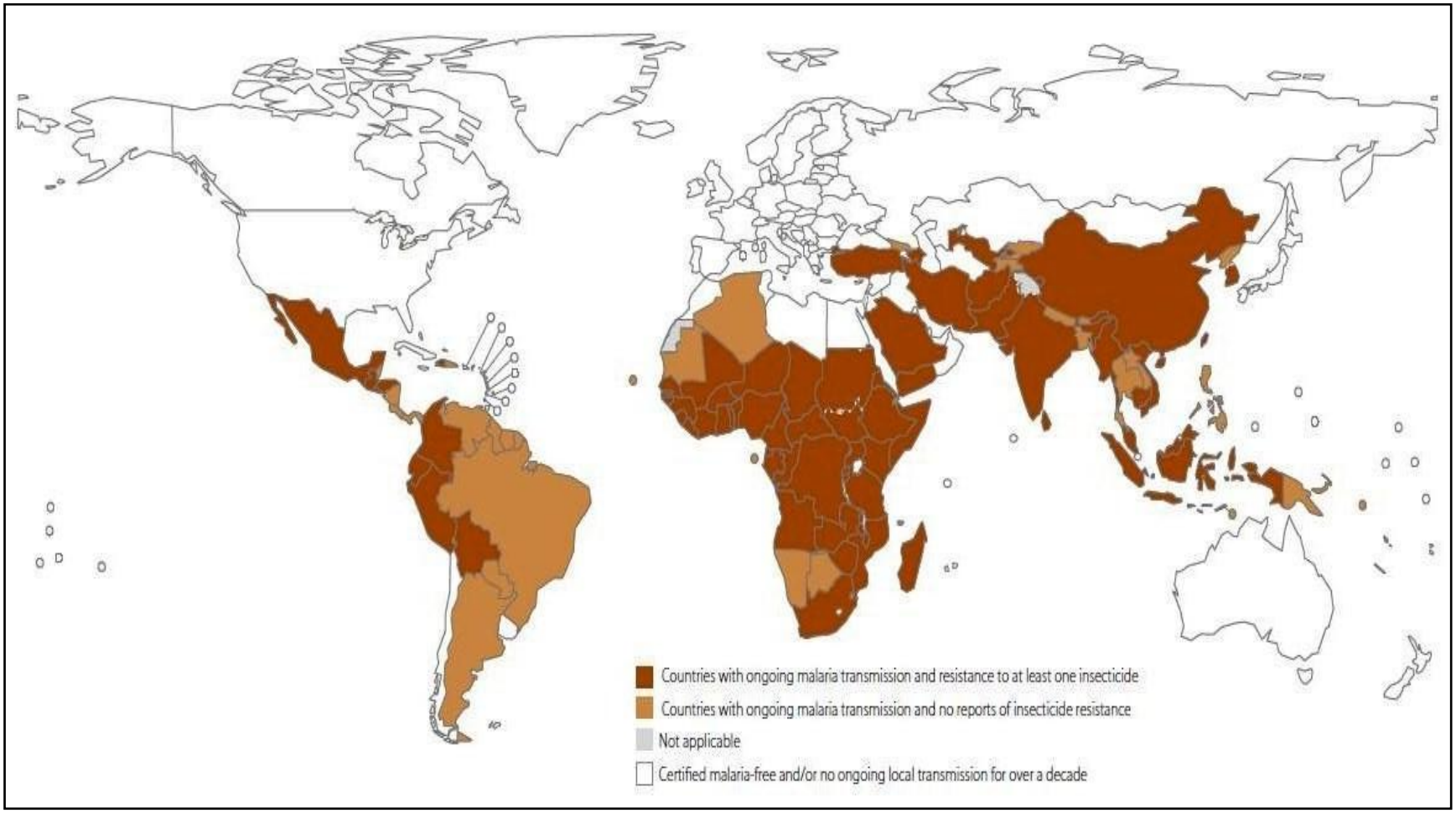

Figure 1.1: Global distribution of malaria transmission risk for 2011. Figure is adapted from WHO (2012a).

6 % deaths in the South-East Asia and 2 % deaths in Eastern Mediterranean region. From this total malaria death globally, 86 % deaths were children under five years old (WHO, 2012a).

1.2.2 Malaysia

Most malaria transmission areas in Malaysia are in Sabah, Sarawak and in the interior central region of Peninsular Malaysia where Perak, Pahang and Kelantan share their borders. In 2011, it is estimated that more than 1 million people live in active malaria transmission areas. Figure 1.2 shows the distribution of confirmed malaria cases in Malaysia. It is estimated that 1.6 million people are suspected with malaria in Malaysia, where *P. vivax* infection was the main cause of the malarial infection followed by *P. falciparum* infection or mixed infections (WHO, 2012a).

Sabah and Sarawak are the major contributors for malaria cases in Malaysia where the highest cases were in Sabah with 12.19 per 10 000 populations followed by Sarawak with 7.28, Kelantan with 1.68 and Pahang with 1.14 (MOH, 2009). In 2009, the Ministry of Health Malaysia (MOH) reported that the number of malaria deaths was the highest in Sabah where 13 people died followed by Sarawak with eight, Selangor with two and one each for Perak, Pahang and Terengganu (MOH, 2009). However, the mortality rate caused by malaria was 0.06 per 100 000 populations in 2011 in Malaysia (MOH, 2012).

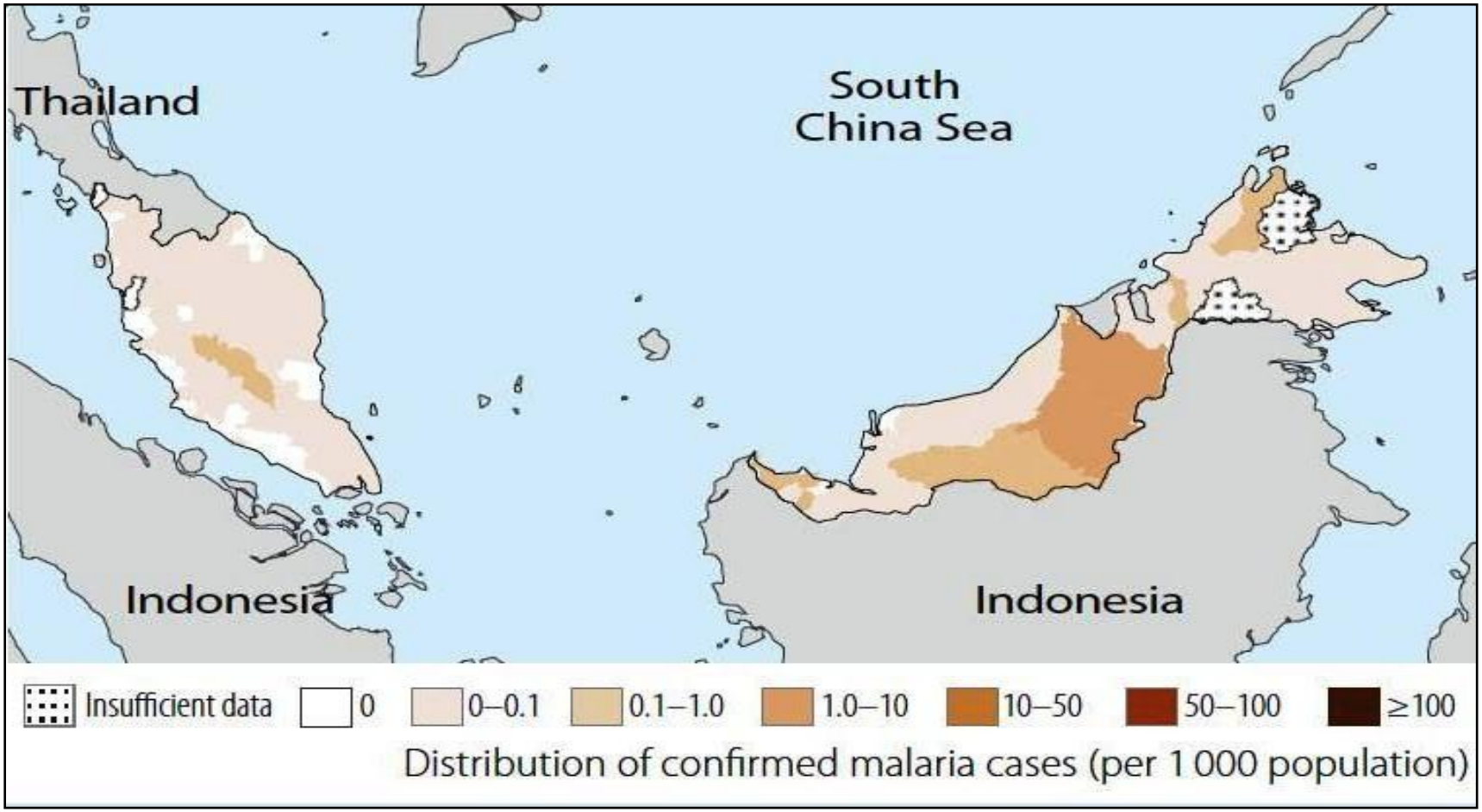

Figure 1.2: Distribution of confirmed malaria cases in Malaysia for 2011. Figure is adapted from WHO (2012).

1.3 Malaria parasite

1.3.1 Characterization

Malaria is caused by protozoan parasites of the genus *Plasmodium*, family *Plasmodiidae*, suborder *Heamosporidiidae*, order *Coccidia*, which can be found in the blood of mammals, reptiles and birds. They are recognized taxonomically by two types of asexual division which are schizogony, in the vertebrate host and sporogony, in the insect vector (Sinden & Gilles, 2002). Five malaria parasites are found in humans. They are *P. falciparum* (Snow *et al.*, 2005), *P. vivax* (Mendis *et al.*, 2001), *P. ovale* (Collins & Jeffery, 2005), *P. malariae* (Collins & Jeffery, 2007), *and P. knowlesi* (Singh *et al.*, 2004). Each of them differs in the severity of the disease (Wipasa *et al.*, 2002) and their microscopic appearance in blood stage (Figure 1.3). Specific name is used to describe the disease such as falciparum malaria, for *P. falciparum* infection and vivax malaria, for *P. vivax* infection. However, malaria cause by *P. malariae* infection is known as quartan malaria (Sinden & Gilles, 2002).

Althougth *P. falciparum* and *P. vivax* are the major causes in malaria infection, it is *P. falciparum* that causes the most severe form of malaria resulting in major morbidity and mortality (Snow *et al.*, 2005; WHO, 2012). This is because *P. falciparum* multiples rapidly in the blood, thus causing anemia in the subject. A non-immune subject not treated with specific drugs can end in acute malaria and be fatal (Sinden & Gilles, 2002). Thus, over the past years, many studies have been done on this *P. falciparum* species.

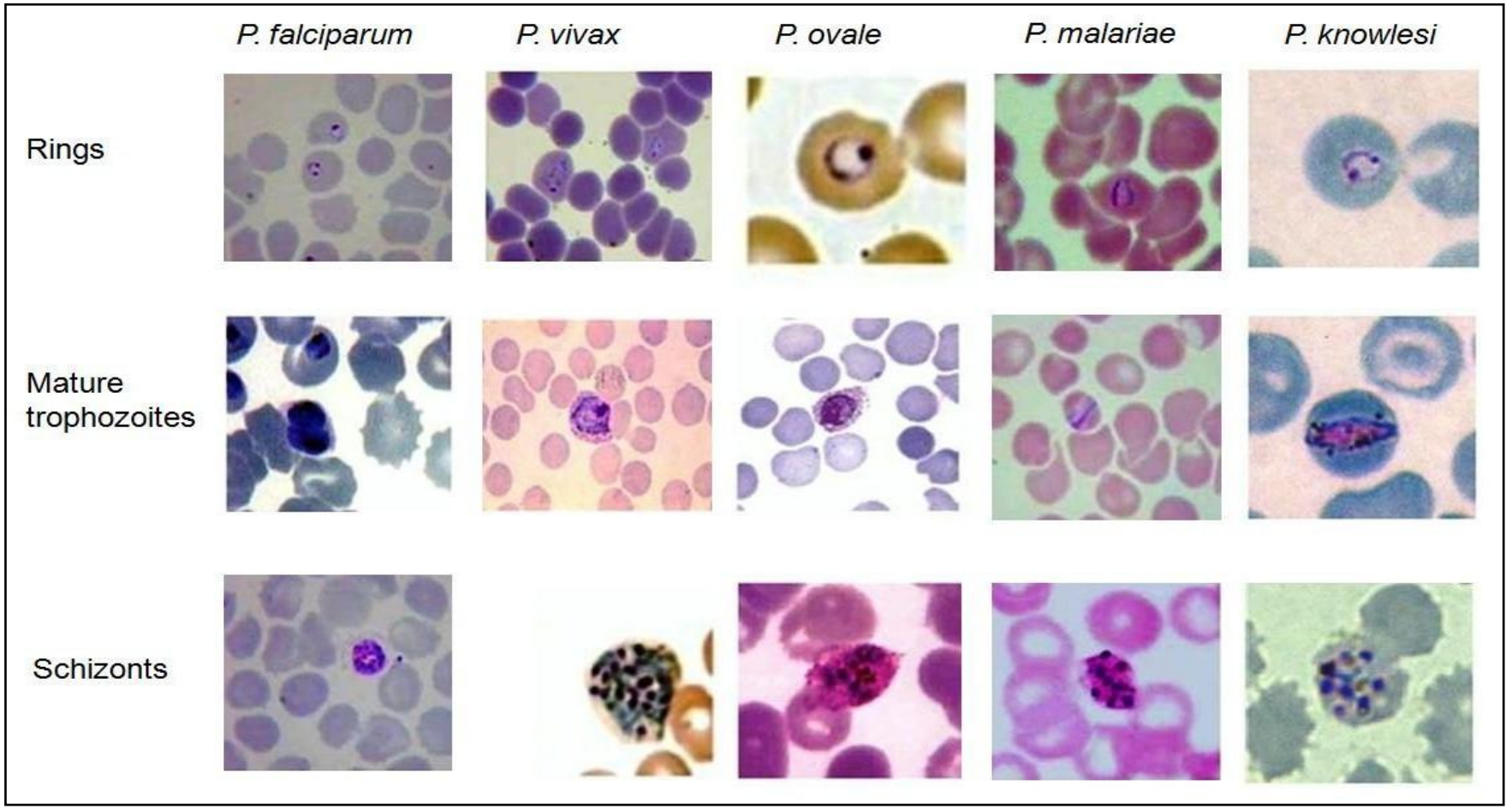

Figure 1.3: Thin blood-films of five human malaria parasites. Figure is adapted from White (2004).

Malaria caused by the other four human malaria parasites is rarely fatal. *P. vivax* and *P. ovale* can relapse, activate and invade the blood for several months or years after infection because of their unique life cycle which they have dormant liver stages called hypnozoites (Cogswell, 1992). However, *P. ovale* differs from *P. vivax* because it can infect individuals who are negative for the Duffy blood group (Mercereau-Puijalon & Menard, 2010). *P. malariae* can remain in blood after infection for a life time without any symptoms and as for *P. knowlesi*, it causes various clinical symptoms and can rapidly progress from uncomplicated to severe malaria because of its 24 hours replication cycle (Greenwood *et al.*, 2008).

1.3.2 Life cycle

All malaria parasites have a complex life cycle involving two types of host as illustrated in figure 1.4. The female *Anopheles* mosquito is the main host in which sexual stage of parasite life cycle occurs and human is the intermediate host in which asexual development occurs. During a blood meal, an infected female *Anopheles* mosquito transmits the *Plasmodium* parasite into the human bloodstream by injecting the sporozoites in their salivary glands (Eyles *et al.*, 1960; Paul *et al.*, 2004). The sporozoites rapidly enter the hepatocytes and replicate into pre-erythrocytic schizont containing 10,000 to 30,000 merozoites or more in the case of *P. falciparum* (Amino *et al.*, 2006). This pre-erythrocytic phase last from 5 to 15 days depending on the *Plasmodium* species, where 5 to 6 days for *P. falciparum*, 8 days for *P. vivax*, 9 days for *P. ovale*, 13 days for *P. malariae* and 8 to 9 days for *P. knowlesi* (Singh *et al.*, 2004). In *P. vivax* and *P. ovale*, hypnozoite may occur when the sporozoite remain dormant for weeks to months in the liver before developing into pre-erythrocytic schizonts. This is due to the genotypically different form of the hypnozoites from the

sporozoites that cause acute infection soon after injection of *Plasmodium* (Cogswell, 1992; Collins, 2007).

The merozoites that develop in the hepatocyte are transferred from the liver to the bloodstream by merosome, a host cell-derived vesicle, which protects the merozoites from phagocytosis by Kupffer cells (Silvie *et al.*, 2008). Merozoites invade the erythrocytes (red blood cells) and undergo erythrocytic phase, in which each merozoite develops through the stages of ring, trophozoite, and schizont. At the end of erythrocytic phase, the infected erythrocytes rupture to release the merozoites. These then invade new erythrocytes to repeat the erythrocytic cycle which occur every 48 hours for *P. falciparum, P. vivax, and P. ovale*, 72 hours for *P. malariae,* and 24 hours for *P. knowlesi* (Greenwood *et al.,* 2008). The rupture of erythrocytes releases toxins that activate macrophages to produce cytokines, resulting in the symptoms of malaria.

Some merozoites can differentiate into the sexual stages gametocytes. The male and female gametocytes in human host are then transmitted to mosquito host via its bites. The sexual phase of malaria parasite's life cycle is continuing in this vector. The male and female gametes fertilize to form zygotes, which later develop into ookinetes. The ookinetes enter the midgut wall of the mosquito to develop into oocysts, which later produce thousands of active sporozoites. The sporozoites are released and travelled to the mosquito salivary gland and transmitted to new human host by another mosquito bite, where the parasite's life cycle starts over again (Matuschewski, 2006).

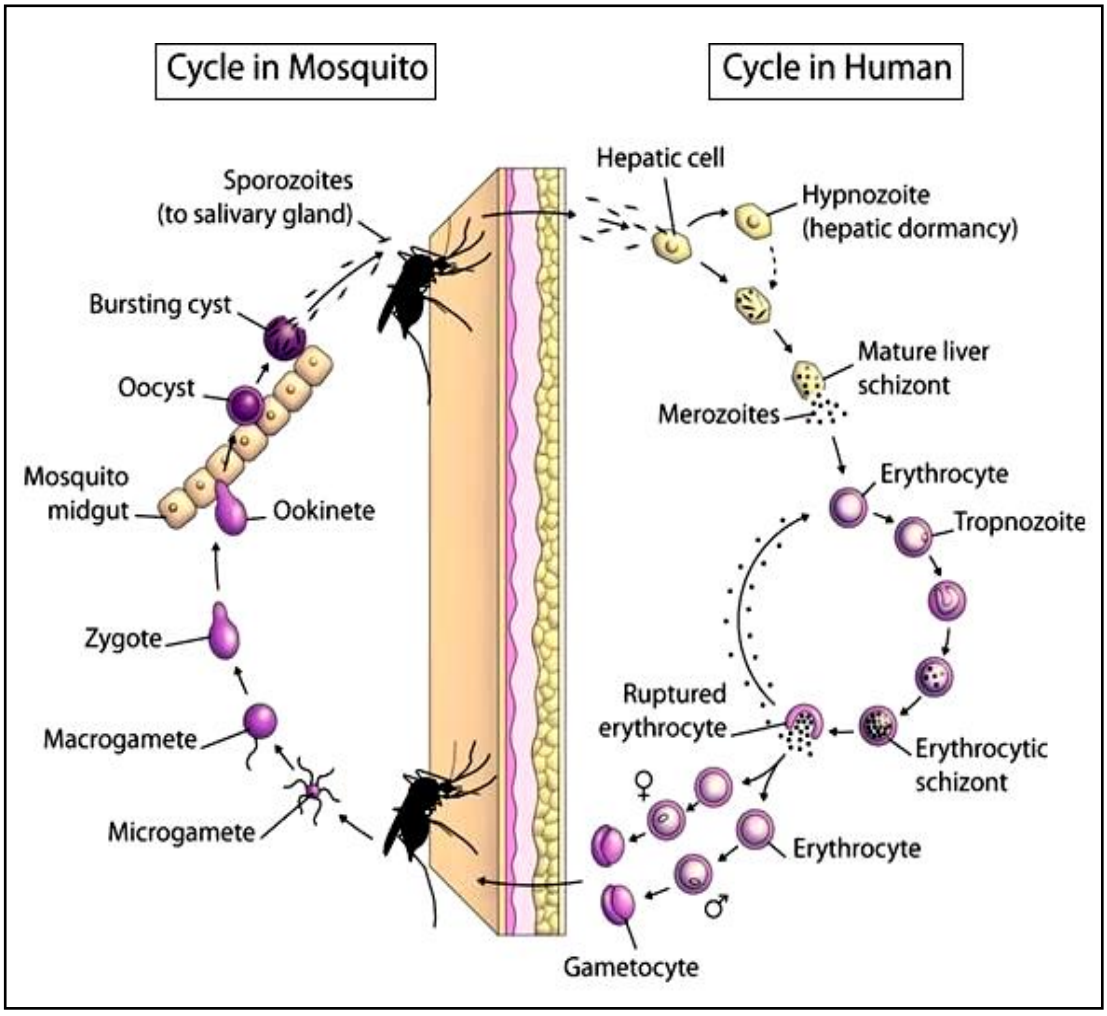

Figure 1.4: The life cycle of malaria parasite. Figure is adapted from Johns Hopkins Bloomberg School of Public Health (2006).

1.4 Pathophysiology of malaria parasites

1.4.1 Pathogenesis of malaria

The infections of the erythrocytes by malaria parasite that undergoes asexual blood cycle are the cause of malarial illness (Tilley *et al.*, 2011). All types of malaria have common symptoms such as fever which are normally accompanied by nausea, aching joints and muscles, headache and chills which is due to the release of parasite products from rupture host cells activating inflammatory cells such as macrophages (Metzger *et al.*, 1995). However, most cases with *P. falciparum* may progress into severe malaria.

Severe malaria occurs when the erythrocytes that are infected with malaria parasite lead to progressive and dramatic structural and biochemical changes which can be worsen into life-threatening complications. The majority of severe malaria is caused by *P. falciparum* infection. However, there are also reports on death by *P. vivax* and *P. knowlesi* infections (Anstey *et al.*, 2009; Cox-Singh *et al.*, 2010). Parasite biomass, malaria toxins and inflammatory response, cytoadherence, rosetting and sequestration, deformability and rigidity of erythrocytes, endothelial activation, dysfunction and injury, and altered trombostasis are the pathophysiological factors of malaria. These pathophysiological factors may result in complications of severe malaria including cerebral malaria, hypoglycemia, metabolic acidosis, renal failure, and respiratory distress (Anstey *et al.*, 2009).

A structural change in the infected erythrocytes leads to adhesiveness of the erythrocytes, where the infected erythrocytes adhere to the capillary and endothelium and also adhere to uninfected erythrocytes resulting in red cell rosettes. This cytoadherence promotes sequestration of the parasites in the heart, lung, brain, liver, kidney, and placenta. Sequestration increases the adhesion of the infected erythrocytes to endothelium which allows them to avoid from clearance by the spleen, thus blocking blood flow and limits the local oxygen supply. These encourage the development of severe malaria disease (Ho & White, 1999; van der Heyde *et al.,* 2006; Horata *et al.,* 2009). Figure 1.5 shows the cytoadherence and rosetting in postcapillary vasculature.

1.4.2 Immunity against malaria

Normally, malaria infection leads to only a partial or short lived immunity that is unable to protect the individual against a new infection. Immunity to malaria is a multi-factorial process that involves both innate and adaptive immune system (Doolan *et al.,* 2009; Vasan & Tsuji, 2010). Most of these studies have been concentrated at pre-erythrocytic (sprorozoites) and asexual erythrocytic (merozoites) stages. However, in this study the focus is on inflammatory mediator in antiparasitic immune responses caused by the parasite in asexual erythrocytic stage. Figure 1.6 shows the mechanisms of immunity to asexual erythrocytic stage of malaria.

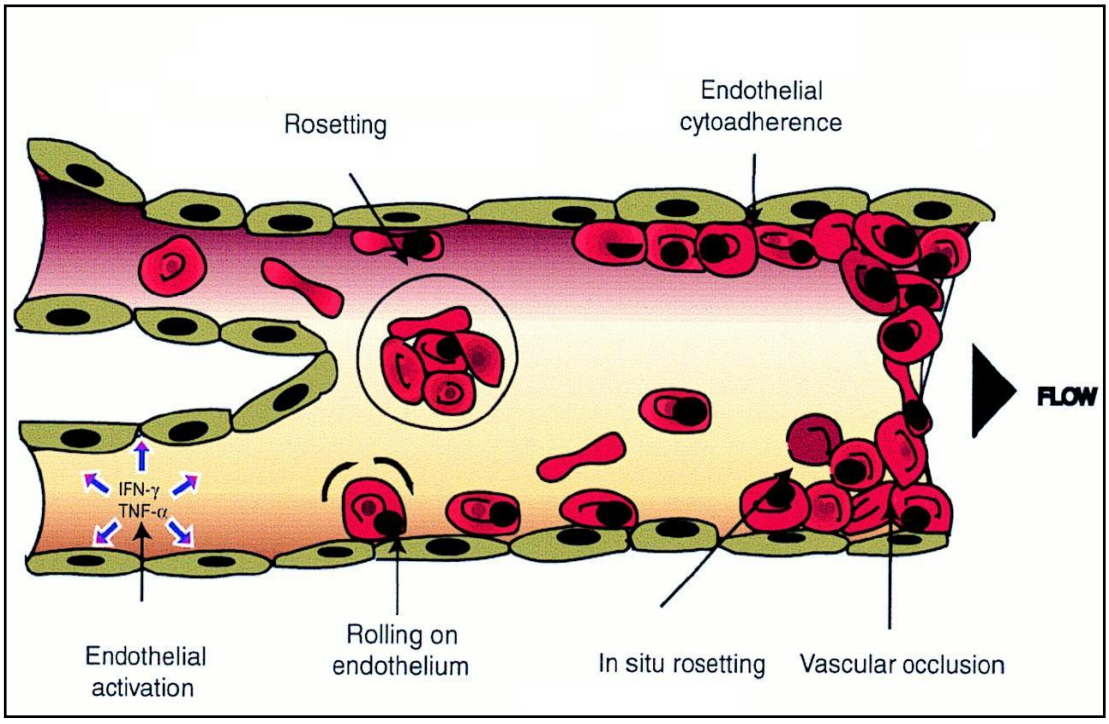

Figure 1.5: The cytoadherence and rosetting in postcapillary vasculature. Figure is adapted from Chen *et al.* (2000).

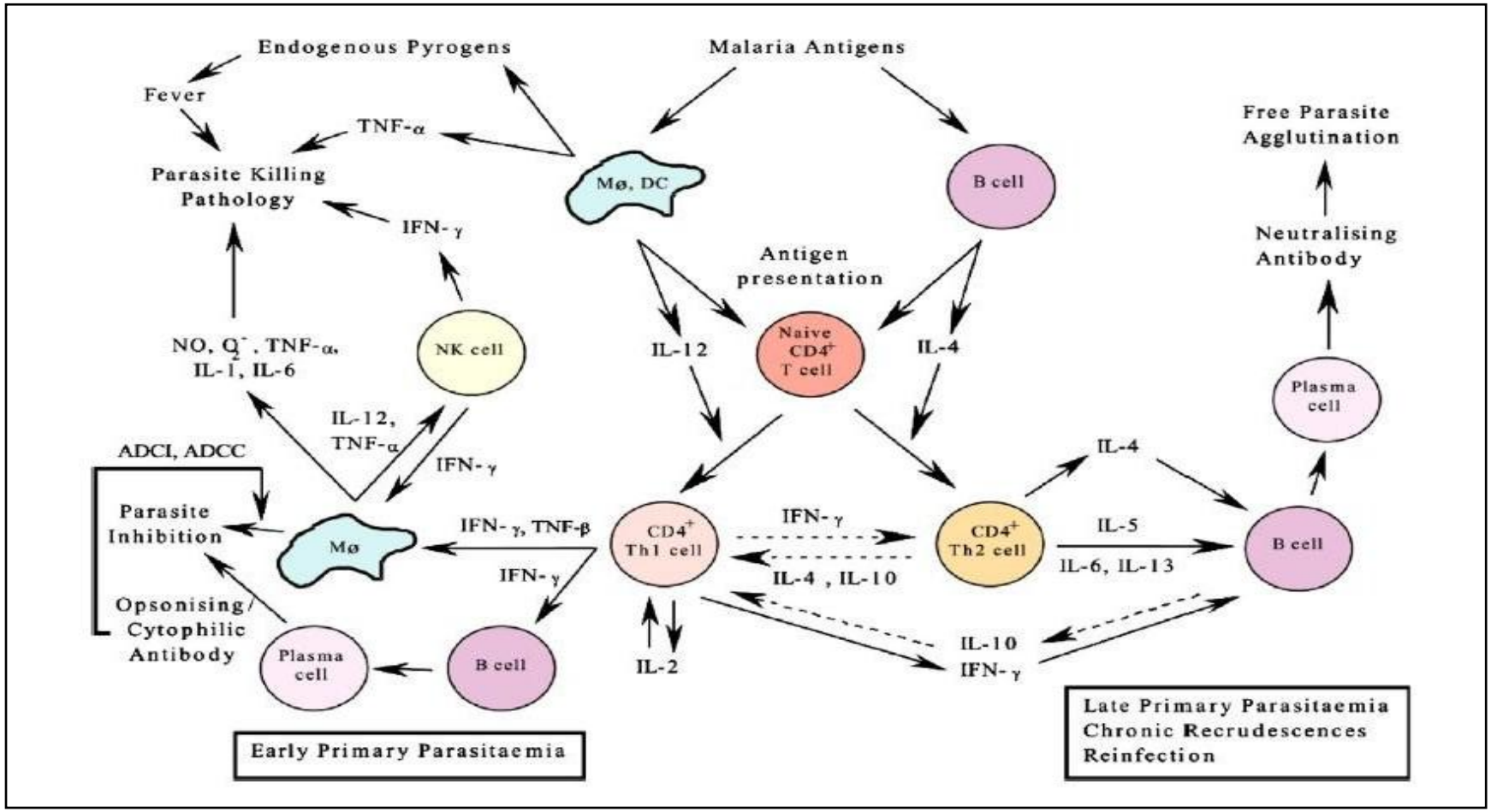

Figure 1.6: The mechanisms of immunity to asexual erythrocytic stage of malaria. Full arrows represent positive regulation, while broken arrows reprerent negetive regulation. Figure is adapted from Taylor-Robinson (2010).

1.4.2.1 Innate immunity

Innate immunity has a crucial role in eliminating malaria parasites from the infected host. Several studies have found that monocytes, macrophages, NK cells and neutrophils appear to be involved in clearing malaria parasites in innate immunity. Moreover, most studies have considered the effectiveness of monocyte-macrophage at phagocytosing infected erythrocytes compared to neutrophils (Taverne *et al.,* 1982).

At asexual erythrocytic stage, the newly formed merozoite and waste such as red cell membrane and parasite products, including glycosyl phosphatidyl inositol (GPI) (Schofield & Hackett, 1993) and malaria pigment (hemazoin) (Pichyangkul *et al.,* 1994), are released into the blood by the rupture of infected erythrocytes. It is found that GPI activates monocytes, neutrophils and macrophages through the toll-like receptor (TLR) 2 and to a lesser extent TLR4 (Krishnegowda *et al.,* 2005), whereas hemazoin recognition by TLR9 is still controversial (Shio *et al.,* 2010). Activated macrophages lead to secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-12, IL-6, and macrophage colony–stimulating factor, as well as superoxide and nitric oxide (NO), where they are largely attributed to the manifestation of malaria symptoms (Hunt & Grau, 2003; Dhangadamajhi *et al.,* 2009) as well as to control parasite growth (Stevenson & Riley, 2004).

TNF-α is typically involved in pathology and immunity to malaria infection. Although, morbidity and mortality in individuals with malaria are associated with the high level of TNF-α, the cytokine also plays an important role in killing parasite and

inhibiting parasite replication (Grau *et al.,* 1989; Kern *et al.,* 1989; Kwiatkowski *et al.,* 1989; Clark *et al.,* 1990; Kwiatkowski *et al.,* 1990). Moreover, TNF-α also stimulates macrophages to produce inducible nitric oxide synthase (iNOS), through generation of NO, which is able to kill the parasites directly (Rockett *et al.,* 1991; Rockett *et al.,* 1992). Most of the malaria symptoms such as fever, headache, nausea, diarrhea and thrombocytopenia are associated with the production of TNF-α (Schwartz *et al.,* 1989). Furthermore, along with interferon (IFN)-γ, which is mainly produced by NK cells and Th1 cells, TNF-α appears vital in controlling parasitemia (Kremsner *et al.,* 1995) and stimulating macrophage phagocytic activity, which enhances parasite clearance (Newsome, 1984). However, over production of these inflammatory mediators together with NO will lead to malarial anemia (Clark & Cowden, 2003; Lyke *et al.,* 2004).

Another endogenous pyrogen that promotes an acute inflammatory response is IL-1β, which contributes as the first line defence against infected erythrocytes (Dinarello, 2004). Together with TNF-α, IL-1β enhances the production of NO and IFN-γ in *in vivo* study (Rockett *et al.,* 1994). A previous study showed that IL-1β protects against the development of cerebral malaria as well as controlling parasitemia (Curfs *et al.,* 1990). However, excess production of IL-1β is likely to be involved in inflammatory disease, and aids in anemia (Dinarello, 2005; Pascual *et al.,* 2005). Interestingly, it is reported that haplotypes of IL-1β promoter polymorphisms promote the development of severe malaria anemia and are correlated with the reduction of IL-1β production, whereas haplotypes that promote protection against severe malaria anemia produce elevated IL-1β (Ouma, 2008).

IL-12, a heterodimeric protein composed of the 35 and 40 kDa subunits, is secreted from dendritic cells, monocytes, macrophages and B-cells in response to bacterial cell wall components (Gately *et al.,* 1998; Trinchieri & Frank, 1998; Mosser & arpt, 1999). IL-12 promotes production of IFN-γ from NK cells and Th1 cells (Gately *et al.,* 1998; Trinchieri & Frank, 1998), which then induce macrophage microbicidal functions including elevation of IL-12 production. It has been demonstrated that high level of IL-12 is effective in inducing protective immunity against blood-stage infection in the murine model (Crutcher *et al.,* 1995; Mohan *et al.,* 1997; Sam & Stevenson, 1999). However, it is shown that low level of IL-12 may aid in anemia and dyserythropoiesis (Mohan & Stevenson, 1998). Other studies show that suppression of IL-12 in children with severe malaria anemia was associated with high hemazoin concentration, where phagocytosis of hemazoin induces up-regulation of IL-10. This anti-inflammatory cytokine in turn suppresses IL-12 production (Luty *et al.,* 2000; Keller *et al.,* 2006).

NO is both protective and pathogenic in malaria. Generation of NO from iNOS occurs in monocytes, macrophages and neutrophils (Perkins *et al.,* 1999). Basically, pro-inflammatory cytokines such as IL-12, IFN-γ and TNF-α increase iNOS-generated NO production, whereas anti-inflammatory cytokines include IL-10 and TGF-β down-regulate iNOS expression (Geller & Billiar, 1998). NO has potent parasiticidal properties against *P. falciparum* (Rockett *et al.,* 1991) and serves as an important effector in limiting parasitemia (Kremsner *et al.,* 1996). However, excess NO production promotes anemia (Keller *et al.,* 2004). As with NO, the reactive oxygen species (ROS) is also both protective and pathogenic in malaria. ROS seems to control parasitemia (Postma *et al.,* 1996; Greve *et al.,* 1999) but at the same time

may cause damage to the erythrocytic membrane, which then lead to severe malaria anemia (Griffiths *et al.,* 2001; Narsaria *et al.,* 2012).

1.4.2.2 Adaptive immunity

The $CD4^+$ T and $CD8^+$ T cells as well as B cells, which are involved in adaptive immunity, are rapidly activated during erythrocytes infection by merozoites. However, $CD4^+$ T cells play more roles in protective immunity against asexual erythrocytic stage (Li *et al.,* 2001). $CD4^+$ T cells can be separated into two functional subsets, T helper (Th) 1 and Th2. The Th1 cells mediate cellular or innate immune response, while the Th2 cells mediate humoral immune response. It seems that production of cytokine such as IL-2, IFN-γ, and TNF-α by Th1 cells can activates macrophages, while Th2 produces anti-inflamatory cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13 which involved in maturation of B cells to plasma cells (Mosmann & Sad, 1996). Thus, both the cellular and humoral immune responses are required to control the parasites infection (Troye-Blomberg *et al.,* 1994; Helmby *et al.,* 1998; Torre *et al.,* 2002). Previous studies revealed that Th1 cells give protection via NO-dependent mechanism, while Th2 cells give protection by the production of parasite-specific IgG1 (Taylor-Robinson *et al.,* 1993).

1.5 Malaria diagnosis

Malaria diagnosis is usually performed by light microscopic examination of Giemsa stained blood slides for the detection of *Plasmodium* parasites and is currently remain as the gold standard (Makler *et al.,* 1998; Hawkes & Kain, 2007; Tek *et al.,* 2010). However, this method requires special training and is not reliable when performed by non experts, which may result in unnecessary or inappropriate treatment of the patient (Coleman *et al.,* 2002; Bates *et al.,* 2004; Rafael *et al.,* 2006).

The presence of malaria parasites also can be detected by other malaria diagnostic methods, such as quantitative buffy coat (QBC) (Moody, 2002) for staining the malaria parasites, enzyme-linked immunosorbant assay (ELISA) (Noedl *et al.,* 2006) which is based on detection of anti-malarial antibodies or polymerase chain reaction (Hanscheid & Groubusch, 2002; Snounou, 2007) that detects the DNA of malaria parasites. However, these methods require expensive and special equipments which most endemic areas have limited resources (Hanscheid & Groubusch, 2002; Daar *et al.,* 2007; Mens *et al.,* 2008).

These limitations have prompted the development of new simple methods to detect the presence of *Plasmodium* parasites which are known as the rapid diagnostic tests (RDTs). These tests do not rely on special equipment, fast, easy to perform and cost less whereby suitable for endemic and poor areas (Moody, 2002; Wongsrichanalai *et al.,* 2007; Murray *et al.,* 2008; Drakeley & Reyburn, 2009; Mawili-Mboumba *et al.,* 2010). RDTs are immunochromatographic assays and are based on the recognition of *Plasmodium* parasite antigen by monoclonal antibodies incorporated into a test strip. Currently, three types of antigens are used in commercialized RDTs, which are

parasite-specific aldolase, found in all malarial species, parasite lactate dehydrogenase (pLDH) and histidine-rich protein 2 (HRP-2), found in *P. falciparum* only (Cheng & Bell, 2006; Hopkins *et al.,* 2007; Wongsrichanalai *et al.,* 2007).

1.6 Prevention of malaria

Vector control or avoidance of mosquito bites is one of the effective ways to prevent malaria. As recommended by World Health Organization, insecticide treated nets (ITN) and indoor residual spraying (IRS) are the most potential tools to control malaria parasites transmission by reducing lifespan of female *Anopheles* mosquito and human contact (WHO, 2012). Each year, the use of ITN such as long lasting insecticidal net (LLIN) has been expended to all people in risk area for malaria. It is reported that LLIN is the most effective and cost-effective prevention for malaria. IRS is highly recommended for high risk area for malaria transmission.

There are 12 insecticides for IRS which is used base on resistance, the residual efficacy of the insecticide, cost, safety and the type of surface to be sprayed. In areas where IRS is the main vector control, the used of pyrethroid for IRS is not recommended to be use with LLIN. This is because pyrethroid is the only class of insecticide that used on LLIN. The combination of IRS and ITN effectively increase vector control (Kleinschmidt *et al.,* 2009). Therefore, when using the combination of IRS and ITN, a non-pyrethroid insecticide should be used for IRS.

1.7 Treatment against malaria

A patient infected with malaria can has a complete recovery if early and proper treatment has been given. Antimalarial drugs may have several uses and effects, thus treatment for malaria depends on several factors such as the species of the malaria parasite, the sensitivity of the parasites to the antimalarial drugs, the severity of infection, the immunity status of the host, and drug cost (Warrell *et al.,* 2002; Griffith *et al.*, 2007).

Uncomplicated *P. falciparum* malaria is symptomatic malaria without signs of severity. According to WHO (2010), the use of artemisinin-based combination therapy (ACT) is the most effective current treatment for uncomplicated malaria. ACT produces rapid clearance of parasitaemia and is able to reduce gametocytes carriage, thus reducing the transmission of malaria parasites (WHO, 2010).

Severe *P. falciparum* malaria patient may rapidly progress to death if not treated with prompt and appropriate treatment (Trampuz *et al.*, 2003). Other than symptomatic malaria, a patient who is suffering from severe *P. falciparum* malaria has one or few severity signs such as impaired consciousness, prostration, respiratory distress, hypoglycaemia and severe anaemia. Rather than quinine, parenteral antimalarial treatment in full doses of artesunate (an artemisinin derivative) can reduce the risk of death from severe malaria in adult and children followed by effective and complete treatment using a full course of ACT (WHO, 2010).

Malaria infection with *P. vivax, P. ovale, P. malariae* or *P. knowlesi* is usually treated as non severe malaria. Both blood and liver stages should be given treatment for *P. vivax* and *P. ovale* infections. For blood stage infection, *P. vivax* and *P. ovale* can be treated with chloroquine or amodiaquine, mefloquine, quinine and ACT if they are resistant to chloroquine. While for liver stage infection, *P. vivax* can be treated with primaquine to prevent the relapse (Galappaththy *et al.,* 2007; WHO, 2010). For infection with *P. malariae* and *P. knowlesi,* treatment with chloroquine is considered to be sufficient. However, they should be treated with the standard regimen for chloroquine-resistant malaria to achieve radical cure (Maguire *et al.,* 2002; WHO, 2010).

1.8 Development of malaria vaccine

Vaccine development is essential in improving the public health and to protect human against malaria due to the emergence of drug-resistant parasites and insecticide-resistant vectors in many parts of the world (Malkin *et al.,* 2006; Ro *et al.,* 2006). In 2002, the *P. falciparum* genome project has completed. From here, researchers have identified hundreds of parasite protein that could be developed as malaria vaccine candidates (Tongren *et al.,* 2004). Figure 1.7 shows several malaria vaccine candidates that have been evaluated in clinical trials.

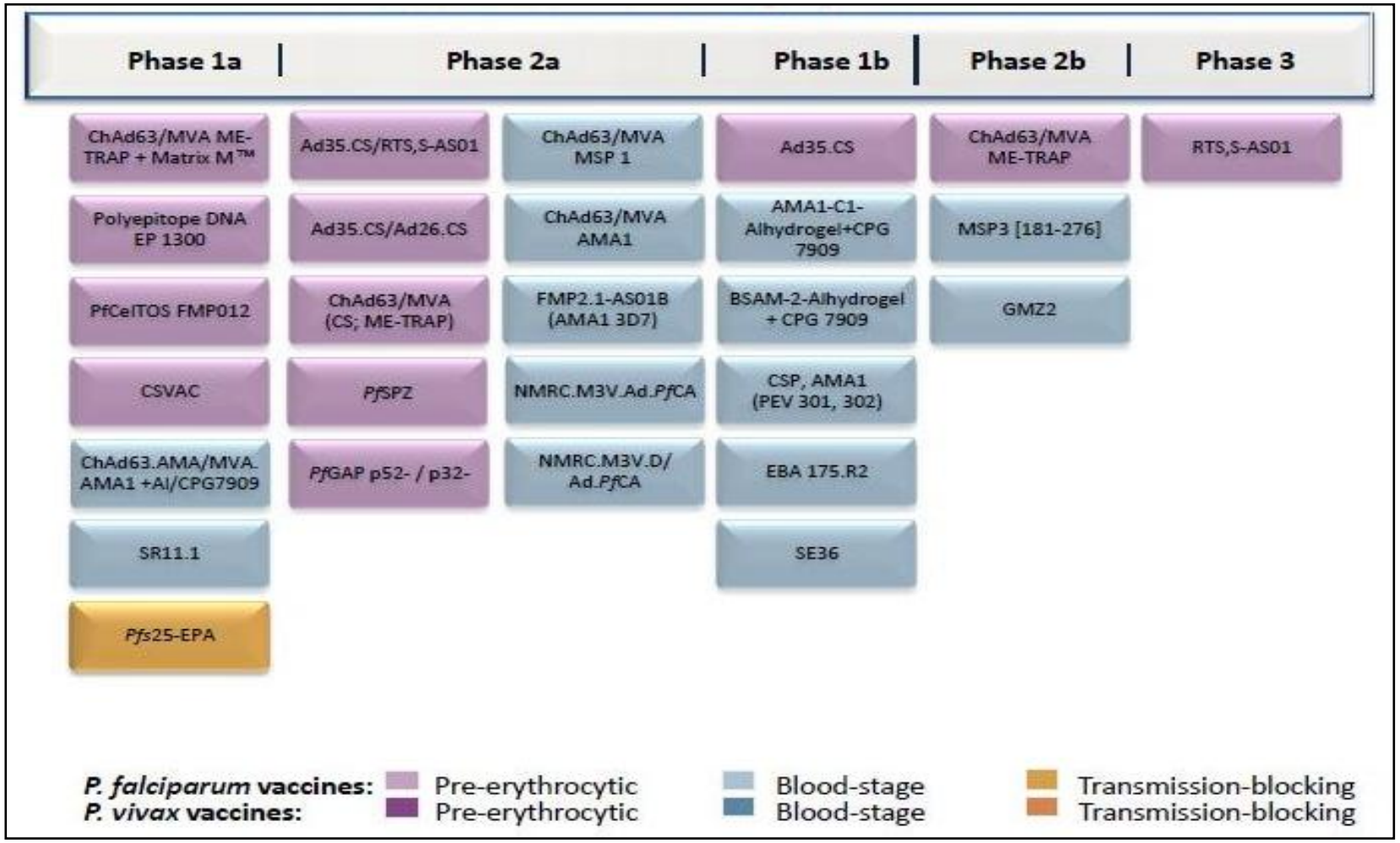

Figure 1.7: Malaria vaccine candidates that have been evaluated in clinical trials. Figure is adapted from WHO (2012b).

1.8.1 Different target stages of malaria vaccine

Because of the complexity of malaria parasite life cycle, the development of effective malaria vaccine should be targeted at different parasite stages which are pre-erythrocytic stage (sporozoites and hepatic stages), asexual erythrocytic stage (merozoites stage) and sexual stage (Girard *et al.*, 2007; Thera *et al.*, 2012). Of those target stages, the asexual erythrocytic stage based vaccines are the most critical (Figure 1.8), since at this stage the infected patient will develop severe malaria and clinical malaria illness (Doolan *et al.*, 2009).

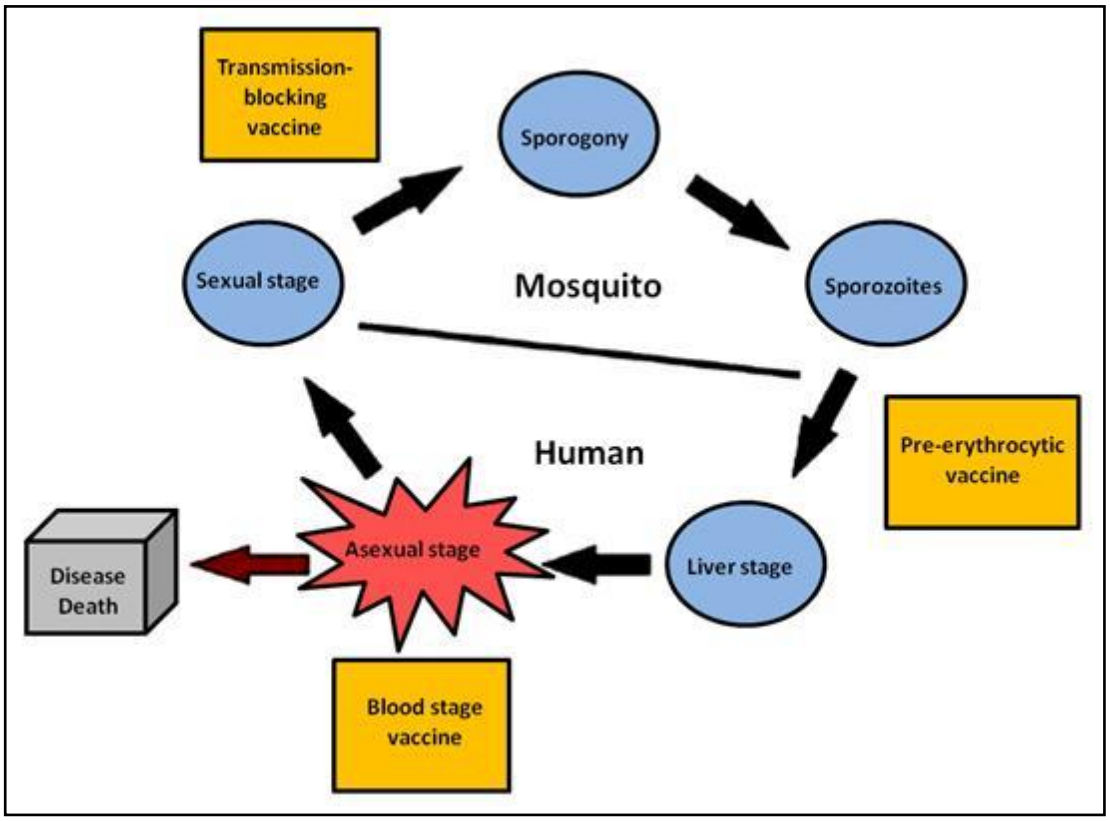

Figure 1.8: Different target stages of malaria vaccine development. The pathological asexual stages are highlighted as a critical target for vaccine development. Figure is adapted from Taylor-Robinson (2010).