PROINFLAMMATORY RESPONSES OF THP-1 MACROPHAGE CELL LINE INFECTED WITH RECOMBINANT BCG (rBCG) expressing the MSP-1C of *Plasmodium falciparum*

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by

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Dissertation submitted in partial fulfillment of the requirement for the degree of the Bachelor of Health Sciences (Biomedicine)

2013

CERTIFICATE

This was to certify that the dissertation entitled "Proinflammatory responses of THP-1 macrophage cell line infected with recombinant BCG (rBCG) expressing the MSP-1C of *Plasmodium falciparum*" was the bonafide record of research work done by Ms Pong Sze Yen during the period from July 2011 to May 2012 under my supervision.

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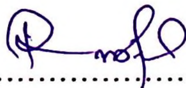

....

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LIST OF SYMBOL, ABBREVIATION AND ACRONYMN

%	Percent
&	And
<	Less than
>	More than
±	Plus/minus
©	Copyright sign
®	Registered sign
°C	Degree Celcius
µ	Micro
µL	Microlitre
Ab	Antibody
AFB	Acid fast bacilli
Ag	Antigen
APC	Antigen presenting cells
Bp	Basepair
BCG	*Bacillus Calmette–Guérin*
CDC	Centers for Diseases Control and Prevention
DNA	Deoxyribonucleic acid
DC	Dendritic cells
DPX	Distyrene Plasticiziel & xylene
EDTA	Ethyleneediamine tetra-acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal bovine serum
g	gram

GNI	Gross National Income
HRP	Horseradish peroxidase
IFN	Interferons
Ig	Immunoglobulin
Il	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRS	Indoor residual spraying
KAHRP	*P. falciparum* Knob-associated Histidine-rich Protein
kb	Kilobase
kD	Kilo Dalton
L	Litre
LPS	Lipopolysaccharides
MΦ	Macrophages
M	Molar
MOI	Multiplicity of infection
MHC	Major Histocompatibility Complex
Min	Minutes
mL	Milliliter
mM	Milimole
MOH	Ministry of Health
MSP-1	Merozoite Surface Protein-1
MSP-1C	C-terminus Merozoite Surface Protein-1
mRNA	Messenger ribonucleic acid
NIH	National Institute of Health
NK	Natural killer cells

NO	Nitric oxide
OD	Optical density
p	Page
PAWE	Power of Association with Error
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PfEMP-1	*P.falciparum* erythrocyte membrane protein 1
PI	Phagocytic Index
PMA	Phorbol 12-myristate 13-acetate
pRBC	Parasitized red blood cells
PVDF	Polyvinylidene difluoride *membrane*
rBCG	Recombinant *Bacillus Calmette–Guérin*
RDTs	Rapid diagnostic test
RESA	Ring-infected erythrocyte surface antigen
RIPA	Radioimmunoprecipitation assay
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
SD-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
Sec	Seconds
SPSS	Stastical Packages for Social Science
Th	T helper
TLR	Toll-like receptor

TNF	Tumor Necrosis Factor
™	Trademark sign
UK	United Kingdom
USA	United States of American
UV	Ultra violet
V	Volt unit for voltage
WHO	World Health Organization
α	Alpha
β	Beta

ABSTRACT

Phagocytosis of macrophage is an important innate defence mechanism against malaria parasites. In the presence of parasites, the macrophage gains its pro-inflammatory responses through the production of pro-inflammatory cytokines and nitric oxide (NO) which allow it to eliminate the parasites from infected red blood cells. In this study, the ability of human macrophage cell line, THP-1 infected with a recombinant BCG (rBCG) clone expressing the 19-kDa C-terminus of the merozoite surface protein-1 (MSP-1C) of *Plasmodium falciparum* in stimulating pro-inflammatory responses indicating by phagocytosis activity, the production of IL-12, NO and inducible nitric oxide synthase (iNOS) were determined. The results demonstrated that the phagoctic activity and expression of NO were higher in cells infected with rBCG compared to cells infected with BCG, while no significant differece was observed on the levels of IL-12 production and iNOS protein expression by these cells. Thus, this study shown that the rBCG vaccine might had the potential to be used as innate immune activator for malaria infection. However, further investigations must be carried out to prove the hypothesis.

ABSTRAK

Fagositosis makrofaj merupakan mekanisma pertahanan semula jadi yang penting terhadap parasit malaria. Dengan kehadiran parasit malaria, makrofaj memperolehi gerak balas pro-inflamatori melalui penghasilan sitokin inflamatori dan nitrik oksida (NO) yang membolehkannya menyingkirkan parasit tersebut daripada sel darah merah yang dijangkiti. Dalam kajian ini, kebolehan sel makrofaj manusia THP-1 yang dijangkiti dengan klon vaksin BCG rekombinan (rBCG) yang mengekspreskan terminus C protein permukaan merozoite-1 19-kDa (MSP-1C) dalam merangsang gerak balas inflamatori. Gerak balas inflamatori dalam kajian ini dicirikan oleh aktiviti fagositosis, penghasilan sitokin IL-12, NO and sintase nitrik oksida indusibel (iNOS). Keputusan kajian menunjukkan bahawa aktiviti fagositik dan pengekspresian NO adalah tinggi dalam sel yang dijangkiti rBCG berbanding dengan sel yang dijangkiti kontrol BCG, manakala tiada perbezaan yang signifikan diperhatikan pada paras IL-12 yang dihasilkan dan pengekspresian protein iNOS oleh kedua-dua sel ini. Oleh itu, kajian ini menunjukkan bahawa vaksin rBCG ini mungkin berpotensi digunakan sebagai aktivator gerak balas imun semula jadi untuk jangkitan malaria. Walau bagaimanapun, banyak kajian lanjut perlu dilakukan bagi membuktikan kenyataan ini.

CHAPTER 1

INTRODUCTION

1.1 Malaria

Malaria is caused by four species of parasites of the genus *Plasmodium* that affect humans (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*). Malaria due to *P. falciparum* is the most deadly form and it predominates in Africa; *P. vivax* is less dangerous but more widespread, and the other three species are found much less frequently (WHO, 2012). Malaria parasites are transmitted to humans by the bite of infected female mosquitoes of more than 30 anopheline species, which occurs mainly between dusk and dawn. Other comparatively rare mechanisms for transmission include: congenitally-acquired disease, blood transfusion, sharing of contaminated needles, and organ transplantation (Filler *at el.*, 2003).

Globally, an estimated 3.3 billion people are at risk of malaria in 2011, with populations living in sub-Saharan has the highest risk of acquiring malaria: approximately 80% of cases and 90% of deaths are estimated to occur in the WHO African Region, with children less than five years of age and pregnant women most severely affected.

Malaria is strongly associated with poverty. Estimated malaria mortality rates are highest in countries with a lower GNI per capita. Countries with higher proportions of their population living in poverty (less than US$ 1.25 per person per day) have higher mortality rates from malaria. Within countries, parasite prevalence rates in children are highest among poorer populations and in rural areas.

1.2 Prevalence of World Malaria

According to World Malaria Report 2012, of the 104 endemic countries in 2012, 79 countries are classified as being in the malaria control phase, 10 are in the pre-elimination phase, 10 are in the elimination phase. Another 5 countries without ongoing transmission are classified in the prevention of re-introduction phase (WHO, 2012) (Figure 1.1 & 1.2).

There are an estimated 219 million cases of malaria (range 154–289 million) and 660 000 deaths (range 610 000–971 000) in 2010. Country level malaria estimates available for 2010 shown that 80% of estimated malaria deaths occur in just 14 countries and approximately 80% of estimated cases occur in 17 countries. Together, the Democratic Republic of the Congo and Nigeria account for over 40% of the estimated total of malaria deaths globally. The Democratic Republic of the Congo, India and Nigeria account for 40% of estimated malaria cases.

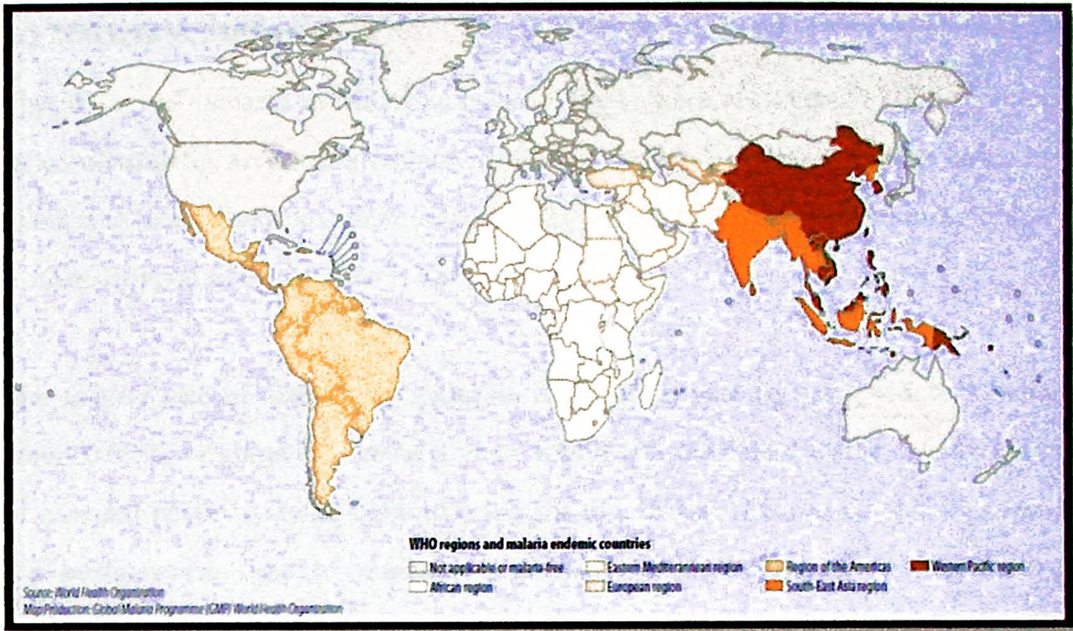

Figure 1.1: World prevalence of malaria (Source: World Malaria Report. 2012).

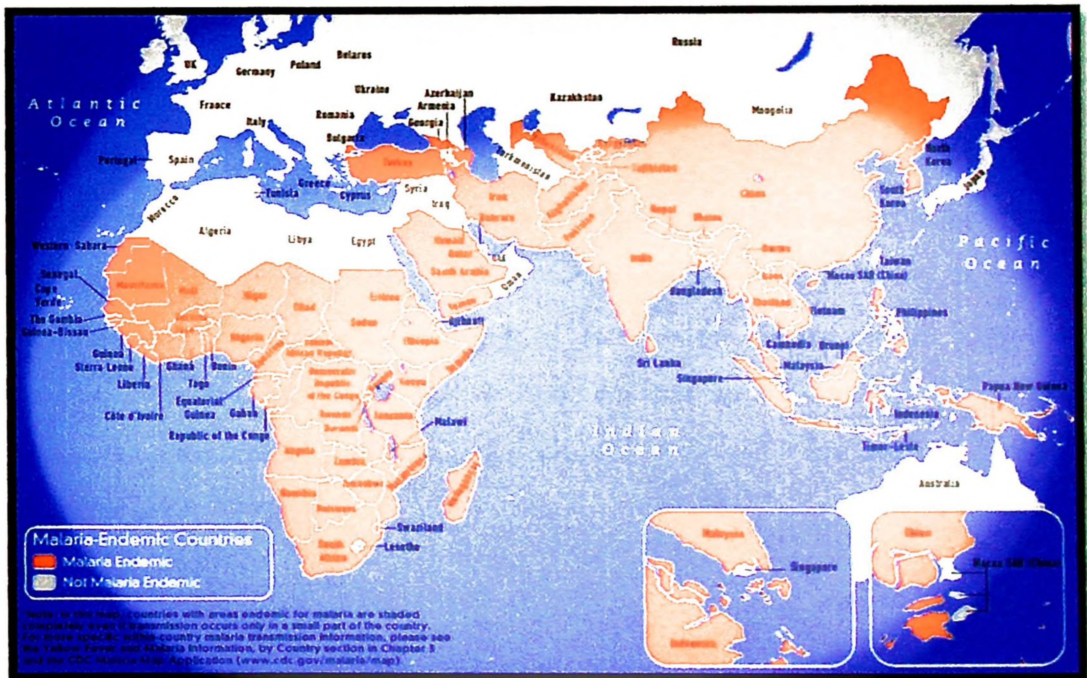

Figure 1.2: Asia prevalence of malaria. (Source: http://www.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/malaria.htm)

1.3 Malaria in Malaysia

The history of malaria in Malaysia back in 1961, there are over 240,000 reported cases of malaria across the country; this was reduced to about 40,000 cases by 1980, due to the measures mentioned briefly above, and in 2008, that number had further dropped to only about 7,000 cases a year.

The fatality rate of malaria in Malaysia is lowering year by year which is being about 0.09%, or about 50 deaths a year, which is a testament to the effectiveness of national health systems at monitoring for the disease. *Plasmodium falciparum*, the most severe type of malaria, is present in Malaysia. These species are responsible for all the reported fatalities in 2008. Malaria transmission still relatively high in rural regions of Malaysia (Figure 1.3). Major *plasmodium* species that present in Malaysia are *P. vivax* (70%) and *P. falciparum* (30%) (WHO, 2012).

Malaysia is fully committed in controlling this disease by introducing the Malaria Elimination Programme in order to achieve malaria elimination status by 2020. The greatest challenge of malaria control in Malaysia is the presence of illegal immigrant from neighbouring endemic countries which impacts on th imported malaria cases (Mali *et al.*, 2006). It is found that approximately 57% of the immigrants are infected with malaria and majorities of them are Indonesians (Jamaiah *et al.*, 2005)

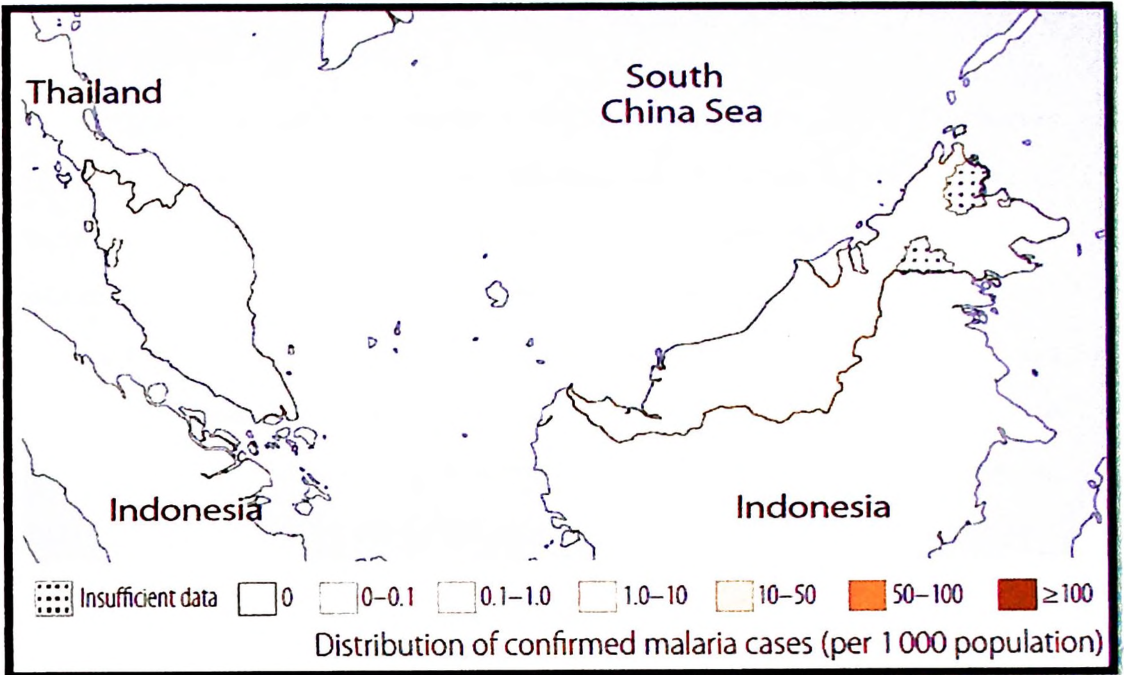

Figure 1.3: Prevalence of malaria in Malaysia (Source: World Malaria Report, 2012)

1.4 Malaria parasite

1.4.1 Life cycle of *P. falciparum*

The life cycle of the malarial parasite is very complex (Figure 1.4). *P. falciparum* and, to a much lesser extent, *P. vivax* (Mendis *et al.*, 2001) are the main causes of disease and death due to malaria. Malaria parasites undergo three distinct asexual replicative stages (exoerythrocytic schizogony, blood stage schizogony, and sporogony) resulting in the production of invasive forms (merozoites and sporozoites). Sexual reproduction occurs with the switch from the vertebrate to invertebrate host and leads to the formation of the invasive ookinete. All invasive stages are characterized by the apical organelles typical of apicomplexan species.

The infection is initiated when sporozoites are injected with the saliva of a feeding mosquito. Sporozoites are carried by the circulatory system to the liver and invade hepatocytes. Recent evidence indicates that sporozoites pass through several hepatocytes before the invasion is followed by parasite development (Mota *et al.*, 2001). The intracellular parasite undergoes an asexual replication known as exoerythrocytic schizogony within the hepatocyte. Exoerythrocytic schizogony culminates in the production of merozoites which are released into the bloodstream.

Merozoites invade erythrocytes and undergo a trophic period in which the parasite enlarges. Trophozoite enlargement is accompanied by an active metabolism including the ingestion of host cytoplasm and the proteolysis of hemoglobin into amino acids. The end of the trophic period is manifested by multiple rounds of nuclear division without cytokinesis resulting is a schizont. Merozoites bud from the mature schizont, also called a segmenter, and the merozoites are released following rupture of the infected erythrocyte. Invasion of erythrocytes initiates another round of the blood-stage replicative cycle (Baer *et al.*, 2007).

The blood stage is responsible for the pathology associated with malaria. The intermittent fever paroxyms are due to the synchronous lysis of the infected erythrocytes. As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro- or microgametocytes. The gametocytes are large parasites which fill up the erythrocyte, but only contain one nucleus. Ingestion of gametocytes by the mosquito vector induces gametogenesis (i.e., the production of gametes) and escape from the host erythrocyte (CDC, 2010).

Factors which participate in the induction of gametogenesis include a drop in temperature, an increase in carbon dioxide, and mosquito metabolites. Microgametes, formed by a process known as exflagellation, are flagellated forms which will fertilize the macrogamete leading to a zygote (CDC, 2010).

The zygote develops into a motile ookinete which penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication resulting in the production of sporozoites. Rupture of the mature oocyst releases the sporozoites into the hemocoel (i.e., body cavity) of the mosquito. The sporozoites migrate to and invade the salivary glands, thus completing the life cycle (CDC, 2010).

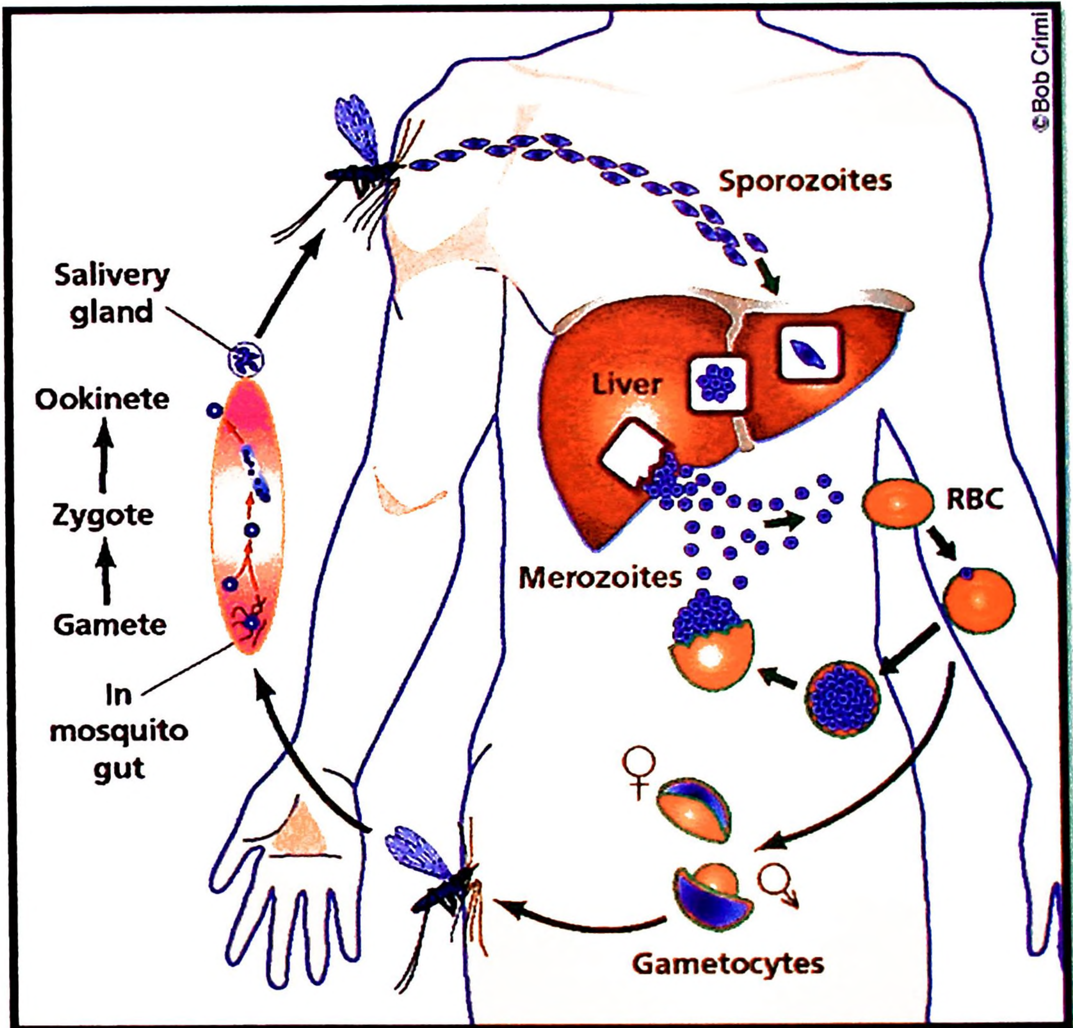

Figure 1.4: Life cycle of malaria parasite (Source: Cowman & Crabb, 2002).

1.5 Pathophysiology of malaria parasites.

1.5.1 Pathogenesis of malaria

Cytoadherence to human cell surfaces is an important component of *P. falciparum* pathogenesis. As *P. falciparum* parasites mature from rings to trophozoites within red blood cells, they induce the formation of sticky knobs on the surface of erythrocytes (Newbold *et al.*, 1999). These structures become involved in the adhesion of infected erythrocytes to vascular endothelium, and they may be possibly involved in erythrocyte rosetting (Waller *et al.*, 1999). The knobs are composed of both host and parasite proteins, including the parasite proteins KAHRP (*P. falciparum* Knob-associated Histidine-rich Protein), PfEMP-1 (*P. falciparum* erythrocyte membrane protein-1) (Waller *et al.*, 1999), PfEMP-2, and RESA (ring-infected erythrocyte surface antigen) as well as human proteins including spectrin, actin, and band 4.1 (Aikawa, 1988; Sharma, 1991; Sharma, 1997). Each *P. falciparum* parasite has ≥60 different var genes; of these, one protein product is present in individual parasites (Chookajorn *et al.*, 2008).

Endothelial binding leads to sequestration of infected red cells within these small vessels (thereby removing parasites from the peripheral circulation during a prolonged period of the life cycle). This leads to partial blood flow obstruction, endothelial barrier breakdown, and inflammation (Newbold *et al.*, 2009). Sequestration can be demonstrated in any organ of a patient infected with *P. falciparum*. The most catastrophic clinical manifestation of sequestration is cerebral malaria. Renal failure in the setting of malaria may occur in part as a result of mechanical obstruction by infected erythrocytes; immune mediated glomerular pathology and fluid loss due to alterations in the renal microcirculation also probably contribute to renal failure (Das, 2008).

Rosetting is another mechanism of microvascular disease. The infected red cells stick to uninfected red cells and form rosettes that clog the microcirculation (Chen

et al., 2000; Rowe *et al.*, 1997). Rosetting is mediated by an interaction between PfEMP-1 within knobs and receptors on the surface of uninfected red cells, such as complement-receptor 1 (CR1)(Chen *et al.*, 2000; Rowe *et al.*, 1997).

1.5.2 Immunity against malaria

1.5.2.1 Innate immunity

Malaria has a complex and multi-stage life cycle, it not only expresses a great variety of proteins at different stages, but these proteins also keep changing often. Thus, an infection with malarial leads to a partial and short lived immunity that is unable to protect the individual against a new infection. Innate immunity against malaria is an inherent refractoriness of the host that prevents the establishment of the infection or an immediate inhibitory response against the introduction of the parasite.

Acute malarial infection induces immediate, non-specific immune response that tends to limit the progression of disease. The humoral and cellular mechanisms of this 'nonspecific' defense are poorly defined. Neutrophils, mononuclear phagocytes and NK cells appear to play roles in innate immunity early in malaria infections. Natural killer (NK) cells have been shown to increase in numbers and to be able to lyse *P. falciparum*-infected erythrocytes in vitro (Doolan & Hoofman, 1999). NK cells in peripheral blood produce Interferon-gamma (IFN-γ) in response to *Plasmodium* infected erythrocytes, leading to parasiticidal macrophages activation, and this may be of greater importance for innate malaria immunity than their potential to lyse infected host erythrocytes. These cells are also important in the initiation and development of adaptive immune responses. NK cells induce the production of the proinflammatory chemokine Interleukin-8(IL-8), that in turn plays its role in the recruitment and the activation of other cells during malaria infection. Dendritic cells, macrophages, gamma delta T cells and NKT cells also sense the presence of the parasite and participate in the

immune response. Malaria infection gives rise to strongly elevated blood concentrations of non-malaria-specific immunoglobulin, but the importance of the underlying polyclonal B-cell activation for innate immunity is not known (Manoor *et al.*, 2001; Roetynck *et al.*, 2006; Stevenson & Riley., 2004).

1.5.2.1a Interleukin 12 (IL-12)

IL-12 production is an important pro-inflammatory indicator in various infections including malaria. IL-12 is originally termed NK cell stimulatory factor or cytotoxic lymphocyte maturation factor (Kobayashi *et al.*, 1989; Stern *at al.*, 1990). It is a heterodimer of 70 kD (p70) formed by two covalently linked glycosylated chains of approximately 40 kD (p40) and 35 kD (p35) (Kobayashi *et al.,* 1989). The p40 subunit is homologous to the extracellular part of the IL-6 and Granulocyte colony-stimulating factor (G-CSF) receptor (Brunda, 1994; Merberg *et al.*, 1992; Gearing & Cosman, 1991).

It has been shown recently that IL-12 plays a decisive role in host-defense against intracellular pathogens. It is produced by infected monocytes/macrophages as one of the first host responses to infection and, together with TNF, induces IFN-3r production by NK cells (Hsieh *et al.*, 1993; D'Andrea *et al.*, 1992; Chan *et al.*, 1991). It showed that the cytokines secreted by DCs are primarily involved in inducing anti-mycobacterial T cell mediated responses, implicated IL-12 with Thl induction (Giacomini, 2001).

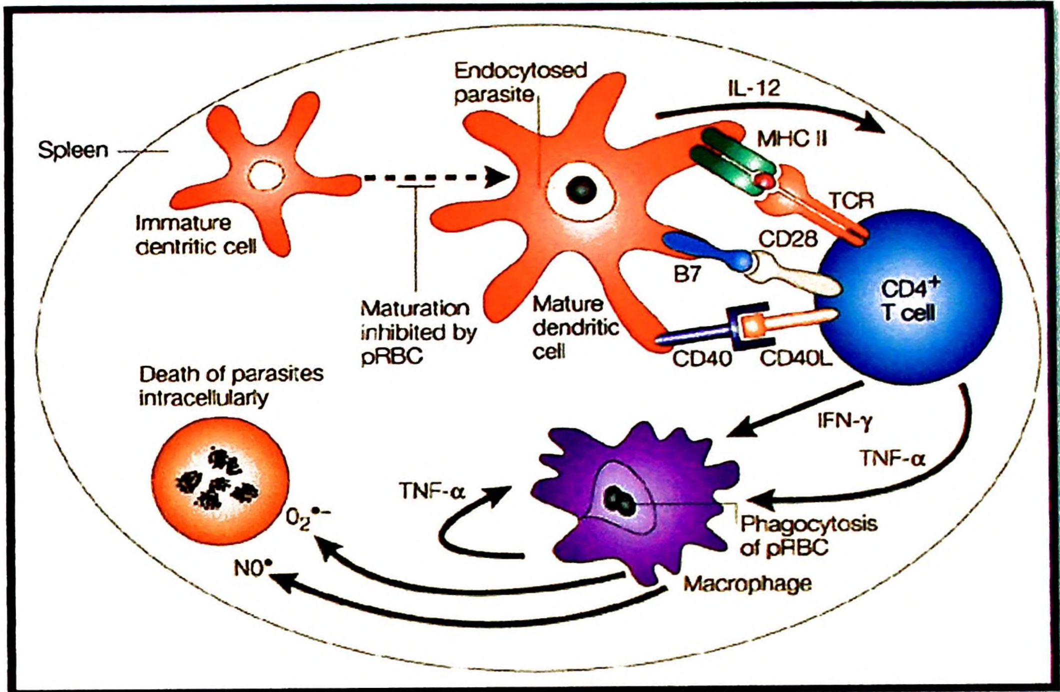

Figure 1.5: Antibody-independent (T-cell) immunity against malaria. Activation of CD4+ T cells by mature dendritic cells leading to macrophages activation, phagocytosis of parasitized red blood cells (pRBC), and elaboration of cytokines and small inflammatory molecules (such as nitric oxide and oxygen redicals)(Source: Good, 2001)

1.5.2.1b Nitric oxide (NO) & inducible Nitric Oxide Synthase (iNOS)

Nitric oxide (NO) is an important intracellular and intercellular signalling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems. It is a free oxygen radical (NOS) and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (Bogdan, 2001; Alderton *et al.*, 2001; Dawn & Bolli, 2002).

Nitric oxide synthases is a family of enzyme which catalyzed the production of NO. NOS enzymes consist of different subtypes depending on the tissue type, although all share between 50–60% sequence homology (Alderton *et al.*, 2001). Currently, at least three distinct isoforms of NOS have been isolated and cloned: eNOS (endothelial NOS, NOS I), iNOS (inducible NOS, NOS II) and nNOS (neuronal NOS, NOS III). nNOS and iNOS are soluble whereas eNOS is membrane bound, with its N-terminal myristoylated (Liu *et al.*, 1995). Nitric oxide synthase (NOS2 or iNOS) isoform is lies at the interface between the innate and adaptive immune systems (MacMicking *et al.*, 1997).

NO is not only synthesized enzymatically, but can also be produced nonenzymatically from nitrite at low pH under reducing conditions. Nonenzymatic NO production may play a role in similar biological events as when NO is generated from L-arginine by NOS enzymes, but its importance in regulation of iNOS activity and NO production is unclear (Weitzberg and Lundberg, 1998).

The overproduction of NO independently of intracellular calcium concentrations is due to iNOS that can result in either protective or damaging effects, although activation of NO production depends on the cell type. Many cell types can express iNOS for their function in host defense against microbial and viral pathogens

(Bogdan, 2001), leading to the formation of NO radicals or S-nitrosothiols or ONOO- in the host cell or in the microbe itself. In addition to the protective effects of iNOS, iNOS expression in macrophages is activated by particular inducers, participating in the pathology of inflammatory diseases leading to cell death (Hubbard and Giardina, 2000; Luoma *et al.*, 1998).

iNOS activity in macrophages is first regulated and modulated by cellular receptor molecules such as Toll-like receptors (TLR) and CD14 (Figure 1.6). CD14 is the receptor for lipopolysaccharide (LPS) and plays an essential role in pro-inflammatory responses in monocytes and macrophages via activation of the NF-kB pathway (Schroder *et al.*, 2000). CD14 has two distinct forms; mCD14 (GPI-anchored form) and sCD14 (soluble CD14). mCD14 is believed to directly effect LPS simulation via interaction with Toll-like receptor-4 (TLR-4). (Du & Low, 2001).

The regulation of NO synthesis by iNOS differs according to the strain and species of animals and depends on the inducers. Bovine and murine macrophages generate considerable amounts of iNOS in response to cytokine stimulation, but human and pig macrophages are resistant (Jungi *et al.*, 1996). Recent developments are reviewed and shown that NO biosynthesis is regulated by a variety of mechanisms at the transcriptional and posttranslational levels in activated macrophages and other cells (Aktan, 2004).

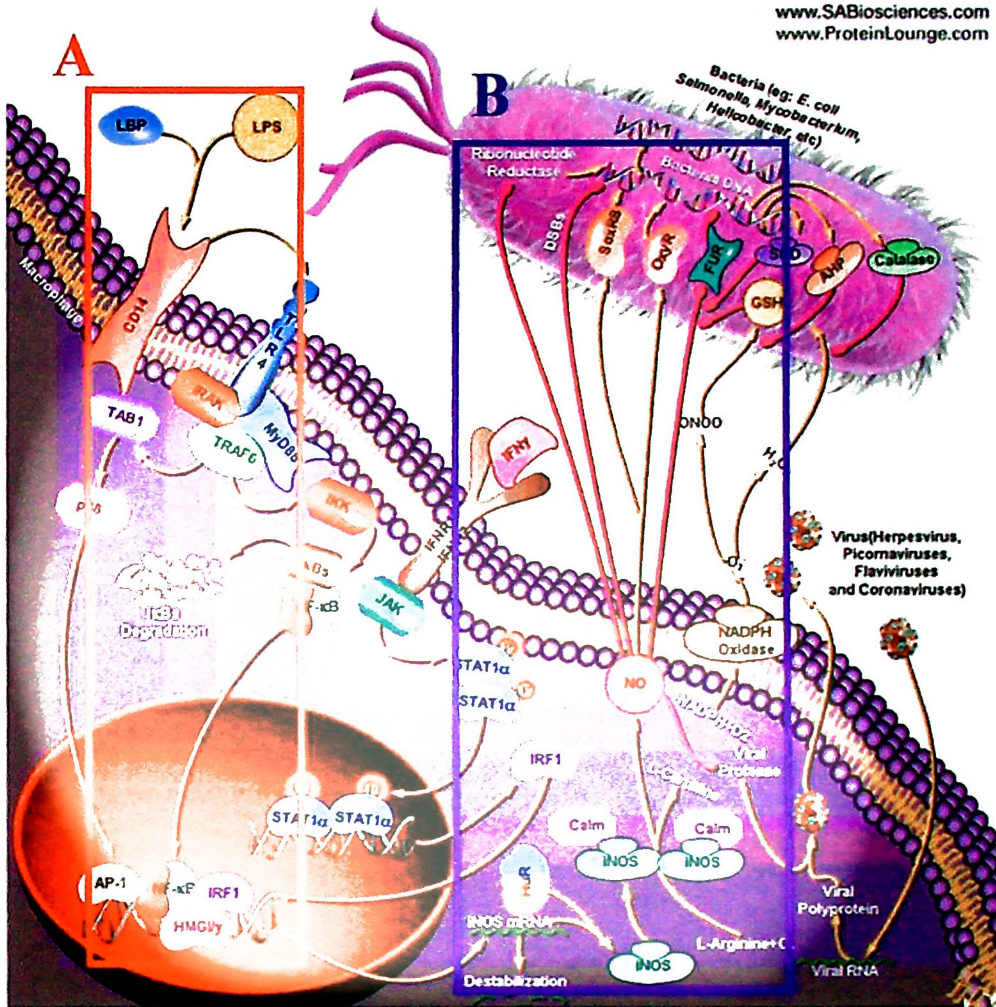

Figure 1.6: Relation of the iNOS and NO. (A) the activation of macrophages by the LPS and other microbacterial. (B) the macrophages produce iNOS after stimulated. iNOS then undergo transcription and NO is biosynthesised by iNOS in the present of L-arginine. (Source: http://www.sabiosciences.com/pathway.php?sn=iNOS_Signaling)

1.5.2.2 Acquired immunity

Acquired or adaptive immunity against malaria develops after infection and its protective efficacy varies depending on the characteristics of the host, place of stay, number of infections suffered and other factors. It has been graded as anti-disease immunity and sterilizing immunity (protects against new infections by maintaining a low-grade, asymptomatic parasitemia), with a considerable overlap between these. The underlying mechanisms and antigenic specificity of protective immunity against malaria are not well understood. The acquired anti malaria immunity has been demonstrated to be strain specific and stage specific, with cross reactivity. Immune response has been documented against the various parasite antigens in pre-erythrocytic (sporozoite), asexual erythrocytic (merozoite) and sexual stages (gametocyte). Natural exposure to sporozoites does not induce complete antiparasite and antidisease immunity but only limit the density of parasitemia and thereby decrease the malaria-associated morbidity and mortality. The acquired immunity is directed predominantly against the asexual erythrocytic stage, the primary targets being the extracellular merozoites in circulation. Although the preerythrocytic stage is also targeted by protective immune responses, it does not effectively block sporozoite invasion or intrahepatic development of the parasite (Doolan *et al.*, 2009)

Antibodies may protect against malaria by a variety of mechanisms. They may inhibit merozoite invasion of erythrocytes and intra-erythrocytic growth or enhance clearance of infected erythrocytes from the circulation by binding to their surface, thereby preventing sequestration in small vessels and promoting elimination by the spleen. Opsonization of infected erythrocytes significantly increases their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various effector cells such as neutrophils and monocytes/macrophages. Interaction of opsonized erythrocytes with these effector cells induces release of

factors such as TNF which may cause tissue lesions but which are also toxic for the parasites (Perlmann *et al.*, 2002).

Cell-mediated immune responses induced by malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages. CD4 T cells are essential for immune protection against asexual blood stages in both murine and human malaria. However the role of $CD8^+$ T cells, which have important effector functions in pre-erythrocytic immunity and which contribute to protection against severe malaria, is less clear. It has been proposed that $CD8^+$ T cells may regulate immunosuppression in acute malaria and down-modulate inflammatory responses. As human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by $CD8^+$ T lymphocytes has no role in the defense against blood-stage parasites (Perlmann *et al.*, 2002).

Malaria parasites not only escape the host's immune response, owing to their antigenic diversity and clonal antigenic variation, but also modulate the immune response and cause significant immune suppression. The parasitized red cells, with the deposited hemozoin inside, have been found to inhibit the maturation of antigen presenting dendritic cells, thereby reducing their interaction with T cells, resulting in immunosuppression. Immune suppression in malaria increases the risk of secondary infections (such as nontyphoidal Salmonella, herpes zoster virus, hepatitis B virus, Moloney leukemia virus and nematode infections and reactivation of Epstein-Barr virus) and may also reduce the immune response to certain vaccines (Hisaeda *et al.*, 2005; Millington *et al.*, 2006).

1.6 Management of malaria

1.6.1 Diagnosis

The first symptoms of malaria most often are fever, chills, sweats, headaches, muscle pains, nausea and vomiting which are often not specific and also found in other diseases. In severe malaria which caused by *P. falciparum*, clinical findings such as confusion, coma, neurologic focal signs, severe anemia and respiratory difficulties are more striking and may increase the index of suspicion for malaria. If possible, clinical findings should always be confirmed by a laboratory test for malaria (CDC, 2012).

The most commonly used diagnostic test for malaria is microscopy to detect malaria parasites in stained blood films. Thick blood films are used in routine diagnosis and as few as one parasite per 200 μl blood can be detected (Cross, 2004). Prior to examination, the specimen is stained with the Giemsa stain to give the parasites a distinctive appearance. This method can be used to differentiate between different parasite species and stages of the life cycle and remains as the gold standard for laboratory confirmation of malaria (Cross, 2004; CDC, 2012).

Various test kits are available to detect antigens derived from malaria parasites. Such immunologic ("immunochromatographic") tests most often use a dipstick or cassette format, and provide results in 2-15 min. Rapid diagnostic 'dipstick' tests are easy to perform and do not require trained personnel or special equipment. However, they are relatively expensive and, although *P. falciparum* can be diagnosed, *P. ovale*, *P. malariae* and *P. vivax* cannot be distinguished from one another using this method. "Rapid Diagnostic Tests" (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available (Cross, 2004; CDC, 2012).

Parasite nucleic acids can be detected using polymerase chain reaction (PCR). Although this technique may be slightly more sensitive than smear microscopy, it is of limited utility for the diagnosis of acutely ill patients in the standard healthcare setting. PCR results are often not available quickly enough to be of value in establishing the diagnosis of malaria infection. PCR is most useful for confirming the species of malarial parasite after the diagnosis has been established by either smear microscopy or RDT (CDC, 2012).

Serology tests such as indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA) detects antibodies against malaria parasites. But these methods does not detect the current infection rather measures past exposure (CDC, 2012).

1.6.2 Treatment

Anti-malarial combination therapy (ACT) are recommended as the first-line treatment for malaria caused by *P. falciparum*, the most dangerous of the *Plasmodium* parasites that infect humans. By 2011, 79 countries and territories had adopted ACTs as a first-line treatment for *P. falciparum* malaria. *P. vivax* malaria should be treated with chloroquine where it is effective, or an appropriate ACT in areas where *P. vivax* is resistant to chloroquine. Treatment of *P. vivax* should be combined with a 14-day course of primaquine to prevent relapse.

ACT s is the simultaneous use of two or more blood schizontocidal medicines with independent modes of action and, thus, different biochemical targets in the parasite. The rationale is twofold which the combination is often more effective and in the very rare event that a mutant parasite resistant to one of the medicines arises *de novo* during the course of the infection, this resistant parasite will be killed by the other antimalarial medicine. This mutual protection is thought to prevent or to delay the emergence of resistance. To realize the two advantages, the

partner medicines in a combination must independently be sufficiently efficacious in treating malaria (WHO, 2010). Artemisinin-based combination therapy is combinations in which one of the components is artemisinin and its derivatives (artesunate, artemether, dihydroartemisinin). The artemisinins produce rapid clearance of parasitaemia and rapid resolution of symptoms, by reducing parasite numbers 100- to 1000-fold per asexual cycle of the parasite (a factor of approximately 10000 in each 48 h asexual cycle), which is more than the other currently available antimalarials achieve.

This long duration of treatment with the artemisinins can be reduced to 3 days when given in combination with slowly eliminated antimalarials. With this shorter 3 day course, the complete clearance of all parasites is dependent on the partner medicine being effective and persisting at parasiticidal concentrations until all the infecting parasites have been killed (WHO, 2010).

1.7 Malaria vaccine

Malaria, unlike many diseases, has evolved as a result of millions of years of interactions at various levels with the mammalian system and hence has found ways to gather resistance to drugs and insensitivity to other treatment modalities. Co-evolution and co-adaptation of the parasite, recrudescence, recurrence, drug resistance, and the complex pathophysiology of the infection process complicate treatment regimens, most of which are ineffective. This brings us to the conclusion that vaccination is the ultimate eradicative strategy (WHO, 2012).

Resistance to antimalarial drugs is proving to be a challenging problem in malaria control in most parts of the world. Since early 1960s, the chloroquine which is the best and most widely used drug for treating malaria have start to shown declined sensitivity to the parasite. More antimalarials drugs are discovered in an effort to tackle this problem, but all these drugs are either expensive or have undesirable

side effects (Lqbal *et al.*, 2002). For nowadays, the parasite shown resistance to artemisinins have detected in 4 countries of the Greater Mekong subregion: Cambodia, Myanmar, Thailand and Vietnam. Despite the observed changes in parasite sensitivity to artemisinins, ACTs continue to cure patients provided that the partner drug is still efficacious (WHO, 2012). To date, there have been no reports of delayed parasite clearance during routine therapeutic efficacy studies conducted in Africa.

However, clearly it is only after a variable length of time, the parasites, *P.falciparum* species, started showning resistance to these drugs too (Lqbal *et al.*, 2002). The important factors that are associated with resistance are longer half-life, single mutations for resistance, poor compliance, host immunity, and number of people using these drugs. The characteristics of a drug that make it vulnerable to the development of resistance are a long terminal elimination half-life, a shallow concentration-effect relationship, and mutations that confer marked reduction in susceptibility. Drug resistance is most commonly seen in *P. falciparum*. Resistance to chloroquine is most prevalent, while resistances to most other antimalarials like pyrimethamine, quinine, mefloquine, artemesin and quinoline (Ward *et al.*, 1997) compounds have also been reported. These developments further justify the cause and the urgency for formulating an effective vaccine against malaria.

As of now, malaria vaccine development is being pursued by three different methods. The most work has been done and progress achieved in attempts to maximize the magnitude and quality of immune responses to a single or a few key antigens, such as the Circumsporozoite protein (CSP), which is a very dominant antigen on the surface of sporozoites and Merozoite Surface Protein (MSP), by immunizing with synthetic peptides or recombinant proteins in an adjuvant (Figure 1.7). These vaccines are primarily designed to induce antibody and $CD4^+$ T cell responses, but there is also interest in eliciting $CD8^+$ T cell responses.

The ideal malaria vaccine will be highly effective in all age ranges, that is, it must elicit protective immunity in infants, children and adults. It has to be immunogenic and at the same time should confer safety and not have any undesirable side effects. In addition to being immunogenic, it should have the property of engendering long-term immunity. The ideal vaccine should go hand in hand with existing immunization programs and schedules and ought to be easy to administer. It should be devoid of other complications such as interference with traditional childhood vaccines. Last, but not the least, the vaccine should be easy and inexpensive to manufacture and be affordable in low-resource settings (Sudhakar & Subramani, 2007).

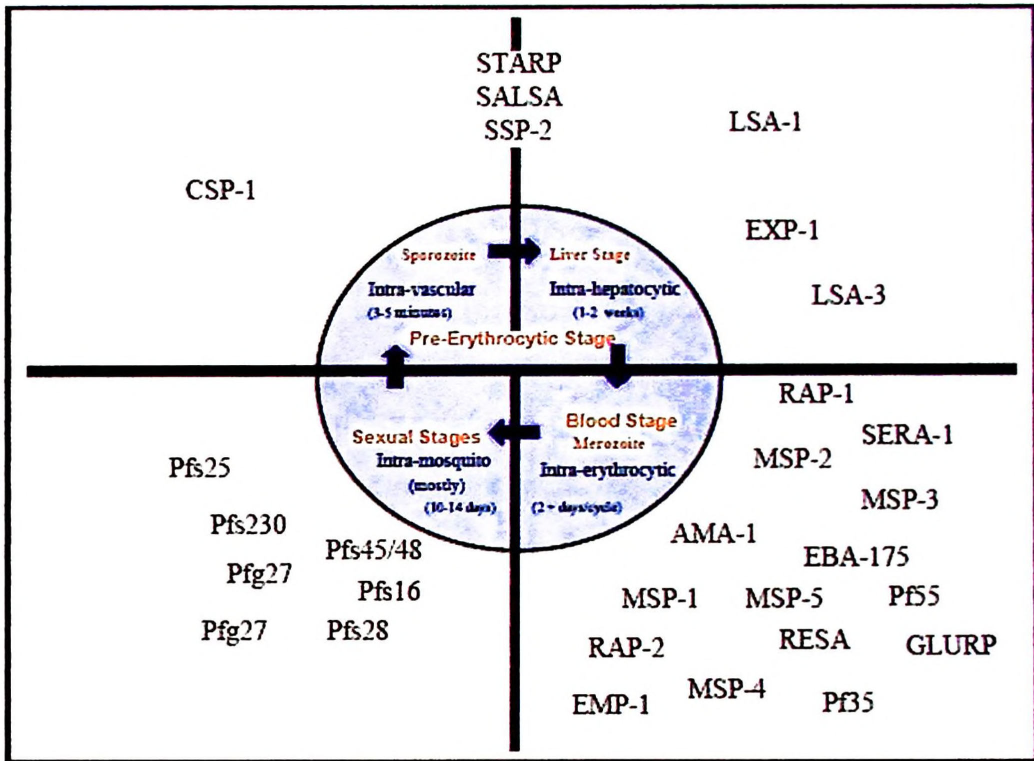

Figure 1.7: Stage-wise distribution of the antigenic profile of the Malarial parasite. (Source: http://www.jyi.org/issue/literature-review-insights-into-formulating-a-protective-malarial-medicine/)

1.7.1 Vaccine development

The development of a malaria vaccine requires more than understanding the molecular, pathogenic, parasitic and epidemiologic aspects of the plasmodium species. A successful vaccine strategy requires an appreciation of how the human host immune response interacts with the parasite. Such an understanding provides vaccine developers with clues on how best to design products that stimulate the proper effector arm of the immune system to achieve the desired goal (Sudhakar & Subramani, 2007).

Malarial parasites are much more complex than other microbes such as viruses or bacteria which have been controlled extensively by vaccination. The complexity of the malarial parasites is reflected by their life cycle which has many stages, each of which is characterized by the expression of different and unique proteins.

The blood-stage cycle of the parasite is responsible for malaria pathogenesis. Intervention at this stage of the parasite's development through vaccination is likely to reduce malaria related clinical symptoms. As a major interface between host and pathogen, the merozoite surface is an obvious target for the development of a malaria vaccine. A number of potential vaccine candidate antigens identified so far are located on or associated with the surface of the merozoite or in apical organelles. These include merozoite surface protein 1 (MSP-1), MSP-2, MSP-3, MSP-4, MSP-5, MSP-8, RAP1/2, AMA-1, and EBA-175, which are implicated in the process of merozoite invasion of the erythrocyte (Mahanty *et al.*, 2003).

1.7.1.1 MSP-1C

MSP-1 is one of the most extensively studied proteins of *P. falciparum* (Holder *et al.*, 1996; Mazumdar *et al.*, 2010). It is synthesized as a 200-kDa precursor and then processed in two steps; the primary processing step produces a complex of four fragments that are present on the merozoite surface, and the secondary