DETECTION OF INTERLEUKIN-1B (IL-1β) AND TUMOR NECROSIS FACTOR (TNF-α) IN MACROPHAGES TREATED WITH RECOMBINANT BCG (rBCG) EXPRESSING THE 19kDa C-TERMINUS OF THE MEROZOITE SURFACE PROTEIN (MSP-1C)

By

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CERTIFICATE

This is to certify that the dissertation entitled

" Detection Of Interleukin-1β(IL-1β) And Tumor Necrosis Factor Alpha (TNF-α) In Macrophages Treated With Recombinant BCG (rBCG) Expressing The 19 kDa C-Terminus Of The Merozoite Surface Protein (MSP-1C) "

is the bonafide record of research work done by

Mr Mohd Abdul Halim Bin Abd Latif

during the period from July 2009 to October 2009 under my supervision.

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ADC	Albumin Dextrose Catalase
ATCC	American type culture collection
APCs	Antigen Presenting Cells
BCG	Bacille Calmette-Guérin
BSV	Blood Stage Vaccine
CTL	Cytotoxic T Cell
CNS	Central Nervous System
ddH_2O	Deionized Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
EPI	Extended Programmed Of Immunization
ELISA	Enzyme link immunosorbent assay
FBS	Fetal Bovine Serum
IL-1β	Interleukin-1β
iNOS	inducible nitric oxide synthase
MHC	Major Histocompatibility Complex
MSP-1C	19kDa C-terminus of merozoite surface protein
NK	Natural killer
OADC	Oleic Acid Albumin Dextrose Complex
PEV	Pre-Erytrocytic Vaccine
PICs	Pro-Inflammatory Cytokines
PBS	Phosphate Buffer Saline

rBCG	Recombinant Bacillus Calmette-Guerin
RES	Rough endoplasmic reticulum
TBV	Transmission blocking vaccine
TNF-α	Tumor necrosis factor alpha
T_H1	T- helper 1
WHO	World Health Organization

ABSTRACT

Malaria remains a serious public health problem and causes an estimated 2 million deaths worldwide each year. The increase of drug resistant parasites and insecticide resistant mosquito vectors in certain areas worsens the situation especially against *P. falciparum*. As such, production of vaccine is very important in malaria prevention. In this study, a recombinant *Mycobacterium bovis* bacille Calmette-Guérin (rBCG) which expressed the 19kDa C-terminus of the merozoite surface protein (MSP-1C) was developed against the blood stage of malaria infection. The inflammatory cytokines such as interleukin-1β(IL-1β) and tumor necrosis factor-α (TNF-α) produced by macrophages are important for elimination of malaria infection. The ability of our rBCG construct to stimulate the production of pro-inflammatory cytokines by macrophages was determined at different incubation times (24 hours, 72 hours and 120 hours) and different concentrations (1:20 and 1:40 (macrophage: BCG or rBCG)). This study showed that the production of IL-1β and TNF-α was significantly higher in macrophages treated with rBCG compared to BCG, which could be observed at all incubation times and concentrations performed. This rBCG construct is able to stimulate the production of cytokine and perhaps preventing malaria infection especially at the blood stage.

ABSTRAK

Malaria merupakan salah satu penyakit yang berbahaya di dunia dan menyebabkan lebih kurang 2 juta orang mati setiap tahun. Peningkatan bilangan parasit yang rintang terhadap ubat dan racun serangga di sesetengah kawasan menambahkan lagi masalah yang dihadapi terutamanya terhadap *P. falciparum*. Oleh itu, penghasilan vaksin sangat penting bagi mengawal penyebaran penyakit malaria. Dalam kajian ini, *mycobacterium bovis* bacille Calmette-Guérin (rBCG) rekombinan yang mengekspreskan terminus C pada protein permukaan merozoite-19kDa (MSP-1C) dihasilkan bagi melawan jangkitan malaria pada peringkat darah. Pro-peradangan sitokin seperti Interleukin-1β (IL-1β) dan tumor necrosis factor (TNF-α) yang dihasilkan oleh makrofaj adalah sangat penting untuk menghindar dari jangkitan malaria. Kebolehan rBCG yang di hasilkan untuk merangsang pro-peradangan sitokin melalui macrofaj ditentukan pada masa pengeraman yang berbeza (24 jam, 72 jam dan 120 jam) dan pada kepekatan yang berbeza (1:20 dan 1:40 (makrofaj : BCG atau rBCG). Dalam kajian ini, penghasilan IL-1β dan TNF-α dari makrofaj yang dieramkan dengan rBCG adalah tinggi berbanding BCG. Ini dapat dilihat pada keseluruhan masa pengeraman dan kepekatan yang di gunakan. Oleh itu, rBCG mampu untuk meransang penghasilan sitokin dan diharap dapat menghalang jangkitan malaria terutamanya pada peringkat darah.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction:

Malaria is responsible for over a million deaths every year, and especially children under 5 years of age in Africa. World Health Organization (WHO) reported that, approximately 2 million people deaths attribute to malaria disease each year. In some endemic areas, the malaria mortality rate remains high for a number of reasons including limited access to healthcare and/or increased drug resistance parasite. There are three different approaches which can be used to control the most important malaria parasite, especially *Plasmodium falciparum (P. falciparum)*, and they are vaccine development, vector control, and also chemotherapy (Katherine *et al.*, 2006).

Malaria is caused due to blood infection by protozoan parasites of the genus *Plasmodium*. It can be transmitted from one human to another human via the bites of infected female anopheles mosquitoes. In human body, the parasites will multiply in the liver, and then it will infect red blood cells (Richard, 2002). Symptoms of malaria include fever, headache and vomiting, and usually appear between 10 to 15 days after the mosquito bite. If not treated, malaria can quickly become life-threatening by disrupting the blood supply to vital organs. In many parts of the world, the parasites have developed resistance to a number of malaria drugs (Katherine *et al.*, 2006).

1.2 History of Malaria

The term malaria is originates from Medieval Italian which is *mala aria* or known as "bad air" and the disease was formerly called ague or marsh fever due to its association with swamps and marshland (Reiter, 2000). Malaria parasites originated in Africa (along with mankind), and fossils of mosquitoes up to 30 million years old, show that the malaria vector, which is the malaria mosquito, was present well before the earliest history. Hippocrates, which is a physician, was born in ancient Greece. Today he was regarded as the "Father of Medicine" and was the first to describe the manifestations of the disease, and relate them to the time of year and to where the patients lived. Before this, the supernatural was blamed. The association with stagnant waters (breeding grounds for the Anopheles mosquito) led the Romans to begin drainage programs, the first intervention against malaria (Sherman, 1998).

The first recorded treatment dates back to 1600, when the bitter bark of the Cinchona tree in Peru was used by the native Peruvian Indians. By 1649, the bark was available in England, as "Jesuits powder," so that those suffering from "agues" might benefit from the chemical substance quinine, which it contained. Not until 1889 was the protozoa (single celled parasite) cause of malaria discovered by Alphonse Laveran working in Algeria, and only in 1897 was the Anopheles mosquito demonstrated to be the vector for the disease by Ronald Ross (Oliver *et al.*, 2008).

Malaria transmission and prevalence is influenced by many factors, such as climate conditions. With increasing weather variability and ability to forecast weather, there is an interest in developing systems for malaria forecasting that incorporate weather related factors as explanatory variables. It has been noted for more than 4000 years (Oliver *et al.*, 2008). In the tropical and subtropical regions in the world, malaria recognized as the major cause of mortality and morbidity. However, the deaths can be reduced by effective use of standard treatment procedures. Patients who require hospitalization and those who need intensive care can be identified promptly and treated before they develop complications. (Pasvol, 2005).

The disease is become the main causes of death which is almost one to three million deaths annually, mostly in Saharan Africa. It is important to note that Colombia's malaria surveillance programs since 1960 have been based on blood tests and clinical follow-ups of positive cases. This is an important difference from many of the African countries where the predominant tool for malaria diagnosis is based on clinical symptoms, such as febrile illness, for treatment decisions. This symptom-based method usually results in over-diagnosis of malaria cases (Gilma *et al.*, 2009).

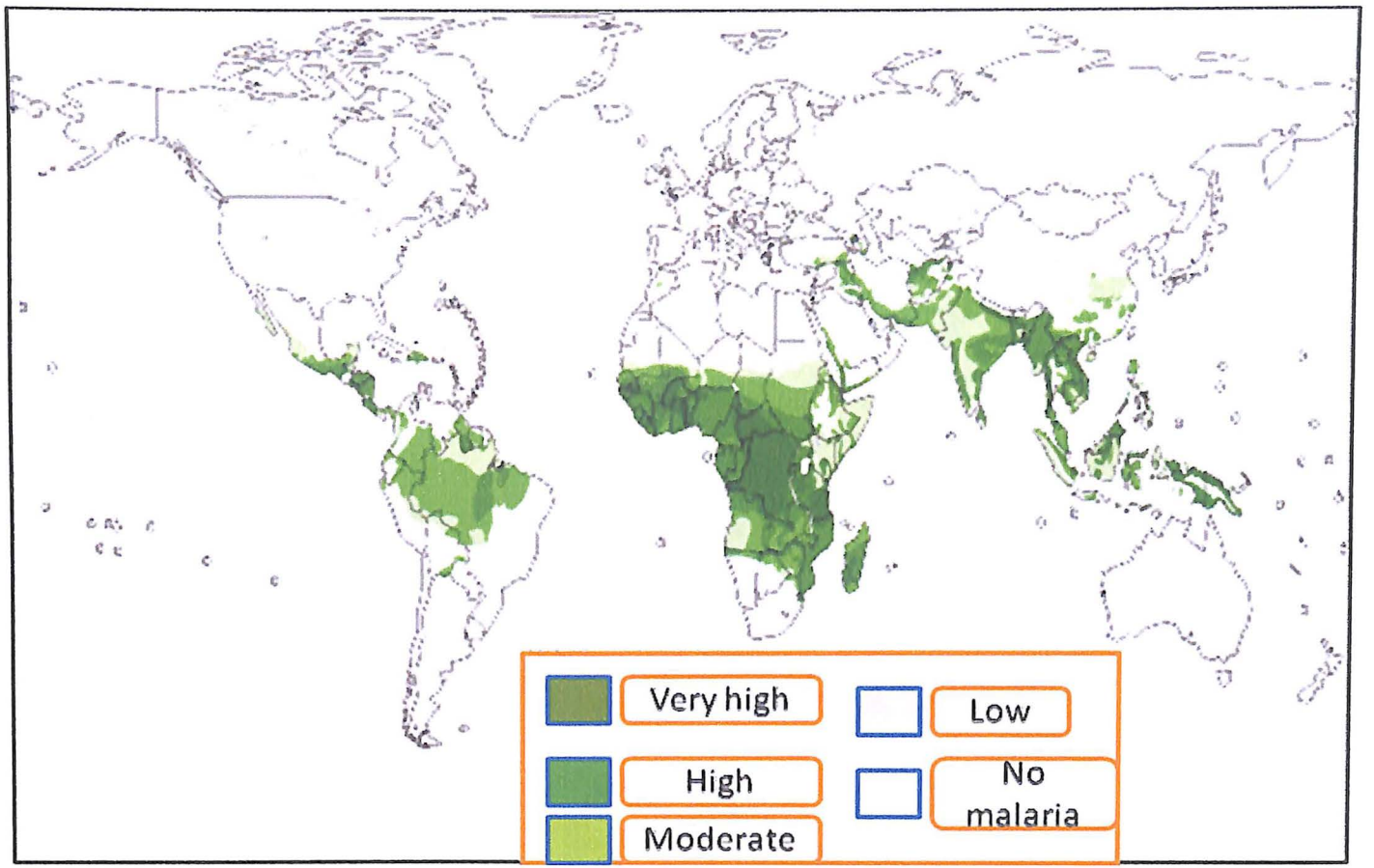

Figure 1.1: Global distribution of malaria transmission risk in 2003 (WHO 2005).

1.4 The Prevalence of Malaria in Malaysia

Malaria was present more about 3000 years. But, it still poses a medical challenge to mankind. Due to previous statistic, in early 50's, approximately 250 million cases with 2.5 million death are occurred annually. In Malaysia, this disease is focal, with about 1.7 million people living in malarious areas and 1.2 million living in malaria prone areas. This incidence rate in 2000 was 4.9 per 10,000 with a case fatality rate of 0.23%. majority of the cases comes from sabah (40%) followed by peninsular Malaysia (31.5%). In peninsular Malaysia, Pahang reported the highest incidence rate of 9.6 per 10,000 followed by Kelantan (5.9 per 10,000) and Perak (2.7 per 10,000). Most of the cases reported in peninsular Malaysia were among orang Asli's which involved 64.4%. Males was constituted about 70.5% from all the cases. The highest incidence of malaria was among those between 5-9 years old (7.o per 10,000). Due to information that has been reported, the *P. falciparum* was the predominant species which composed about 50% of the cases (Gurpreet, 2003).

Table 1.1: Reported malaria cases by selected subnational area in Malaysia (WHO, 2005).

State	2000	2001	2002	2003	%
Sarawak	3 011	3 145	2 496	2 615	41
Sabah	5 776	6 050	5 096	1 770	28
Pahang	1 301	1 544	1 563	850	13
Johor	710	671	579	284	4
Perak	852	470	280	276	4
Selangor	271	172	159	119	2
Pulau Pinang	209	197	76	106	2
Kelantan	386	184	333	99	2
Kedah	12	26	82	92	1
Terengganu	94	76	140	47	1
Negeri Sembilan	37	205	180	45	1
W.P.Kuala Lumpur	27	20	15	20	<1
W.P. Labuan				7	<1
Melaka	18	15	16	7	<1
Perlis	1	5	4	1	<1

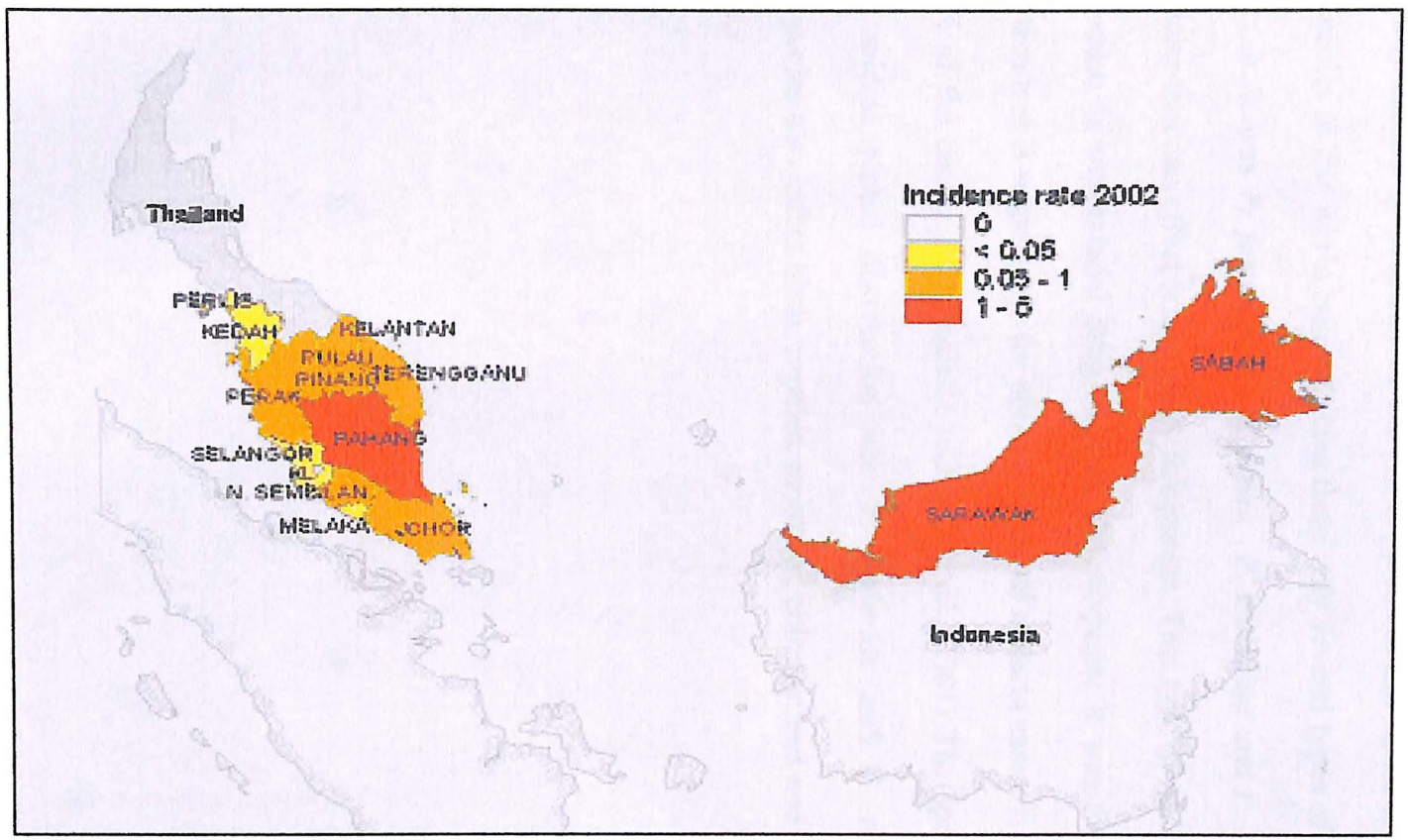

Figure 1.2: Incidence rate of confirmed malaria cases by province Malaysia, 2003 (WHO, 2005).

1.5 Malaria Parasite

Malaria is mainly caused by protozoa of the genus plasmodium. There are severe type of plasmodium species in this world but until now there only several types of malaria parasite can infect human. It was *P. falciparum* , *P. vivax, P. malariae and P. ovale.* The most dangerous species that can effect human is *P. falciparum.* This type of malaria parasite was resistance to some of antimalarial drug such as choloroquine. It was the most common cause of infection and is responsible for about 80% of all malaria cases. It also responsible for about 90% of the deaths from malaria (Kamini *et al.*, 2001) The species that almost achieved the widest global distribution was *P. malariae* and *P. vivax.* Parasitic Plasmodium species also infect birds, reptiles, monkeys, chimpanzees and rodents (Kamini *et al.*, 2001).

Table 1.2: Geographical Distributions of *plasmodium* species (Ivo *et al.*, 2007)

Vivax	***Falciparum***	***Malariae***	***Ovale***
➢ widespread in tropical and subtropical areas ➢ range extends into temperate areas ➢ relatively uncommon in Africa	➢ widespread, but primarily in tropics and subtropics	➢ broad, but spotty geographical distribution	➢ primarily tropical Africa, especially western coast

1.6 Malaria Life Cycle

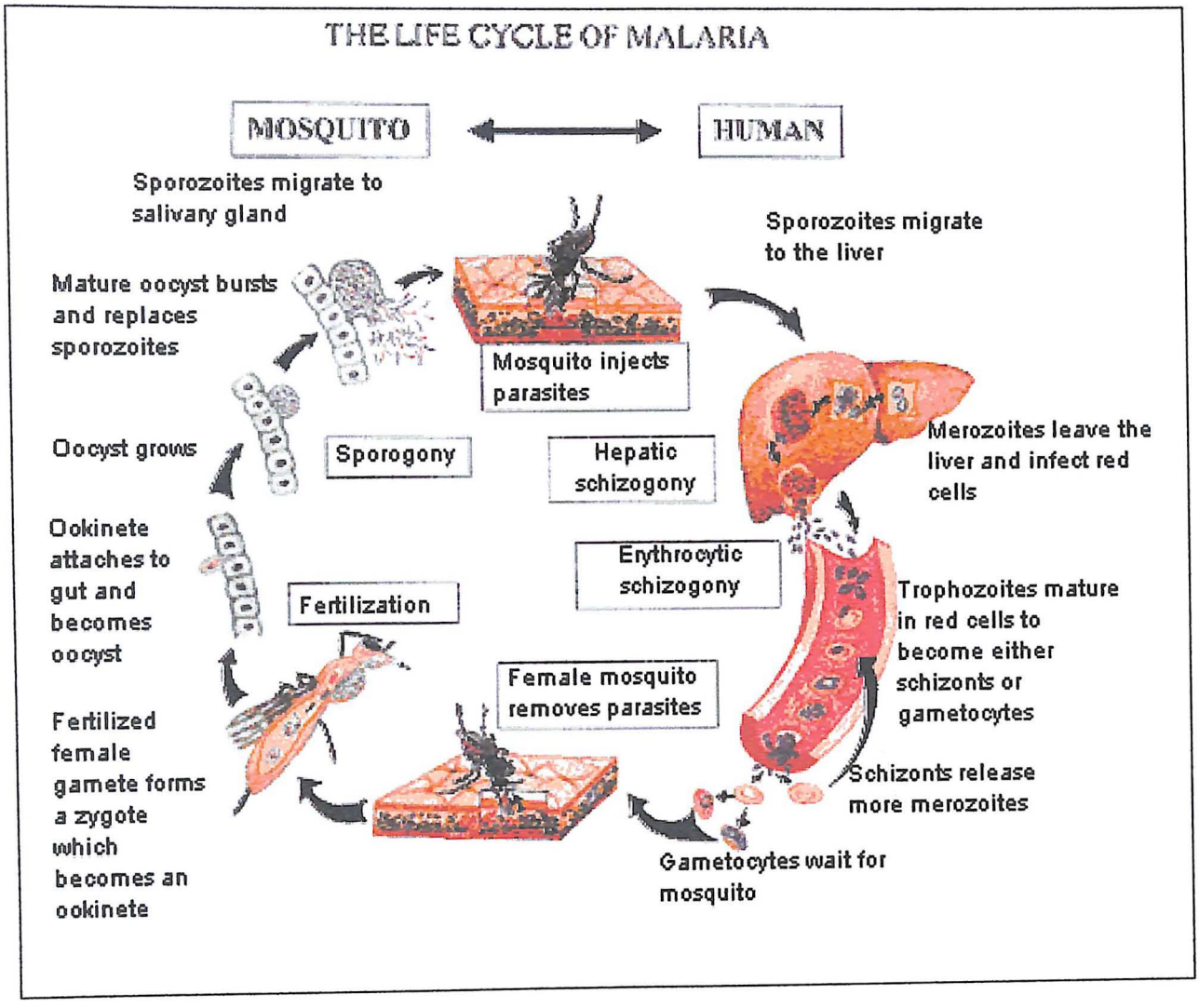

Figure 1.3: Life cycle of *P. falciparum* (WHO, 2007)

Plasmodium species has a complex life cycle because it requires two different host: human and females anopheles mosquito (Perry and stanley, 1997). The complexity of the malaria life cycle means that, there is a number of different stages of the parasite can be targeted. The candidate that is most advanced in clinical development targets pre-erythrocytic stages of the parasite (Fabrizio *et al.,* 2009). The *plasmodium* parasites enter the human host when an infected mosquito takes a blood meal. The parasites in a form of sporozoites enter the bloodstream and migrate to the liver where they replicate without causing any symptoms. They infect the liver cells (hepatocytes), where they multiply into merozoites, rupture the liver cells and escape back into bloodstream. After that, the merozoites will infect the red blood cells, where they developed into ring forms. Then it will developed to trophozoites (a feeding stage).

Eight to nine days later, schizont (a reproduction stage) are released from the liver cells and invade red blood cells. Here, merozoites are produced which then infect red blood cells. Sexual forms called gametocytes are also produced which, if taken up by mosquito, it will infect the insect and continue the life cycle. The recurring flu-like symptoms of malaria are caused by this cyclical increase in parasitemia (parasites in the blood) and should be treated promptly with anti-malarial drugs to prevent the development of potentially fatal complications. Infections with *P. falciparum* in particular can cause anemia by destroying the red blood cells and can damage vital organs (including the brain) by blocking the capillaries that supply them with blood (Blaise *et al.*, 2008).

1.7 Immunity to Malaria

Immunity is a biological term that describe a state of having sufficient biological defense to avoid infection, disease or other unwanted biological invasion. There is 2 type of immunity; innate immunity and adaptive immunity. This immunity are involved in protection against malaria parasites. An innate immunity is composed of skin, cilia, neutrophils, macrophages and some other type of cells. On the other hand, the adaptive immunity is involved the humoral immunity (involve B-cell) and cellular immunity (involve T-cell).

Previous study show that, immunity to blood stage malaria is actually depends primarily on humoral immune responses. However, innate immune responses and cell mediated immune response also play a supporting role in protective immunity against the parasite. Following blood stage infection, infected red blood cells will activate the innate immune responses which hinder the growth of early blood stage parasite. On the other hand, the innate immune response will interacts with and modulate adaptive immune response allowing the host to control parasetemia (Yazdani *et al.*, 2006).

Malaria parasite multiply in red blood cells during the blood stage of the parasite life cycle. This due to red cell unable to present antigens since it does not express histocompatibility complex molecules (MHC) class I and II. Because of that, humoral immune responses are primarily responsible for naturally acquired protective against blood stage *P. falciparum* parasite (Yazdani *et al.*, 2006). Previous study, showed that the *plasmodium* infected red