THE EFFECT OF TLR4 AGONIST (CRX-527) ON IL-10 PRODUCTION IN MICE IMMUNIZED WITH BCG-MSP-1C

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Dissertation submitted in partial fulfillment of the requirements for the degree of Bachelor of Health Science (Honours) (Biomedicine)

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DECLARATION

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

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(TABASSUM IRIN ZAMAN)

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µL	Microliter
mL	Milliliter
µg/mL	Microgram per milliliter
ANOVA	Analysis of Variance
BCG	*Bacillus Calmette-Guérin*
BCG-MSP-1C	*Bacillus Calmette-Guérin* - Merozoite Surface Protein 1C
CDC	Centers for Disease Control and Prevention
CSP	Circum-sporozoite Protein
CO2	Carbon Dioxide
EBP	Enhancer Binding Protein
DCs	Dendritic Cells
DIC	Disseminated Intravascular Coagulation
EBA	Erythrocyte Binding Antigen
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
ESAT-6 Region	Early Secreted Antigenic Target 6 Region
F2R	Coagulation Factor II (Thrombin) Receptor
FBS	Fetal Bovine Serum

Foxp3	Forkhead Box Protein P3
GC	Germinal Center
GPI	Glycosylphosphatidylinositol
H2SO4	Sulfuric Acid
ICAM1	Intercellular Adhesion Molecule 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFNs	Interferons
iNOS	Inducible Nitric Oxide Synthase
IL	Interleukin
iRBCs	Infected Red Blood Cells
ITNs	Insecticide-Treated Nets
IACUC	Institutional Animal Care and Use Committee
K13	Kelch 13
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MAVS	Mitochondrial Antiviral Signaling Protein
MBCs	Memory B Cells
MD-2 receptor	Myeloid Differentiation Protein 2 receptor
MDA5	Melanoma Differentiation-Associated Protein 5
MEK	Mitogen-Activated Protein Kinase Kinase
MHC	Major Histocompatibility Complex
MSP	Merozoite Surface Protein
MyD88 pathway	Myeloid Differentiation Primary Response 88 Pathway

NF-kB	Nuclear Factor Kappa B
NK Cells	Natural Killer Cells
NO	Nitric Oxide
Nos2	Nitric Oxide Synthase 2
P38	P38 Mitogen-Activated Protein Kinase
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
P.falciparum	*Plasmodium falciparum*
p-value	Probability Value
PBS	Phosphate-Buffered Saline
PRRs	Pattern Recognition Receptors
RBC	Red Blood Cell
RAP 1	Repressor/Activator Protein 1
RIG-I	Retinoic Acid-Inducible Gene I
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RTS,S/AS01	Recombinant Sporozoite Protein-based Vaccine with AS01 Adjuvant
R21	Recombinant Sporozoite Protein-based Vaccine with Matrix-M Adjuvant
SES	Socioeconomic Status
STEVOR	Sub-telomeric Variable Open Reading Frame Antigen

T cells	T Lymphocytes
TAK-242	Toll-Like Receptor 4 Inhibitor
Tfh	Follicular Helper T Cells
Th cells	T Helper Cells
TLR	Toll-Like Receptor
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-β
Treg	Regulatory T Cells
Tr1	Type 1 Regulatory T Cells
T-20	Tween-20

KESAN AGONIS TLR4 (CRX-527) TERHADAP PENGELUARAN IL-10 PADA TIKUS YANG DIIMUNISASI DENGAN BCG-MSP-1C

ABSTRAK

Malaria, yang disebabkan oleh parasit *Plasmodium*, masih menjadi beban kesihatan global yang signifikan, terutamanya di kawasan dengan akses terhad kepada penjagaan kesihatan. Vaksin malaria semasa menghadapi cabaran seperti keberkesanan yang tidak optimum dan halangan logistik untuk pelaksanaan secara meluas. Salah satu antigen yang berpotensi untuk pembangunan vaksin malaria ialah domain terminal-C merozoit surface protein 1 (MSP-1C), iaitu bahagian terpelihara daripada MSP-1, protein permukaan utama *Plasmodium* merozoit yang penting untuk pencerobohan sel darah merah dan pengelakan tindak balas imun hos. Kajian ini menilai kesan agonis reseptor Toll-like 4 (TLR4), CRX-527, terhadap pengeluaran IL-10 pada tikus yang diimunisasi dengan vaksin BCG-MSP-1C, yang dihasilkan di makmal kami dengan mengkonjugasi antigen MSP-1C dengan vaksin Bacillus Calmette–Guérin (BCG), yang secara utama digunakan untuk pencegahan tuberkulosis (TB). Kumpulan rawatan termasuk PBS-T80, LPS, BCG, dan BCG-MSP-1C. Hasil kajian menunjukkan peningkatan signifikan dalam pengeluaran IL-10 dalam semua kumpulan, baik dalam sampel hati mahupun nodus limfa, dengan kehadiran CRX-527. Antara kumpulan ini, kumpulan BCG-MSP-1C menunjukkan tahap IL-10 tertinggi, diikuti oleh BCG, LPS, dan PBS-T80. IL-10, sitokin anti-radang, memainkan peranan penting dalam mengawal tindak balas imun, memastikan pengawalan jangkitan yang berkesan sambil mengurangkan kerosakan tisu. Penemuan ini menonjolkan potensi CRX-527 sebagai adjuvan imunomodulator yang berkesan untuk strategi vaksin malaria. Penggabungan CRX-527 dengan vaksin BCG-MSP-1C menawarkan pendekatan imunisasi berganda, yang memungkinkan perlindungan

terhadap kedua-dua malaria dan tuberkulosis dengan satu dos imunisasi. Strategi ini berpotensi mengurangkan beban keperluan dos vaksin berganda dan meningkatkan liputan imunisasi di kawasan yang mempunyai sumber terhad. Kajian ini menggariskan potensi CRX-527 dalam meningkatkan keberkesanan vaksin dan mempromosikan imuniti jangka panjang, menyokong peranannya dalam memajukan pelaksanaan vaksin dwi-tujuan yang menjimatkan kos untuk kawasan endemik.

THE EFFECT OF TLR4 AGONIST (CRX-527) ON IL-10 PRODUCTION IN MICE IMMUNIZED WITH BCG-MSP-1C

ABSTRACT

Malaria, caused by the *Plasmodium* parasite, continues to be a significant global health burden, particularly in regions with limited access to healthcare. Current malaria vaccines face challenges of suboptimal efficacy and logistical barriers for widespread implementation. One promising antigen for malaria vaccine development is the C-terminal region of merozoite surface protein 1 (MSP-1C), a conserved fragment of MSP-1, the major surface protein of *Plasmodium* merozoites, which is essential for red blood cell invasion and evasion of host immune responses. This study evaluates the impact of the Toll-like receptor 4 (TLR4) agonist CRX-527 on IL-10 production in mice immunized with the BCG-MSP-1C vaccine, developed in our laboratory by cloning the MSP-1C antigen with the *Bacillus Calmette–Guérin* (BCG) vaccine, which is primarily used for tuberculosis (TB) prevention. Treatment groups included PBS-T80, LPS, BCG, and BCG-MSP-1C. The results revealed a significant enhancement of IL-10 production across all groups in both liver and lymph node supernatant samples in the presence of CRX-527. Among these, the BCG-MSP-1C group exhibited the highest IL-10 levels, followed by the BCG, LPS, and PBS-T80 groups. IL-10, an anti-inflammatory cytokine, plays a pivotal role in regulating the immune response, ensuring controlled infection management while minimizing tissue damage. These findings highlight CRX-527's potential as an effective immune-modulatory adjuvant for malaria vaccine strategies. The incorporation of CRX-527 with the BCG-MSP-1C vaccine offers a dual immunization approach, enabling protection against both malaria and tuberculosis with a single immunization. This strategy could significantly reduce the burden of multiple vaccine

doses and improve immunization coverage in resource-limited settings. The study underscores the potential of CRX-527 to enhance vaccine efficacy and promote long-term immunity, supporting its role in advancing cost-effective, dual-purpose vaccine solutions for endemic regions.

CHAPTER ONE: INTRODUCTION

1.1 Study Background

Malaria is a life-threatening parasitic disease primarily transmitted to humans through the bites of infected female *Anopheles* mosquitoes. In rare cases, malaria can also spread through blood transfusion or the use of contaminated needles. It is endemic to tropical and subtropical regions and persists as a major public health challenge, despite being both preventable and treatable (World Health Organization, 2024).

Malaria is caused by five distinct protozoan species: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*, with *P. falciparum* and *P. vivax* posing the greatest threats to global health. The remaining species, *P. malariae*, *P. ovale*, and *P. knowlesi*, also contribute to the global malaria burden but are less commonly implicated in severe cases (World Health Organization, 2024). The morbidity and mortality associated with malaria result from the parasite's invasion of erythrocytes, leading to hemolysis. In *P. falciparum* infections, the sequestration of infected red blood cells within the microvasculature of critical organs further exacerbates disease severity. Most malaria-related deaths are attributed to *P. falciparum* in sub-Saharan Africa, whereas *P. vivax*, more common in Asia and South America, predominantly causes anemia due to relapsing infections (Poespoprodjo *et al*., 2023).

Malaria represents a major public health challenge, putting almost half of the global population at risk of infection. In several malaria-endemic countries, it is the primary

cause of mortality. According to the World Health Organization (WHO), there were 249 million malaria cases in 2022, a slight increase from 244 million in 2021 (World Health Organization, 2024).

Malaria presents with a range of symptoms, typically emerging 10 to 15 days after an individual is bitten by an infected *Anopheles* mosquito. Early symptoms often include fever, headache, and chills. For individuals with prior malaria infections, these symptoms may appear mild, leading to delays in seeking medical care. Given the nonspecific nature of these early manifestations, prompt diagnostic testing is crucial for timely intervention (World Health Organization, 2024).

Effective treatment of malaria is critical for reducing disease severity, preventing fatalities, and limiting transmission. The World Health Organization (WHO) emphasizes the need for early diagnosis, recommending that all suspected cases undergo parasite-based diagnostic testing, either through microscopy or rapid diagnostic tests. As a serious infection, malaria requires prompt medical intervention with appropriate antimalarial medications tailored to the specific characteristics of the case. Treatment strategies vary depending on factors such as the *Plasmodium* species responsible for the infection, the presence of drug resistance, the patient's age or weight, and pregnancy status (Centers for Disease Control and Prevention, 2024a)

Artemisinin-based combination therapies (ACTs) have been the frontline treatment for malaria and have significantly contributed to reducing the disease burden. However, their reliability is now undermined by the emergence and spread of artemisinin resistance, first

reported in Cambodia in 2008. Resistance has since expanded across the Greater Mekong sub-region, posing a major challenge to malaria control efforts. Unlike resistance to other antimalarial drugs, such as chloroquine, artemisinin resistance does not show a marked increase in the in vitro inhibitory concentration (IC50) of resistant strains compared to sensitive ones. Instead, it is characterized by delayed parasite clearance in vivo and the ability of resistant parasites to enter a temporary quiescent state in vitro. This resistance is primarily linked to mutations in the Kelch propeller domain of the *Plasmodium falciparum* K13 (pfK13) gene. The reduced efficacy of artemisinin places additional stress on the partner drugs used in combination therapies, increasing the likelihood of resistance to these drugs and treatment failures. ACT failure is now recognized as a combination of reduced susceptibility to artemisinin and resistance to partner drugs, highlighting the urgent need for alternative treatments and interventions (Erhunse and Sahal, 2020).

Malaria vaccine development has been ongoing since the 1960s, with significant advancements made over the past decade. A landmark moment occurred on October 6, 2021, when the World Health Organization (WHO) recommended the widespread use of the RTS,S/AS01 (RTS,S) vaccine for children in sub-Saharan Africa and other areas with moderate to high transmission of *P. falciparum* malaria. Two years later, the WHO approved a second malaria vaccine, R21/Matrix-M, for use in countries where malaria is endemic (Centers for Disease Control and Prevention, 2024b).

Despite the promising safety profiles and ability to reduce disease severity, the RTS,S/AS01 malaria vaccine has significant limitations. While it offers some protection,

it does not provide full sterile immunity, requiring three booster doses to achieve a reasonable level of efficacy. Moreover, the vaccine's immunogenicity is insufficient to prevent the survival of *Plasmodium* gametocytes, which allows the parasite to continue circulating in the population and be transmitted through mosquitoes, thereby maintaining malaria's endemicity. Additionally, the efficacy of RTS,S is significantly influenced by the socioeconomic status (SES) of the vaccine recipients, with individuals from higher SES households showing better protection. This discrepancy further contributes to the immunity gap, especially in regions where malaria is most prevalent, as approximately 95% of malaria cases and 96% of deaths occur in lower SES areas (Zakaria *et al*., 2024). Lower SES households face higher malaria exposure due to poor housing, inadequate sanitation, and limited healthcare access, reducing vaccine efficacy (Gyaase *et al*., 2021).

These challenges with the RTS,S vaccine underscore the ongoing need for more effective malaria vaccines. The search for a more potent vaccine remains a key focus in the global effort to combat malaria. Among the various candidates for malaria vaccination, the merozoite surface protein 1 (MSP1) has shown considerable promise. MSP1 is a crucial protein involved in the invasion of red blood cells by the *Plasmodium* parasite, with its C-terminal domain (MSP-1C) identified as a critical target for vaccine development due to its conserved structure and essential role in the parasite’s invasion mechanisms (Mazumdar *et al*., 2009). A promising strategy for advancing vaccine development involves the use of recombinant *Mycobacterium bovis Bacillus Calmette-Guérin* (rBCG) to express malaria antigens, including merozoite surface protein (MSP). MSP1-based vaccines have demonstrated safety, good tolerability, and strong immunogenicity. The development of recombinant vaccines using live bacteria, like BCG, is becoming

increasingly popular, with the BCG-MSP-1C vaccine being one candidate targeting malaria blood-stage infections. This recombinant vaccine aims to stimulate both cellular and humoral immune responses, offering hope for more durable and effective malaria prevention strategies (Zakaria *et al*., 2024).

1.2 Problem Statement

Malaria remains a global health challenge, particularly in regions with limited access to healthcare. Existing malaria vaccines demonstrate limited efficacy in vulnerable age groups, and often necessitate multiple booster doses to achieve moderate immunogenicity. These requirements impose logistical and economic challenges for a large-scale implementation. Additionally, the emergence of *P. falciparum* strains resistant to artemisinin-based combination therapies due to mutations in the Kelch propeller domain of the *P. falciparum* K13 (*pfk13*) gene further undermines current treatment strategies.

The purpose of this study is to address the urgent need for a malaria vaccine capable of eliciting strong and long-lasting immune responses across all age groups. Given the significant morbidity and mortality associated with the disease, this research also seeks to explore more efficient and less painful immunization strategies to reduce its global burden.

1.3 Rationale of Study

Malaria continues to pose a major global health burden, particularly in endemic regions, where it remains a leading cause of morbidity and mortality. In response to this persistent challenge, innovative vaccine strategies are urgently needed. This study explores the development of a recombinant BCG-based vaccine targeting the MSP-1C antigen of *Plasmodium* falciparum, with the potential for dual immunization against tuberculosis (TB) and malaria. *Mycobacterium bovis Bacillus Calmette-Guérin* (BCG), a widely used vaccine for TB, serves as an effective delivery vector due to its established safety profile and its capacity to provide non-specific protection against various pathogens.

The MSP1-based recombinant vaccine targeting malaria blood-stage infections was developed in our laboratory. The C-terminus of the merozoite surface protein-1 (MSP-1C) from *Plasmodium falciparum* was integrated into the genome of the BCG clone to create the BCG-MSP-1C vaccine. Previous studies have demonstrated that BCG-based vaccines can elicit both innate and adaptive immune responses, potentially through the modulation of TLR signaling pathways.

This study specifically investigates the role of the TLR4 agonist CRX-527 in enhancing the immune response to the BCG-MSP-1C vaccine, with a particular focus on IL-10 production. IL-10 is a cytokine critical for regulating immune responses to malaria, and its modulation could have significant implications for vaccine efficacy. However, the effect of CRX-527 on IL-10 production in this context remains unexplored.

By broadening our understanding of the immune mechanisms involved in TLR4 activation and its influence on IL-10 production in the context of recombinant BCG vaccines, this research aims to contribute to the development of a single vaccine providing protection against both TB and malaria. Such an approach could reduce the need for multiple immunizations, offering a more efficient and less invasive solution, particularly for children in endemic regions. This represents a promising advancement in global vaccination strategies, therefore providing safer and more accessible immunization options.

1.4 Objectives

1.4.1 General Objective

1. To investigate the effects of CRX-527 as a TLR4 agonist on Th1 and Th2 cytokine production in mice immunized with BCG-MSP-1C.

1.4.2 Specific Objectives

i. To evaluate the effects of CRX-527, a TLR4 agonist, on the production of IL-10 in liver cell supernatants of mice immunized with BCG-MSP-1C.

ii. To evaluate the effects of CRX-527, a TLR4 agonist, on the production of IL-10 in lymph node supernatants of mice immunized with BCG-MSP-1C.

1.5 Hypothesis

1.5.1 Null Hypothesis (H0)

Stimulation with the TLR4 agonist CRX-527 does not lead to an increase in the production of IL-10 in mice immunized with BCG-MSP-1C vaccine.

1.5.2 Alternative Hypothesis (HA)

Stimulation with the TLR4 agonist CRX-527 will lead to an increase in the production of IL-10 in mice immunized with BCG-MSP-1C vaccine.

1.6 Significance of study

The development of an effective malaria vaccine remains a critical global health priority, particularly in malaria-endemic regions. This study explores a novel approach by utilizing *Mycobacterium bovis Bacillus Calmette-Guérin* (BCG) as a delivery vector for the *Plasmodium falciparum* MSP-1C antigen. BCG, widely recognized for its use as a tuberculosis (TB) vaccine, is an ideal candidate for dual-purpose vaccine development due to its ability to elicit robust innate and adaptive immune responses and its non-specific protective effects against various pathogens.

This research is significant as it focuses on the immunological mechanisms that enhance the immune response to the BCG-MSP-1C vaccine, particularly through Toll-like receptor 4 (TLR4) signaling. While prior studies have shown that the BCG-MSP-1C vaccine can trigger immune responses, the specific role of TLR4 activation in modulating these responses remains underexplored. This study investigates the use of CRX-527, a TLR4 agonist, to enhance cytokine production, with a particular emphasis on IL-10. IL-10 plays a critical role in malaria immunity by regulating the balance between pro-inflammatory and anti-inflammatory responses, yet its role in the context of CRX-527-mediated TLR4 activation has not been thoroughly investigated.

Furthermore, the dual-purpose nature of this vaccine holds the potential to address the burden of multiple immunizations, offering a more efficient and less invasive solution, particularly for children in regions co-endemic for malaria and tuberculosis. The findings from this study could not only advance our understanding of IL-10's role in vaccine-

mediated immunity but also contribute to the development of accessible and cost-effective vaccines that are better suited for resource-limited settings.

1.7 Study Flowchart

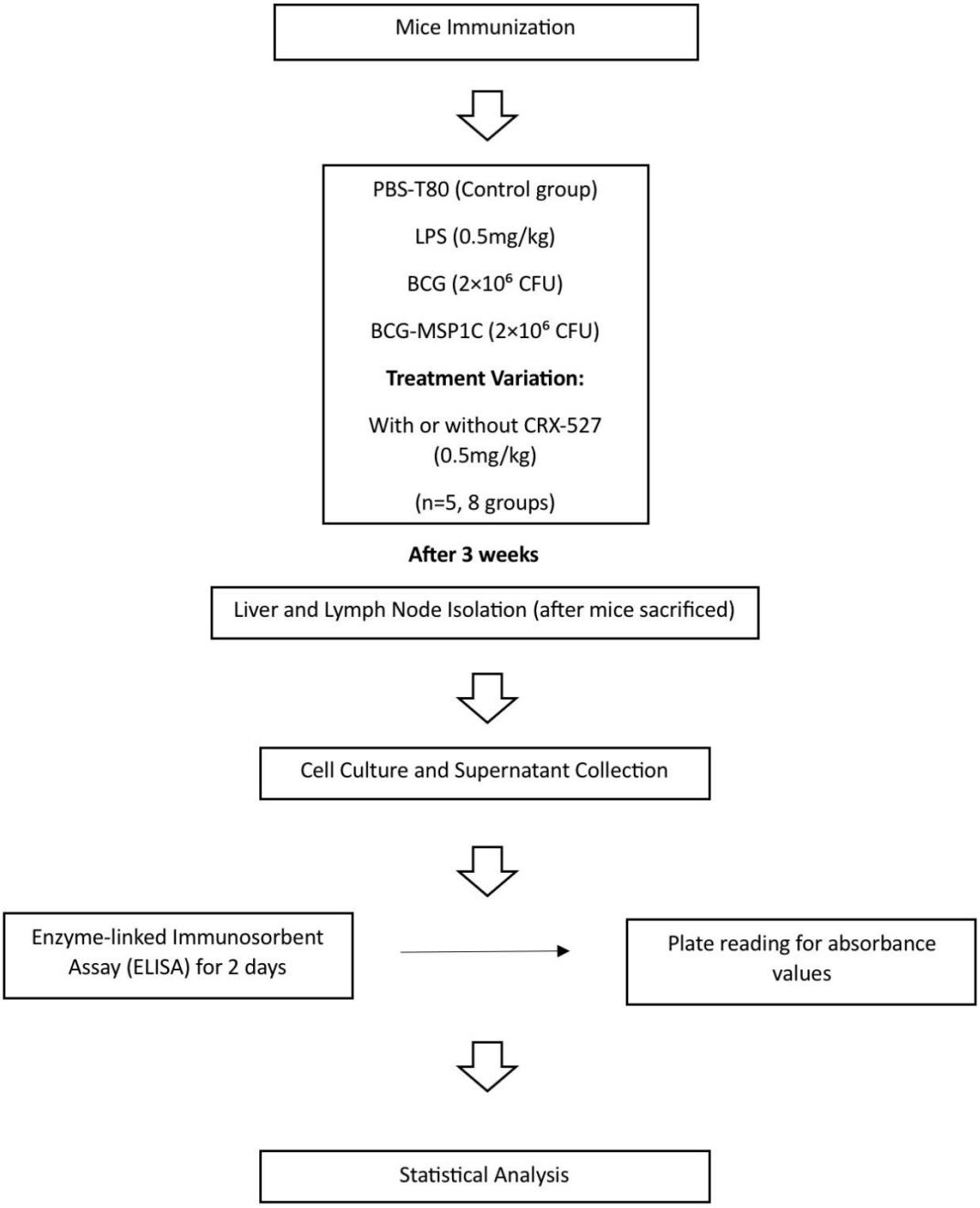

Figure 1.0: Study Flowchart

CHAPTER TWO: LITERATURE REVIEW

2.1 Prevalence of malaria

2.1.1 Global malaria prevalence

According to the most recent World Malaria Report, the number of malaria cases reached 263 million in 2023, an increase from 252 million in 2022. The estimated number of deaths due to malaria was 597,000 in 2023, slightly lower than the 600,000 recorded in 2022 (World Health Organization, 2024).

The WHO African Region continues to bear a disproportionate share of the global malaria burden. In 2023, approximately 94% of all malaria cases and 95% of malaria-related deaths occurred in this region. Children under the age of five represented around 76% of the malaria fatalities in the area. The majority of these deaths were concentrated in four countries: Nigeria (30.9%), the Democratic Republic of the Congo (11.3%), Niger (5.9%), and the United Republic of Tanzania (4.3%) (World Health Organization, 2024).

Africa remains the most heavily impacted region by malaria due to several interconnected factors: The presence of highly efficient mosquito vectors, such as those in the *Anopheles gambiae* complex, facilitates high transmission rates. The dominant parasite species, *Plasmodium falciparum*, is particularly associated with severe malaria and increased mortality. Favorable local climate conditions often enable malaria transmission throughout the year. Limited resources and socio-

economic challenges have obstructed the effective implementation of malaria control measures. While malaria is less of a leading cause of death in other regions, it still leads to significant morbidity and disability, particularly in certain countries in South America and South Asia (Centers for Disease Control and Prevention, 2024c).

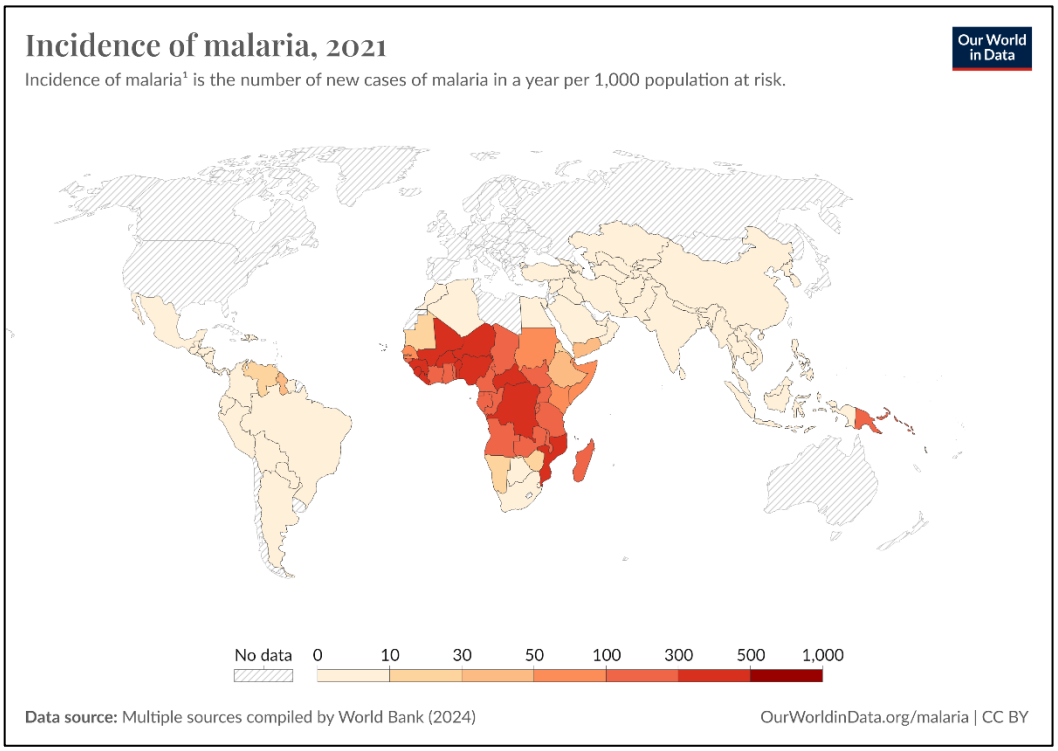

Figure 2.1: Global malaria incidence 2021 (Roser and Ritchie, 2024)

2.1.2. Malaria prevalence in Malaysia

Between 2013 and 2017, Malaysia reported a total of 16,500 malaria cases, with the majority of cases originating from Sabah (7,150; 43.3%) and Sarawak (5,684; 34.4%). The majority of affected individuals were male (13,552; 82.1%). In Peninsular Malaysia, the most frequently affected age group was 20 to 29 years (1,286; 35.1%), while Sabah and Sarawak saw the highest number of cases in the 30 to 39 age group (2,776; 21.6%). The predominant ethnic groups in Sabah and Sarawak were Bumiputera Sabah (5,613; 43.7%) and Bumiputera Sarawak (4,512; 35.1%), while in Peninsular Malaysia, the most common groups affected were other ethnicities (1,232; 33.6%) and Malays (1,025; 28.0%). *Plasmodium knowlesi* was the most common species in Sabah and Sarawak (9,902; 77.1%), while *Plasmodium vivax* was more prevalent in Peninsular Malaysia (1,548; 42.2%) (Hussin *et al.*, 2020).

The overall average incidence rate of malaria in Malaysia from 2013 to 2017 was 0.106 per 1,000 population, with a mortality rate of 0.030 per 100,000 population and a case fatality rate of 0.27%. Sarawak had the highest average incidence rate at 0.420 per 1,000 population, followed by Sabah at 0.383 per 1,000. Other states in Peninsular Malaysia reported incidence rates below the national average, with less than 0.100 per 1,000 (Hussin *et al.*, 2020).

Malaysia has seen a rise in zoonotic malaria cases caused by *Plasmodium knowlesi.* The number of *P. knowlesi* cases increased from 1,960 to over 4,000 between 2016 and 2018. Although this figure slightly decreased in 2019 and 2020 (to 3,213 and

2,609 cases, respectively), *P. knowlesi* infections still led to 6 and 5 fatalities in 2019 and 2020, respectively (Ali *et al.,* 2022).

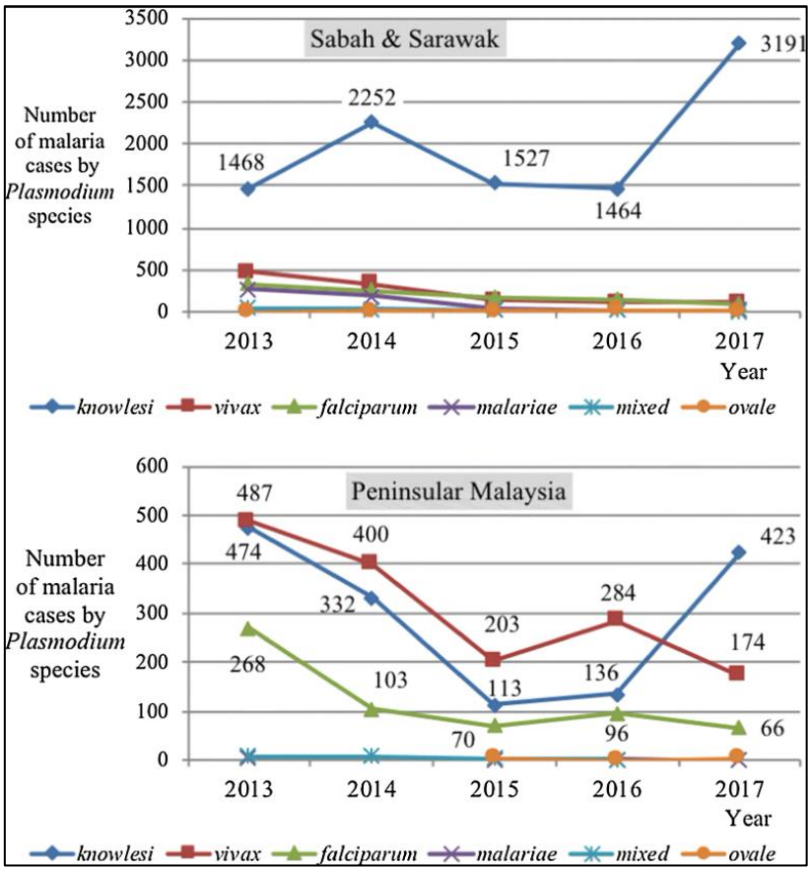

Figure 2.2: Malaria prevalence in Malaysia from 2013-2017 (Hussin *et al.*, 2020)

2.2 Pathogenesis of malaria

The lifecycle of *Plasmodium* species plays a crucial role in understanding the pathology and transmission dynamics of malaria, providing essential insights for disease control and intervention strategies. All *Plasmodium* species exhibit a similar lifecycle, consisting of two main phases: the initial phase, where the parasite infects a vertebrate host, such as a human, and the subsequent phase, where it is transmitted from an infected host to another through an insect vector (Sato, 2021). According to a study, the lifecycle begins in the first phase when sporozoites, produced within the insect vector, are introduced into the bloodstream of a vertebrate host through a mosquito bite. After being deposited in the dermis, these sporozoites swiftly travel to the liver, where they invade hepatocytes and multiply extensively in a process known as schizogony. Consequently, this results in the formation of merozoites, which re-enter the bloodstream and proceed to infect erythrocytes. Within each erythrocyte, a merozoite replicates asexually, producing between 8 and 64 merozoites, depending on the species. These newly formed merozoites are released into the bloodstream, continuing this intraerythrocytic cycle every 48 hours for *P. falciparum*. A proportion of merozoites then transition into the gametocyte stage, which is crucial for the continuation of the lifecycle. This transition represents the conclusion of the *Plasmodium* lifecycle within the human host, as the gametocytes prepare for sexual reproduction upon ingestion by a mosquito. Therefore, the progression of the lifecycle depends on the completion of the sexual phase in the mosquito's midgut, following the ingestion of an infected blood meal, where male and female gametes fuse (Zekar and Sharman, 2023).

A research study centered on the process of meiotic division of malarial parasite demonstrated that the second phase of the *Plasmodium* lifecycle begins when a female Anopheles mosquito ingests a blood meal containing gametocytes from an infected vertebrate host. Within the mosquito's midgut, male and female gametocytes differentiate into microgametes and macrogametes, respectively. Subsequently, the microgamete fertilizes the macrogamete to form a diploid zygote, which soon undergoes meiosis and transforms into a motile ookinete. The ookinete penetrates the midgut wall, forming an oocyst on the outer surface. Within the oocyst, several mitotic divisions occur, producing numerous sporozoites through a process called sporogony. As a result, upon maturation, the oocyst ruptures, releasing sporozoites into the mosquito's haemolymph, which migrate to the salivary glands. This phase of the lifecycle, from gametocyte ingestion to sporozoite maturation, typically completes in 10 to 18 days (Sinden and Hartley, 1985).

A study focused on the liver stage of *Plasmodium* species showed that, upon transmission to a human host through a mosquito bite, the sporozoites enter the bloodstream, quickly migrating to the liver, where they infect hepatocytes and develop as liver schizonts. Through a process called schizogony, the parasites replicate within hepatocytes and release motile merozoites into the bloodstream, where they invade red blood cells (RBCs). Consequently, the lifecycle continues with successive asexual replication cycles, progressing through ring, trophozoite, and schizont stages, each generating daughter merozoites that infect new RBCs, leading to a rapid increase in parasite load (Prudêncio *et al.,* 2006).

According to Meibalan and Marti (2016), *P. falciparum* is notable for producing high quantities of blood-stage parasites and for altering the surface of infected RBCs to promote "stickiness." This modified adhesive phenotype enables sequestration within small and mid-sized vessels, therefore allowing the parasite to avoid splenic clearance. However, this sequestration also damages endothelial cells and obstructs microvascular blood flow. (Meibalan and Marti, 2016). In contrast, during the asexual blood stage of *Plasmodium falciparum*, infected erythrocytes express a range of variant surface antigens (VSAs) on their membranes. These antigens play a critical role in the pathogenesis of malaria by facilitating immune evasion. The primary VSA, PfEMP1, along with other protein families such as RIFIN and STEVOR, interact with host receptors, including EPCR, ICAM1, and CD36. These interactions result in the sequestration of infected erythrocytes in microvascular structures, particularly in organs such as the brain, where they obstruct blood flow and hinder parasite clearance by the spleen. The formation of rosettes, where uninfected erythrocytes surround infected ones, further exacerbates microvascular blockage, promoting parasite survival and disease progression (Gonzales *et al.*, 2020). A subset of intra-erythrocytic parasites undergoes sexual differentiation, producing male and female gametocytes, which, upon ingestion by a mosquito, restart the cycle of transmission by enabling fertilization, ookinete formation, and eventual sporozoite production in the mosquito (Zekar and Sharman, 2023).

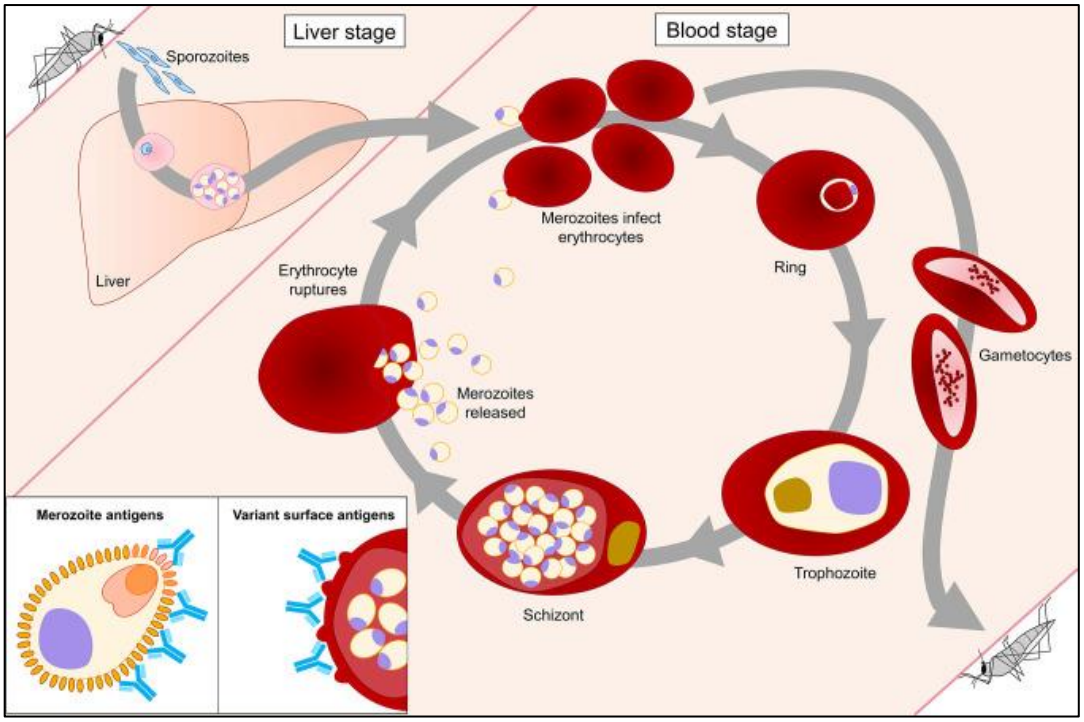

Figure 2.3: Pathogenesis of Malaria (Gonzales *et al.*, 2020)

2.3 Immune responses against Malaria

Malaria immunity involves a combination of innate, adaptive, humoral, and acquired responses to combat *Plasmodium* infection. The innate immune system provides an immediate, non-specific defense, while the adaptive immune system generates a targeted response involving T cells, B cells, antibodies, and memory cells that ensure long-lasting immunity.

2.3.1 Innate Immunity against Malaria

A study by Pohl and Cockburn (2022) highlighted the importance of innate immune response in the initial response to *Plasmodium falciparum* infection. Upon *Plasmodium* transmission, sporozoites are predominantly cleared by the innate immune system, with only a fraction reaching the liver. Dendritic cells (DCs) in draining lymph nodes are instrumental in presenting antigens to T cells, essential for protective immunity, while gamma-delta T cells (γδT cells) contribute to early immune responses. At the liver stage, type I interferons (IFNs) are induced, facilitating the recruitment of natural killer (NK) cells and aiding parasite control. This process involves the mitochondrial antiviral-signaling protein (MAVS) pathway, activated by RNA sensors such as melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene I (RIG-I), which is critical for type I IFN production. Additionally, autophagy plays a role in clearing parasites from hepatocytes (Pohl and Cockburn, 2022).

Moreover, the study addressed the role of Toll-like receptor 2 (TLR2) and how it detects sporozoite surface patterns and triggers inflammatory cytokine production. In TLR2-deficient models, liver-stage parasite burdens are elevated, underlining its significance. During blood-stage infection, parasite-derived ligands, including glycosylphosphatidylinositols (GPIs) and hemozoin, activate immune cells, leading to pro-inflammatory cytokine release. TLR2, along with RNA-sensing receptors like MDA5 and TLR8, enhances immune activation, collectively supporting an effective innate immune response to malaria (Pohl and Cockburn, 2022).

2.3.2 Adaptive Immunity against Malaria

I. Cell-mediated immunity against Malaria

Cell-mediated immunity is essential in combating malaria, particularly during the pre-erythrocytic stage of *Plasmodium falciparum* infection. CD8+ T cells play a key role in protection at this stage, as they recognize parasite-derived peptides presented by MHC class I molecules on infected hepatocytes. These cells exert their effects through cytotoxic activity, the release of cytokines such as interferon-gamma (IFN-γ), and the induction of nitric oxide production, which collectively eliminate infected hepatocytes. CD4+ T cells also contribute to pre-erythrocytic immunity by recognizing parasite peptides presented by MHC class II molecules and providing helper signals. However, the limited number of sporozoites inoculated during natural infections may not always induce a robust T-cell response, and experimental evidence suggests that achieving strong sterile immunity often requires high doses of sporozoites (Seder *et al.*, 2013).

During the erythrocytic stage, T cells play a less direct role in parasite clearance, as infected red blood cells (RBCs) lack MHC molecules. Nonetheless, antigen-specific αβ T cells are primed, and low levels of cytokines like IFN-γ and tumor necrosis factor-alpha (TNF-α), produced by γδ T cells, NK cells, and macrophages, help reduce parasitemia. CD8+ and CD4+ T cells are more prominently involved in severe malaria outcomes, such as experimental cerebral malaria (ECM), where CD8+ T cells act as effectors and CD4+ T cells play a priming role. The sequestration of activated CD8+ T cells in cerebral microvasculature has been linked to neurological symptoms