THE EFFECT OF CRX-527 ON PRO-INFLAMMATORY CYTOKINE PRODUCTION AGAINST *Mycobacterium bovis* BCG CLONE EXPRESSING THE C-TERMINUS OF MEROZOITE SURFACE PROTEIN-1 OF *Plasmodium falciparum*

WAN RAIHAN BINTI WAN YUSOFF

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by

WAN RAIHAN BINTI WAN YUSOFF

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CERTIFICATE

This is to certify that the dissertation entitled THE EFFECT OF CRX-527 ON PRO-INFLAMMATORY CYTOKINE PRODUCTION AGAINST *Mycobacterium bovis* BCG CLONE EXPRESSING THE C- TERMINUS OF MEROZOITE SURFACE PROTEIN-1 OF *Plasmodium falciparum* is the bona fide record of research work done by Ms Wan Raihan bt. Wan Yusoff during the period from March 2021 to September 2021 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Master of Science (Biomedicine) Mixed Mode.

Assoc. Prof. Dr. Rapeah Suppian
Deputy Dean (Postgraduate, Career and International)
School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150 Kubang Kerian
Kelantan, Malaysia
Date: 6/10/2021

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

Signature

WAN RAIHAN BT WAN YUSOFF

Date: 6/10/2021

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percentage
˚C	Degree celcius
μg	Microgram
ml	Millilitre
μl	Microliter
APCs	Antigen-presenting cells
BCG	*Mycobacterium bovis bacille Calmette–Guérin*
BCG-MSP1C	Recombinant BCG expressing the MSP-1C of *P. falciparum*
DCs	Dendritic cells
IL-12	Interleukin 12
MAPKs	Mitogen-activated protein kinases
MSP-1C	C-terminus of the merozoite surface protein-1
NF-κB	Nuclear factor-κB
NK	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
rBCG	Recombinant BCG
TB	Tuberculosis
TLR-4	Toll-like receptors 4
TNF-α	Tumor necrosis factors
TRIF	TIR-domain-containing adapter-inducible interferon-β

TRAM	TRIF-related adaptor molecule
WHO	World Health Organization

KESAN CRX-527 PADA PENGELUARAN SITOKIN PRO-RADANG TERHADAP *Mycobacterium bovis* BCG CLONE MENGEKSPRESKAN C-TERMINUS PROTEIN PERMUKAAN MEROZOITE-1 *Plasmodium falciparum*

ABSTRAK

Jangkitan malaria adalah penyebab utama kematian dan morbiditi di negara-negara Afrika dan Asia Tenggara. TLR-4 telah terbukti penting dalam imuniti malaria dan boleh digunakan sebagai adjuvant untuk meningkatkan tindak balas imun yang tahan lama terhadap BCG-MSP1C. Penyelidikan ini telah dijalankan untuk menentukan kesan reseptor seperti TLR-4 agonis, CRX527, pada tindak balas imun selular dan humoral terhadap *Mycobacterium bovis* bacille Calmette-Guérin (rBCG) yang menyatakan C-terminus protein permukaan merozoite-1 *Plasmodium falciparum* (BCG-MSP1C). Dalam kajian ini, enam kumpulan tikus (n=6 setiap kumpulan) telah disuntik dengan 200 µl intraperitoneal fosfat diimbalkan saline T80 (PBS-T80), 200 µl PBS-T80 yang mengandungi 2 x 10^6 cfu/ml BCG atau rBCG dengan kehadiran atau ketiadaan CRX527. Darah dikumpulkan dari urat ekor tikus sebelum permulaan imunisasi dan 4 minggu selepas setiap imunisasi. Enzyme yang dikaitkan dengan assay immunosorbent (ELISA) telah dijalankan untuk mengukur pengeluaran TNF-α dan IL-12 dalam sera tikus yang diimunisasi. Hasil kajian menunjukkan bahawa sera tikus dalam kumpulan imunisasi rBCG tanpa CRX menghasilkan peningkatan yang paling banyak IL-12 diikuti oleh tikus yang disuntik rBCG dengan CRX. Sebaliknya, semua kumpulan imunisasi tikus menunjukkan penurunan pengeluaran untuk TNF-α selepas diimunisasi. Oleh itu, gabungan rBCG dengan Agonis TLR4 ini boleh digunakan

untuk membuat vaksin yang mampu mendorong imuniti yang kuat dan tahan lama untuk membolehkan sistem imun bersedia untuk kemunculan penyakit malaria yang boleh menyebabkan ketahanan ubat.

THE EFFECT OF CRX-527 ON PRO-INFLAMMATORY CYTOKINE PRODUCTION AGAINST *Mycobacterium bovis* BCG CLONE EXPRESSING THE C- TERMINUS OF MEROZOITE SURFACE PROTEIN-1 OF *Plasmodium falciparum*

ABSTRACT

Malaria infection is a leading cause of mortality and morbidity in African and Southeast Asian countries. TLR-4 has been shown to be important in malaria immunity and can be possibly use as an adjuvant to enhance the long-lasting immune response against BCG-MSP1C. This research was conducted to determine the effects of toll-like receptor 4 (TLR-4) agonist, CRX527, on cellular and humoral immune response against *Mycobacterium bovis* bacille Calmette-Guérin (BCG) expressing the C-terminus of merozoite surface protein-1 of *Plasmodium falciparum* (BCG-MSP1C). In this study six groups of mice (n=6 per group) were injected with 200 µl intraperitoneal phosphate buffered saline T80 (PBS-T80), 200 µl of PBS-T80 containing 2 x 10^6 cfu/ml of BCG or rBCG in the presence or absence of CRX527. Blood was collected from the tail veins of the mice before the start of the immunisation and 4 weeks after each immunisation. Enzyme linked immunosorbent assay (ELISA) was carried out to measure the production of TNF-α and IL-12 in the sera of the immunized mice. The findings showed that the sera of rBCG without CRX immunised group yielding the most increase in IL-12 followed by the mice injected with rBCG with CRX. In contrast, the entire mice immunised groups showed decrease production for TNF-α after the immunisation. Therefore, this combination of rBCG with TLR4 agonist can be used to create a vaccine that capable of inducing a strong and long-

lasting immunity to allow the immune system to be prepared for the emergence of multidrug-resistant strains of malaria diseases.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Chapter one covers the background of the study about malaria infection and how rBCG strain is capable of stimulating phagocytic activity and pro-inflammatory cytokines production and how the present study was based on the finding that TLR-4 activation is a major pathway involved in a class of malaria. In addition the general objective of the research and specific objective of the research also will be covered in chapter one.

1.2 Background of the Study

Malaria infection, particularly from *Plasmodium falciparum*, is a leading cause of mortality and morbidity in African and Southeast Asian countries (Snow *et al*., 2005). It annually affects millions of people throughout the world, especially older people and pregnant ladies. Mostly, children under the age of five years are vulnerable to life-threatening anemia and cerebral malaria (World Health Organization, 2015). An estimated 229 million cases of malaria have been reported in 2019, with 409,000 deaths (WHO, 2020). A total of 16,500 malaria cases were notified in Malaysia from 2013-2017. The cases were mainly contributed by Sabah and Sarawak (Hussin *et al*., 2020). *Plasmodium knowlesi* was the commonest species in Sabah and Sarawak (Hussin *et al*., 2020). This problem might be due to various contributing factors such as genetic diversity, the emergence of multidrug-resistant strains, and environmental factors (Hay *et al*., 2003).

MSP-1C is a 19 kDa blood-stage antigen produced by proteolysis of a high molecular weight precursor, 195 kDa MSP-1 protein. During merozoite invasion of red blood cells, the protein is processed by proteases and released from the parasite surface except for a 19 kDa C-terminal region of MSP-1 which remains on the surface of the invading merozoites. This protein is responsible for protective immunity against malaria infection and is one of the most promising malaria vaccine candidates. Previous research had cloned and expressed a synthetic gene encoding the C-terminus of the merozoite surface protein-1 (MSP-1C) in a recombinant BCG (rBCG) construct. (Holder *et al*., 1985; Nurul & Norazmi, 2011).

Mycobacterium bovis bacille Calmette–Guérin (BCG), currently available vaccine for the prevention of tuberculosis. It is the most commonly used vector for developing recombinant vaccines for other diseases, including malaria. Due to its unique safety profile, BCG is used in vaccines for other human pathogens. It is easily recognised and rapidly phagocytosed, eliciting specific adaptive responses such as antibody formation and T cell responses. (Stover *et al*., 2004; Abbas & Suppian, 2019). A previous study showed that rBCG strain is capable of stimulating phagocytic activity and pro-inflammatory cytokines production in macrophages at different incubation times (Nurul & Norazmi, 2011). Moreover, the rBCG strain also stimulated higher cellular and humoral immune responses in an animal model. Our laboratory had earlier constructed a recombinant BCG expressing the MSP1C of *P. falciparum* which elicited robust cellular and humoral immune responses through the activation of toll-like receptor 4 (TLR4) (Abbas & Suppian, 2019).

The TLRs are primary components of the innate immune system that recognize pathogen-associated molecular patterns present on bacterial, fungal, or viral pathogens. The present study was based on the finding that TLR-4 activation is a major pathway involved in a class of malaria (Barboza *et al*., 2017). TLR-4 recognises glycosyl phosphatidylinositol from P. *falciparum* and is activated through both the MyD88-dependent and MyD88-independent pathways leading to cytokine release and induction of adaptive immunity (Abbas & Suppian, 2019). Thus, the properties of TLR agonists suggest that these innate immunity activators could be used as adjuvants.

CRX-527 is a synthetic lipid A mimic belonging to the aminoakyl glucoaminide 4-phosphate (AGP) family (Stover *et al*., 2004). CRX-527 activates Toll-like receptor 4 (TLR4) (Stover *et al*., 2004). Subsequently, it initiates MyD88- and TRIF-dependent pro-inflammatory signaling cascades. Importantly, CRX-527 does not require the TLR4 co-receptor CD14 for activation of either signaling pathway. CRX-527, along with other AGPs, is less toxic than LPS and importantly retains its immunostimulatory properties. Therefore, AGPs have great potential as future vaccine adjuvants as well as stand-alone immunostimulants (InvivoGen,).

1.2 Problem Statement and Rationale of the study

Due to the genetic diversity, the emergence of multidrug-resistant strains of malaria diseases, a vaccine that capable of inducing a strong and long-lasting immunity need to be discovered. The recombinant BCG clone expressing the MSP-1C of *P. falciparum* developed by the previous student from our group generated immune responses by utilizing toll-like receptors 4 (TLR-4). TLR-4 is important in malaria immunity and can be possibly used as an adjuvant to enhance the long-lasting immune

response against BCG-MSP1C. Knowledge regarding the potential use of the TLR-4 agonist, in enhancing the immune response against the BCG-MSP1C is still lacking. Hence, this study was conducted to determine the effect of TLR-4 agonist (CRX527), in enhancing the immune response against the BCG-MSP1C.

1.3 Objective

1.3.1 General objective

To determine the effects of TLR 4 agonist (CRX- 527) on pro-inflammatory cytokine production against recombinant *Mycobacterium bovis* BCG clone expressing the C-terminus of merozoite surface protein-1 (MSP-1C) of *Plasmodium falciparum*.

1.3.2 Specific objectives

The specific objectives are as follows:

1. To confirm the *M. bovis* BCG and BCG-MSP1C colonies using Acid Fast Staining.
2. To determine the stability of the MSP-1C gene in the BCG-MSP1C clone using PCR.
3. To determine the effect of CRX-527 on TNF-α and IL-12 production in mice immunized with BCG-MSP1C.

1.4 Flow chart

The flow chart of the study is shown in Figure 1.1

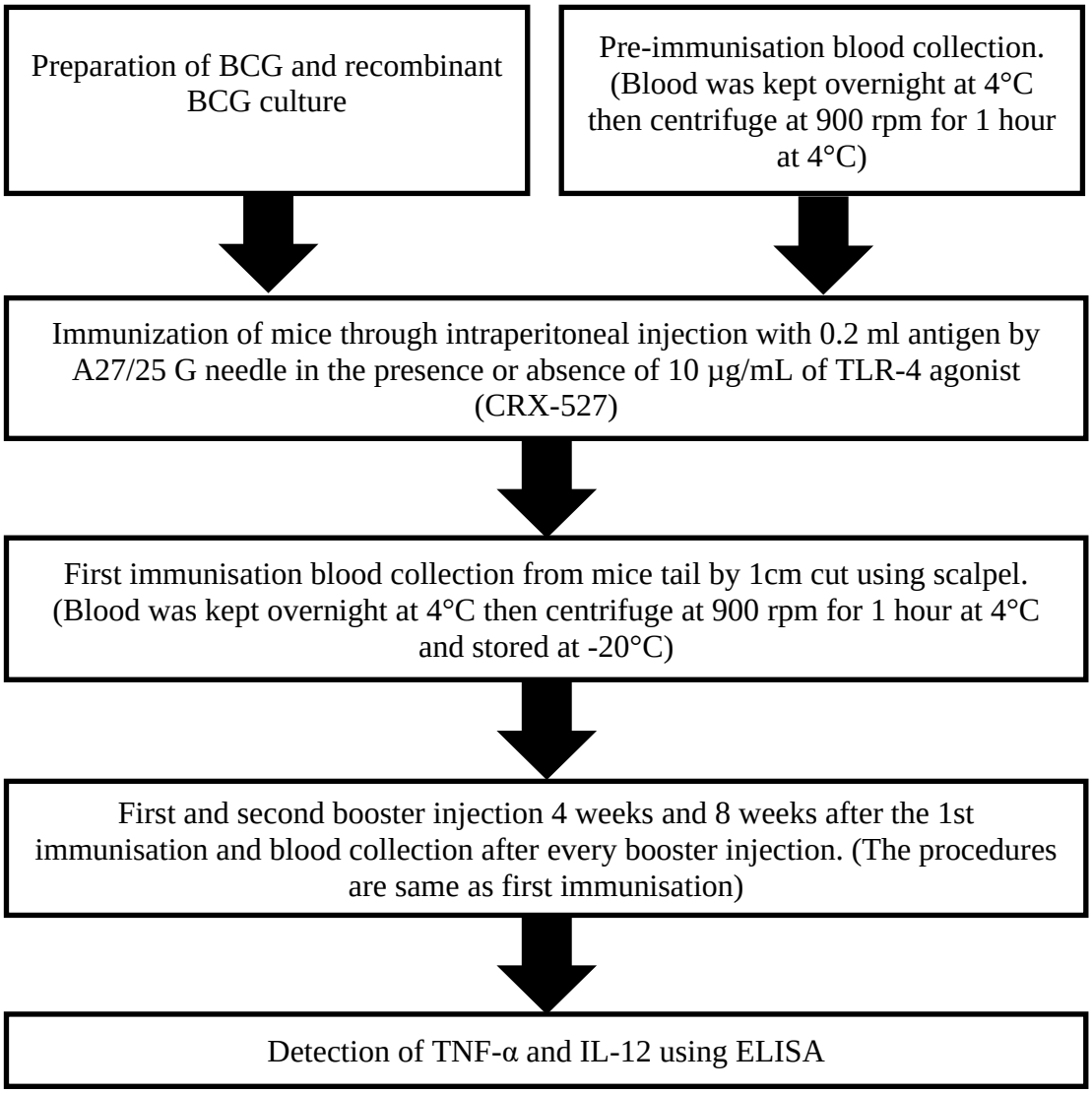

Figure 1.1 Flowchart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The literature review part will focus on malaria diseases and some key factors that were selected to describe the role of TLR and the pathway of TLR-4 activation in innate immune signaling. The use of TLR agonists in the combination of BCG and MSP-1C as a potential vaccine adjuvant will also be discussed in this chapter.

2.2 History of malaria

Malaria is an ancient disease and references to what was almost certainly malaria occur in a Chinese document from about 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC, and Hindu texts as far back as the sixth century BC. The early Greeks, including Homer in about 850 BC, Empedocles of Agrigentum in about 550 BC, and Hippocrates in about 400 BC, were well aware of the characteristic poor health, malarial fevers, and enlarged spleens that were seen in people living in marshy places. For over 2500 years the idea that malaria fevers were caused by miasmas rising from swamps persisted and it is widely held that the word malaria comes from the Italian mal'aria meaning spoiled air although this has been disputed (Cox, 2010). With the discovery of bacteria by Antoni van Leeuwenhoek in 1676, and the incrimination of microorganisms as causes of infectious diseases, and the development of the germ theory of infection by Louis Pasteur and Robert Koch in 1878-1879, the search for the cause of malaria intensified. Scientific studies only became possible after the discovery of the parasites themselves by Charles Louis Alphonse Laveran in 1880 and the incrimination of mosquitoes as the vectors, first for

avian malaria by Ronald Ross in 1897 and then for human malaria by the Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi, and Ettore Marchiafava between 1898 and 1900 (Graves *et al*., 2008).

2.3 Malaria burden

2.3.1 Worldwide

Malaria infection is a serious worldwide health issue that contributes to millions of deaths. According to the latest estimates, there were 229 million cases of malaria in 2019 and 409 000 malarial deaths were reported in 2019 (World Health Organization, 2020). African Region is the most affected region with malaria burden. In 2019, 6 countries accounted for approximately half of all malaria deaths worldwide: Nigeria (23%), the Democratic Republic of the Congo (11%), United Republic of Tanzania (5%), Burkina Faso (4%), Mozambique (4%) and Niger (4% each). Globally, approximately 274 000 children before the age of five years are vulnerable to get malaria and its developing disease (World Health Organization, 2015; (Zulkipli and Norazmi, 2018). The majority of malaria deaths in the African continent were caused by *Plasmodium falciparum* (World Health Organization, 2020).

2.3.2 Malaysia

In Malaysia, a total of 16,500 malaria cases were reported in the 5 years 2013 to 2017 (Hussin *et al.*, 2020). The overall average incidence rate, mortality rate, and case fatality rates for malaria from 2013 to 2017 in Malaysia were 0.106/1000, 0.030/100,000, and 0.27%, respectively. These infections comprise human malaria infections and zoonotic malaria infections. Malaria infections were usually found in

the states of Sabah and Sarawak and *Plasmodium knowlesi* was the commonest species in Sabah and Sarawak. These species accounted 38% of the reported cases of malaria in 2014 (World Health Organization, 2015). In contrast, there were more *Plasmodium vivax* cases in Peninsular Malaysia. The most common age group in Peninsular Malaysia was 20 to 29 years (1286; 35.1%), while Sabah and Sarawak reported the highest number of malaria cases in age group of 30 to 39 years (2776; 21.6%). In addition, Malaria predominantly affected males compared with females.

2.4 Malaria parasites

2.4.1 *Plasmodium* species

The causative agent of malaria is a small protozoon belonging to the group of Plasmodium species, and it consists of several subspecies. Some of the Plasmodium species cause disease in human. The genus Plasmodium is an amoeboid intracellular parasite which accumulates malaria pigment (an insoluble metabolite of hemoglobin). Parasites on different vertebrates; some in red blood cells, and some in tissue. Of the 172 of Plasmodium species, five species can infect humans. These are P. *malariae*, *P. falciparum*, *P. vivax*, *P. ovale*, and *P. knowlesi*. In South-East Asia, the zoonotic malaria *P. knowlesi* is recorded. Other species rarely infect humans. All the mentioned Plasmodium species cause the disease commonly known as malaria. 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 *P. falciparum* due to its ability to multiply rapidly in the patient's blood which contributes to severe anaemia in the patient. *P. falciparum* infection may lead to death if untreated (Sinden & Gilles, 2002; Snow *et al*., 2005).

2.4.2 Life cycle of Plasmodium

The mode of infection and developmental stages in the hosts are similar in all human malaria parasites. As shown in Figure 2.1, 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). During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host. Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites. 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. After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony). Merozoites infect red blood cells. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites and every schizont produces 10,000 to 30,000 merozoites. Some parasites differentiate into sexual erythrocytic stages (gametocytes). Blood stage parasites are responsible for the clinical manifestations of the disease (CDC, 2020).

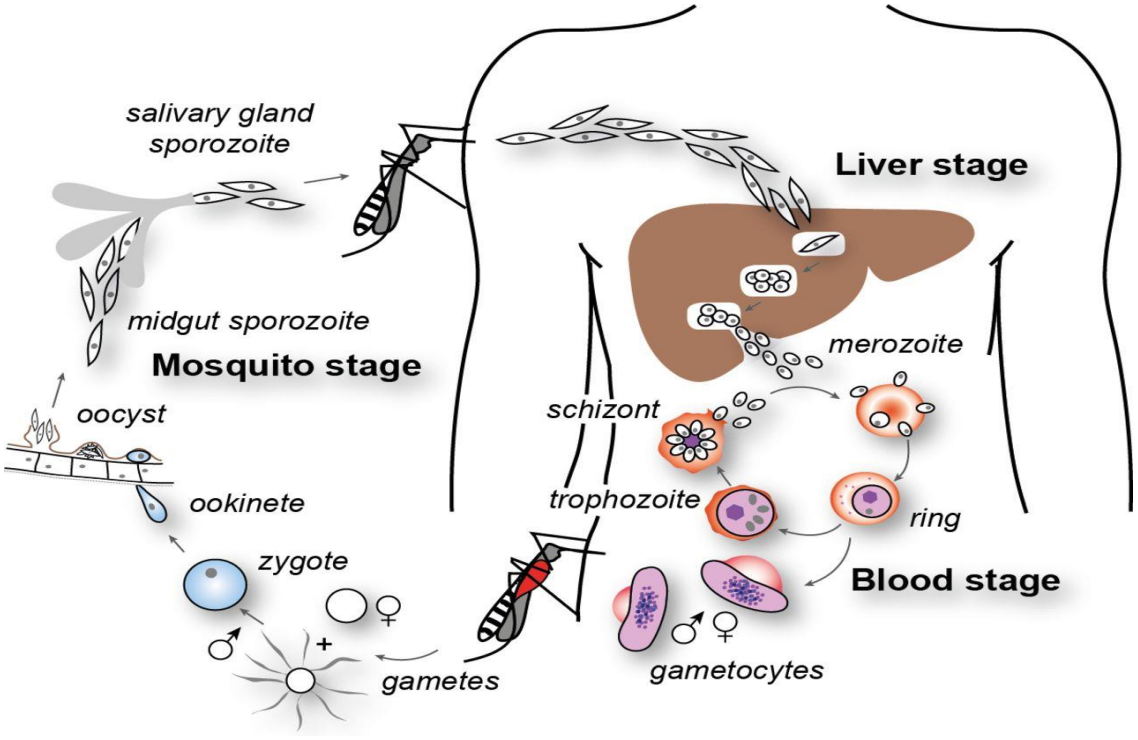

Figure 2.1 Life cycle of Plasmodium. The figure is adapted from Cowman *et al*. (2012).

The sexual cycle is initiated when some of the released merozoites from infected RBC turned into mature gametocytes. The mature gametocytes which are male (microgametocytes) and female (macrogametocytes), are ingested by an Anopheles mosquito during a blood meal circulate in the human bloodstream and can be ingested by the mosquitoes. The parasites multiplication in the mosquito is known as the sporogonic cycle. The maturation process of gametocytes continues in the midgut of mosquito where the union of gametes results in the formation of zygotes. The zygotes in turn become motile and elongated (ookinetes) which invade the midgut wall of the mosquito where they develop into oocysts. Over time, the oocysts expand and rupture which lead to the formation of sporozoites which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (Bousema & Drakeley, 2011).

2.4.3 Clinical symptoms and manifestations

The person infected with malaria parasites remains asymptomatic during the liver stage of parasite life cycle. The most frequent symptoms include fever and chills, which can be accompanied by headache, myalgias, arthralgias, weakness, vomiting, and diarrhea. Other clinical features include splenomegaly, anemia, thrombocytopenia, hypoglycemia, pulmonary or renal dysfunction, and neurologic changes. The clinical presentation can vary substantially depending on the infecting species, the level of parasitemia, and the immune status of the patient. During the erythrocytic cycle, severe forms of malaria pathologies including cerebral anaemia (coma), hypoglycemia, metabolic acidosis, renal failure and severe anaemia were

observed. The majority of complication of severe malaria cases such as kidney failure and coma contribute to deaths (CDC, 2020).

2.4.4 Pathogenesis of malaria

Sequestration is a key event of pathogenesis of malaria leading to severe malaria and can be characterized by the blockage of blood vessel and impaired oxygen supply. 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 describes the process whereby some 12–18 h after merozoite invasion P. *falciparum* parasitized erythrocytes adhere to vascular endothelium and disappear from the circulation. Once adherent they do not detach until schizont rupture and so the parasites do not reappear in the circulation until the next asexual cycle. The adhesion of infected RBC in the endothelium of the brain results in cerebral malaria. Furthermore, the occurrence of cerebral malaria is mediated by the production of NO and other pro-inflammatory cytokines (Clark *et al*., 1992). 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 a severe form of microvascular disease (Udomsangpetch *et al*., 1989; Chen *et al*. 2000).

2.4.5 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 (BHeelan *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 parasites provides great challenges to the treatment of malaria (Cravo *et al*, 2015). Together with treatments of malaria, long-term mosquito abatement was implemented for the reduction of the infected mosquito (vector) population. Several strategies such as the 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 mosquitoes as well as kill sporozoite-infected mosquitoes. Still, this strategy is partially effective because some of the mosquitoes have developed into insecticide-resistant mosquitoes (Bockarie *et al*., 2006).

2.4.6 Malaria vaccine development

The development of a malaria vaccine is a public agenda since the widespread of drug-resistant malaria parasites and insecticide-resistant mosquitoes gave difficulty in combating malaria infection (Phillips, 2001). Several 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). The 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 minimize or prevent the severe form of malaria complications that were developed from RBC invasion at the blood stage. Apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) are leading vaccine candidates that have been extensively studied among other vaccines. (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). In addition, RTS,S which is pre-erythrocytic vaccines is the first malaria vaccine candidate that enters Phase III trials. Vaccination with RTS,S induces 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).

2.5 C-terminus of merozoite surface protein-1

Merozoite surface protein-1 (MSP-1) is a 195 kDa major surface glycoprotein of merozoites. This protein binds to human erythrocytes in a sialic acid-dependent manner. It plays a pivotal role in erythrocyte invasion by merozoites. MSP-1 is synthesised as a large precursor form which subsequently undergoes two independent proteolytic cleavage events. First, the large precursor protein is cleaved into four polypeptides (83, 30, 38 and 42 kDa) that form a non-covalently associated complex. The 42 kDa fragment (MSP-142) is further cleaved into 33 kDa (MSP-133) and 19 kDa (MSP-119) fragments when merozoites invade erythrocyte. Only MSP-119 remains on the merozoite surface and enters into the erythrocyte whereas the others are released from the merozoite. MSP-1 is highly immunogenic. Species-specific natural immune responses against this antigen have been reported in patients naturally exposed to *P. falciparum* and *P. vivax*. In particular, the C-terminal region of MSP-1, MSP-119 or MSP-142, is of particular interest since individuals naturally infected with malaria acquire humoral immune responses against this domain. Antibodies that

recognise the C-terminal 42 kDa region of *P. falciparum* MSP-1 (PfMSP-142) can inhibit the growth of the parasite or invasion of the merozoite into host erythrocytes (Zulkipli and Norazmi, 2018).

2.6 Recombinant BCG as a potential vaccine candidate

Mycobacterium bovis bacille Calmette-Guerin (BCG) is a non-pathogenic and attenuated strain of *M. bovis* that was the first live attenuated vaccine used in humans. BCG has been widely used as tuberculosis (TB) vaccine because it preserves the immunogenic characteristics of TB (Britton & Palendira, 2003). It is the most commonly used vector for developing recombinant vaccines for other diseases, including malaria. BCG is an excellent vehicle for delivering recombinant antigens since it can be readily manipulated to express a range of recombinant proteins. However, mice immunized with BCG only stimulate nonspecific resistance against malaria infection (Murphy *et al*., 1981). 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 (Stover *et al*., 1994). Using this approach, our group was constructed a recombinant BCG expressing the MSP-1C of *P. falciparum* (BCG-MSP1C) in the previous study. Previous studies showed that the clone stimulated immune responses in animal and inflammatory responses in mouse and human macrophages (Nurul & Norazmi, 2011; Mohammad *et al*., 2014) through the activation of toll-like receptor 4 (TLR4) (Abbas & Suppian, 2019).

2.7 Immunity against malaria infection

2.7.1 Innate immunity

Innate immunity is an efficient way to control early infection of infectious agents such as mycobacteria. 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 (Artavanis *et al*., 2003). In humans, innate immune system mainly comprises of innate immune cells such as monocytes/macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, mast cells (MCs), eosinophils, basophils along with newly identified innate lymphoid cells (ILCs) and mucosal-associated invariant T and its humoral components that is circulating complement system proteins/components, cytokines and chemokines secreted by innate immune cells along with various antimicrobial peptides (AMPs). Innate immune cells express various pattern recognition receptors (PRRs) including Toll-like receptors (TLRs,) responsible for the recognition of (PAMPs) and induction of inflammatory immune response. Thus this recognition of pathogens by PRRs plays a very important role in the generation of an effective innate immune response (Vijay, 2018).

2.7.2 Pro-inflammatory cytokines

Pro-inflammatory cytokines are positive mediators of inflammation. In a wide variety of infections, such molecules are released as a host response due to inflammasome activation. This is popularly known as the pro-inflammatory cytokine response. Pro-inflammatory cytokines play a central role in inflammatory diseases of infectious or noninfectious origin. PAMPs and DAMPs trigger a cytokine

cascade that initially is composed of the pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, IFN-γ, IL-18, and TNF itself). These cytokines serve to contain and resolve the inflammatory foci through activation of local and systemic inflammatory responses. TNF also triggers a cytokine cascade of the anti-inflammatory cytokines that block pro-inflammatory cytokine synthesis, as well as cytokine inhibitors that block pro-inflammatory cytokine actions. In most cases the inflammatory response is successfully resolved. Overzealous production of cytokines or the inability to shut down pro-inflammatory cytokine production, however, can lead to increasing concentrations of cytokines in the systemic circulation ("cytokine storm"). This continued cytokine production can have a deleterious effect on the host, with the development of hypotension, intravascular thrombosis, pulmonary edema, and hemorrhage; if this process is left unchecked, it can lead to multiple organ failure and death. This condition often is referred to as the *systemic inflammatory response syndrome* (SIRS). This term describes the clinical manifestations of widespread endothelial inflammation that leads to increased vascular permeability. This condition is the initiating pathologic process in a group of diverse disorders, such as bacterial sepsis, ischemia, burn injury, trauma and tissue injury, and hemorrhagic shock (Srinivasan *et al*, 2017).

2.7.2 (a) Tumor necrosis factor (TNF-α)

Tumor necrosis factors-(TNF) α is cytokines that bind to common receptors on the uppermost layer of target cells and express some usual biological activities. Human TNF-α is of 17 and 25 kDa, respectively. Their corresponding cDNAs were cloned in 1984, and the genes encoding the factors have been plotted to chromosome 6 in humans, (Vilcek & Lee, 1991) inside the area of the major histocompatibility complex

(MHC). TNF-α, or cachectin, subsist as a trimer (Smith & Baglioni, 1987) and is a production of activated macrophages/monocytes, fibroblasts, mast cells, and some T and natural killer (NK) cells (Aggarwal, 1992; Beutler & Cerami, 1988).

TNF-α and IL-1 share some pro-inflammatory characteristics such as fever inducer, either via stimulation of PGE2 formation by the vascular endothelium of the hypothalamus, or via initiating secretion of IL-1 (Warren, 1990). Both cytokines can trigger the formation of collagenase and PGE2 via synovial cells and can cause joint problems in inflammatory situations like rheumatoid arthritis (Warren, 1990). TNF-α have the same vital inflammatory characteristics as IL-6 and IL-11. TNF-α and IL-1 exercise secondary inflammatory effects by triggering IL-6 formations in some cell types. IL-6 then arbitrates its own effects and those of TNF-α and IL-1 in initiating fever and the acute phase reaction, so sustaining the inflammatory reactions via a cascade of cytokines with overlapping characteristics. In general, the effects of cytokines are exercised locally at their production location (autocrine and paracrine).

TNF-α , IL-1, and IL-6 have main systemic effects when either synthesize acutely in huge amounts, such as in case of bacterial sepsis or chronically in small amounts, such as in the case of chronic infections. During sepsis with Gram-negative organisms, lipopolysaccharides (endotoxin) secreted from bacteria stimulate the broad formation of TNF-α by macrophages. The systemic secretion of these cytokines has been proved to be accountable for the fever and hypotension that manifest septic shock. Moreover, chronic formation of TNF-α is believed to be accountable for the metabolic alterations which caused in the cachexia.

2.7.2 (b) Interleukin 12

IL-12 family cytokine consists of four heterodimeric proteins, which are synthesised by myeloid cells. IL-12 consist of 2 subunits, IL-12p35 and IL-12p40, which combine form active IL-12p70. IL-12p70 is a strong activator of Th1 cell responses. IL-12p40 is also expressed and released as a monomer and a homodimer. The p40 monomer has no biological role but the homodimer is an antagonist of IL-12p70 by competitively binding the IL-12R. The IL-12p40 homodimer increases alloantigen-specific Th1 progression proposing a similar role activity to IL-12p70 under some situations. Its biological action involves the increase of cytotoxic T cells and lymphokine-activated killer (LAK) cell production and activation, elevates natural killer (NK) cell cytotoxicity, initiation of activated T cell and NK cell proliferation, initiation of IFN-g production by NK cells and T cells, and suppression of IgE synthesis by IL-4-stimulated lymphocytes through IFN-g-dependent and independent mechanisms. IL-12 is released by activated B cells, macrophages, and other antigen-presenting cells (APCs), but its generation is suppressed by IL-4 and IL-10. Moreover, the stimulatory effect of IL-12 on TH1 development is antagonized by IL-4, a cytokine which promotes TH2 cell development. Therefore, IL-12 acts as a vital role in cell-mediated inflammation and also helping in the regulation of immunoglobulin formation (Kalinski *et al*, 2003).

2.7.3 Toll-like receptor

TLRs have been considered evolutionarily conserved proteins and are essentially characterized by an extracellular leucine-rich repeat (LRRs) domain, which mediates recognition of PAMPs, a transmembrane domain along with its cytosolic or intracellular Toll/IL-1R-like (TIR) domains required for downstream signaling

pathways. Toll-like receptors (TLRs) are the PRRs that are expressed predominantly in the host antigen-presenting cells (APCs) and play crucial roles in deciding the fate of a pathogenic infection. To date, 11 functionally different TLRs have been identified in human and categorized into two groups such as transmembrane (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) and intracellular (TLR3, TLR7, TLR8 and TLR9) where they recognize microbial genetic material that is DNA or RNA. However, TLR2 and TLR4 are also present as intracellular TLRs in DCs, epithelial and endothelial cells. As these intracellular TLRs are present in vesicles or endosomal compartments so they do not come in contact with host cell-derived nucleic acids (DNA or RNA) under most physiologic conditions and do not cause activation of an innate immune response against self DNA or RNA (Vijay *et al*., 2016).

Intriguingly, the number of TLRs within the mammals (eg, mice possesses 12 TLRs) and their emergence through the course of evolution are not conserved. The receptors possess an extracellular domain comprising leucine-rich repeats (LRR) and an intracellular Toll-interleukin1 (IL-1) receptor (TIR) domain. LRR domain is typically involved in the recognition of distinct pathogen-associated molecular patterns (PAMPs), while the TIR domain plays a crucial role in transmitting the signal to elicit inflammatory responses. LRR and TIR are the two functionally important domains in the 3D structure of each TLR and the presence of conserve regions can be detected in both domains. However, the disposition of each TLR is different. The variations in TLR structure and function primarily result from species-specific co-evolution caused by gene conversion between the receptors resulting in the formation of heterodimers and gene duplications followed by deletions (Mukherjee *et al.*, 2015).

All TLRs expressed by host cells are synthesized in the endoplasmic reticulum (ER) and are transported to the Golgi complex and from there these TLRs are transported to either cell membrane or intracellular compartments (i.e. endosomes). The trafficking of intracellular TLRs to endosomes is controlled and regulated by a multi-pass transmembrane protein called UNC93B1 (Unc-93 homolog B1). The excessive activation of TLR7 is also controlled by UNC93B1 by employing TLR9 to counteract the exaggerated activation of TLR7. Protein associated with TLR4 (PRAT4A) is another ER-resident protein molecule controlling TLR trafficking of TLR1, TLR2, TLR4, TLR7 and TLR9 from ER to their site of location that is plasma membrane and endosomes. gp96 (a member of Hsp90 family) in ER acts as a general chaperone for most of TLRs including TLR1, TLR2, TLR4, TLR5, TLR7 and TLR9. Proteolytic cleavage of nucleic acid-sensing TLRs by Cathepsin B, S, L, H, and K and asparaginyl endopeptidase is required for the functional maturation of TLRs to recognize their competent ligands and mount an effective innate immune response. (Vijay *et al.,* 2016).

2.7.3 (a) Activation of TLR 4 with LPS

LPS is a complex glycolipid that represents a major constituent of the gram-negative bacterial cell wall. LPS consists of three major components: a hydrophilic solvent-exposed repeating oligosaccharide chain known as O-antigen, a membrane-proximal core oligosaccharide, and a membrane-embedded diglucosamine backbone that is attached to a varying number of acyl chains (Chandler and Ernst, 2017). This membrane-embedded structure is known as Lipid A and represents the specific component of LPS that stimulates TLR4. While TLR4 forms weak and minimal contact with LPS (Picard *et al*., 2010), picomolar concentrations of this PAMP are

capable of stimulating TLR4-dependent inflammatory responses in macrophages (Gioannini *et al*., 2004). For these high-sensitivity interactions to occur, additional LPS-binding proteins must act upstream of TLR4 (Gioannini *et al*., 2004; Ryu *et al*., 2017). The extracellular LPS binding protein (LBP) forms direct contact with the bacterial outer membrane (or micelles of LPS) and alters the outer membrane in a manner that facilitates the extraction of a single molecule of LPS by the protein CD14 (Gioannini *et al.*, 2004). CD14 can either exist as a soluble extracellular protein or a GPI-anchored protein embedded in the outer leaflet of the mammalian cell plasma membrane (Frey *et a*l., 1992; Lee *et al*., 1993; Tobias *et al*., 1986; Wright *et al*., 1990). Regardless of its soluble or membrane-bound positioning, CD14 acts to transfer a single molecule of LPS to the protein MD2 (Gioannini *et al*., 2004). MD2 is a small protein that interacts stably with the ectodomain of TLR4, forming TLR4-MD2 heterodimers that represent the functional LPS receptor (Schromm *et al*., 2001; Shimazu *et al*., 1999). Upon CD14-mediated transfer of LPS to MD2, TLR4 dimerization occurs (Akashi *et al*., 2000). Structural analysis of this process explained the molecular basis of TLR4 dimerization, as acyl chains within the Lipid A region of LPS interact with distinct regions of two TLR4-MD2 heterodimers (Park *et al*., 2009). Five of the six acyl chains present in hexacylated Lipid A interact with a hydrophobic pocket present in the MD2 component of a TLR4-MD2 heterodimer. The sixth acyl chain does not interact with the TLR4 component of this heterodimer, but rather interacts with a different TLR4 molecule. LPS structures that contain less than six acyl chains have minimal ability to crosslink distinct sets of TLR4-MD2 heterodimers, thus explaining their weakened inflammatory activities (Park *et al*., 2009).

2.8 TLR molecular signaling

Molecular signals originating from TLR activation are primarily transmitted via two core signaling pathways that result in the activation of nuclear factor-κB (NF-κB) or the mitogen-activated protein kinases (MAPKs). These two diverse pathways are operated through myeloid differentiation primary response protein 88 (MyD88)-dependent and MyD88-independent pathways.

2.8.1 Protein 88 (MyD88) -dependent

MyD88 interacts with interleukin-1 receptor (IL-1R)-associated kinase (IRAK)-4 and activates IRAK-4 which further actuates rest of the IRAK family proteins like IRAK-1 (Figure 2.2). Activated IRAKs also interact with tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6). TRAF6 is an E3 ubiquitin protein ligase that functions with an E2 ubiquitin-conjugating enzyme complex (Ubc) comprising Ubc13 and Uev1A.18 TRAF6-Ubc13 complex catalyses the formation of an isopeptide bond between the carboxyl terminus of one ubiquitin and the ε-amino group of lysine 63 (K63) of the adjacent ubiquitin molecule to form a polyubiquitin chain. This unconjugated K63 polyubiquitin chain activates the complex comprising TGF-β activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and phosphorylates IKK (inhibitor of kappa B kinase) (IKK-α, IKK-β, IKK-γ) and MAP kinase.19 IKK-γ, that is, NF-κB essential modulator (NEMO), activates NF-κB by phosphorylating the inhibitory subunit of NF-κB (IkB). Activated NF-κB enters into the nucleus and induces the expression of proinflammatory cytokine genes by inviting RNA polymerase II at the promoter site. On the other side, transcription factor complex, activator protein-1 (AP-1), gets activated through the MAP kinase cascade and triggers cytokine expression. (Mukherjee *et al.,* 2015).

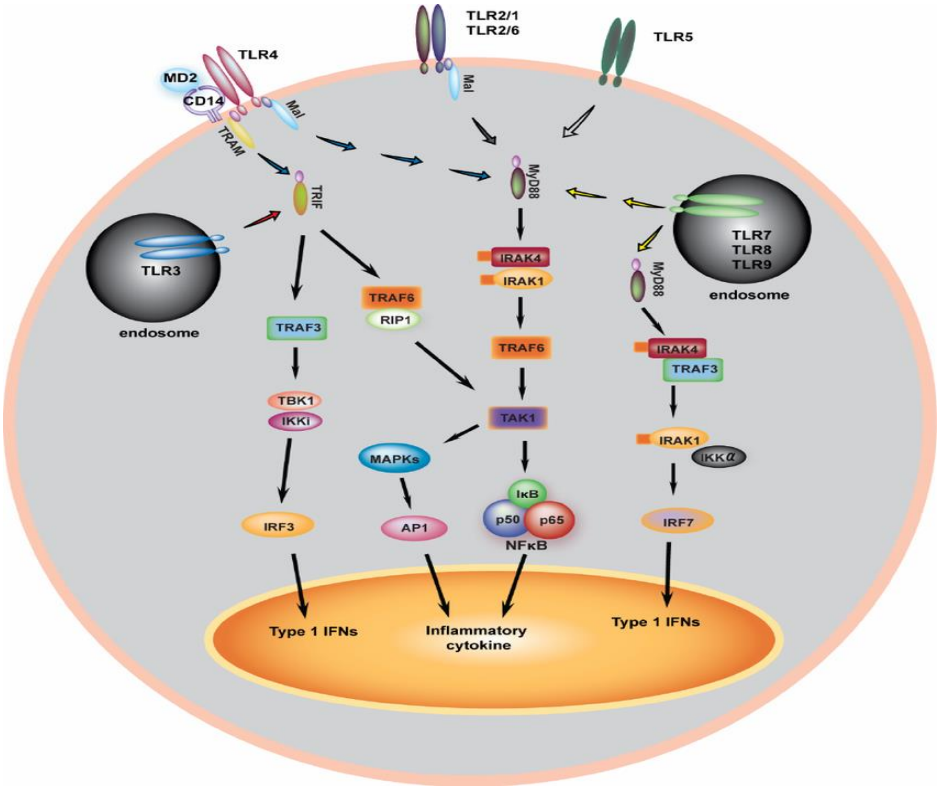

Figure 2.2 Protein 88 (MyD88) -dependent pathway. The figure is adapted from Yuk and Jo, (2011).