IMMUNOMODULATORY EFFECTS OF *PLASMODIUM BERGHEI* EXPRESSED MOUSE INTERFERON GAMMA CULTURE SUPERNATANTS IN MICE CHALLENGED WITH HOMOLOGOUS WILD-TYPE PARASITES

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Immunomodulatory effects of *Plasmodium berghei* expressed mouse interferon gamma culture supernatants in mice challenged with homologous wild-type parasites

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as the University supervisors:

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DEDICATION

I dedicate this thesis to my mum, Eunice and to my siblings. Your constant encouragement and support has enabled me successfully complete my studies.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisin-based combination therapy
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD4	Cluster of differentiation antigen 4
CD8	Cluster of differentiation antigen 8
CO_2	Carbon dioxide
DHFR/TS	Dihydrofolate reductase thymidylate sythase
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DXO	Double cross over
ELISA	Enzyme linked immunosorbent assay
EDTA	Ethylenediamine-tetraacetic acid
EMP-1	Erythrocyte membrane protein 1
HIV	Human immunodeficiency virus
HEPES	Hydroxylethyl piperazine-ethanesulfonic acid
IgG	Immunoglobulin gamma
ITNs	Insecticide treated nets
IRS	Insecticide residual spray
IFN-γ	Interferon gamma
IPR	Institute of Primate Research
KAC	Potassium acetate
KEMRI	Kenya Medical Research Institute
LB	Lysogen broth
mIFN-γ	Mouse interferon gamma
MIM	Multilateral Initiative on Malaria
N2	Nitrogen

NKT cells	Natural killer T cell
ORF	Open Reading Frame
O_2	Oxygen
PBS	Phosphate buffered saline
PRBC	Parasitized red blood cells
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PfHRP-2	*Plasmodium falciparum* histidine rich protein 2
pLDH	Parasitic lactate dehydrogenase
pUC	University of California plasmid
RESA	Ring infected surface antigen
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SXO	Single cross over
TBE	Tris-borate-EDTA
TH1	T helper cell type 1
TH2	T helper cell type 2
TNF-α	Tumor Necrosis Factor-Alpha
TNF-1β	Tumor necrosis factor-1beta
UV	Ultra violoet
WHO	World Health Organization

ABSTRACT

Malaria causes up to 755,000 deaths per year, worldwide. Availability of an affordable, long lasting, safe and effective malaria vaccine would be very appropriate in reducing the disease burden by bridging the gap left by other malaria interventions. Most malaria vaccine development approaches have faced challenges such as the choice of appropriate adjuvant and inconsistent efficacy results during trials. Search for a feasible malaria vaccine continues. Use of whole-organism vaccines such as live attenuated or immunopotentiated parasites could resolve this challenge. Cytokine immunopotentiation of *Plasmodium* parasites may provide an important approach towards development of a potent malaria vaccine. This study aimed at exploring immunomodulatory potential of parasite expressed mouse interferon gamma (mIFN-γ) in a malaria model with a view of contributing to malaria vaccine development strategies. Transfection was used to generate interferon gamma (IFN-γ) producing *Plasmodium berghei* parasites. A randomized controlled study design was adopted. The design constituted four treatment groups; one test group and three control groups. There were three sampling points. Each treatment group constituted five mice at each sapling point. In total, sixty mice were used. The parasites were cultured and IFN-γ culture supernatants collected. Mice were immunized with the parasite expressed mIFN-γ culture supernatants. Fourteen days later, they were later intraperitoneally challenged with wild-type parasites. Sampling for cytokine and antibody assays was done and ELISA performed on the collected samples. Parasitaemia was monitored daily and survival time (days) recorded. Analysis of variance (ANOVA) was used to analyze the results using graphpad instat software. The group pre-treated with IFN-γ *P. berghei* culture supernatants exhibited significantly higher levels of IFN-γ ($p < 0.001$). The level of IL-4 in this group was significantly low ($p < 0.05$). There was no significant difference in the levels of IgG ($p = 0.0682$) amongst all the treatment groups. Mean parasitaemia was significantly reduced ($p = 0.023$) in the group pre-exposed to IFN-γ expressing *P. berghei* culture supernatants compared with the controls.

There was a 4 day delay in onset of patent parasitaemia accompanied by higher parasitaemia suppression (94.15% on day 11 post-infection). The survival time of the mice was 5 days longer than that of the controls. The findings of this study have demonstrated the potential for culture supernatants of IFN-γ expressing *P. berghei* in protecting mice against virulent infection. The study has set a pace for adoption of interferon gamma immunopotentiation of *Plasmodium* as an approach to attenuated malaria vaccine development. However, further studies need to done to shed more light on host protection against active infection. Such studies include purifying the *P. berghei* expressed mouse IFN-γ and determining its effect on murine malaria at different concentrations and time points during infection, in synergy with whole parasite antigen.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

Malaria causes up to 755,000 deaths per year (WHO, 2013), mainly in the tropical and subtropical regions of the world (Mana *et al.,* 2012; Inoue *et al*., 2013; Sosovele *et al*., 2013). Vaccination is effective in reducing disease burden and is also cost-effective (Syaifudin *et al*., 2011). However, there is currently no registered vaccine available for the control of malaria (Syaifudin *et al*., 2011; Chia *et al*., 2014). Though malaria vaccine development has been met with only limited success, there is evidence that it is feasible (Arama &Troye-Blomber, 2010; Syaifudin *et al*., 2011; Schwartz *et al*., 2012; Chia *et al*., 2014).

Most malaria vaccine development approaches have either been using a subunit, Deoxyribonucleic acid (DNA) or irradiated sporozoites (Leitner *et al*., 2009; Robin, 2011; Behet *et al*., 2014; Chia *et al*., 2014). These vaccines have faced challenges including the choice of adjuvant and inconsistent efficacy results during trials (Thera & Plowe, 2012). The most advanced malaria candidate vaccine so far is the subunit vaccine RTS, S/AS01 (Thera & Plowe, 2012). The main challenges facing the RTS, S/AS01 vaccine candidate are low efficacy rates, reactogenicity and low immunogenicity (Valupadasu & Maleti, 2012). The vaccine has shown low efficacy rates of about 30-50% prolonged protection from infection in field trials (Ellis *et al*., 2010; Fitchett and Cook, 2010; Sauerwein *et al*., 2011; Spring *et al*., 2013).

Use of whole-organism vaccines such as live attenuated or immunomodulated parasites could resolve this challenge (Thera & Plowe, 2012; Behet *et al*., 2014). Whole-organism vaccines raise interesting questions such as ability for mass production, possibility of

generating a live infection as well as mode of delivery (Luke and Hoffman, 2003; Engwerda and Good, 2008; Ellis *et al*., 2010; Pinzon-Charry, 2010; Thera and Plowe, 2012). However, they present a near total repertoire of antigens to the immune system. In addition, the antigen is delivered in its native conformation and no precise antigen or epitope determination is required (Pinzon-Charry, 2010).

Increase in genomic and biological knowledge especially the genetic manipulation of *Plasmodium* parasites by transfection has facilitated the possibility of developing genetically attenuated malaria parasites through immunopotentiation by expression of host cytokines (Khan *et al*., 2012). One such cytokine is interferon gamma, which apart from its immunomodulating activity (Giavedon *et al*., 1997), is a key effector cytokine in protection against malaria (Ozwara *et al*., 2003). Previous work has shown that *Plasmodium* transgenically expresses bioactive host interferon gamma (Ozwara *et al*., 2003). This study aimed at exploring the immunomodulatory potential of *P. berghei* expressed mouse IFN-γ culture supernatants in a murine malaria model.

1.2 Global distribution of Malaria

Malaria is a parasitic disease that is transmitted by mosquitoes. The causative agent of the disease is the *Plasmodium.* This parasite puts half of the world's population at risk and causes up to 755,000 deaths per year (WHO, 2013), mainly in resource-poor tropical and sub-tropical regions of the world (Fig. 1).

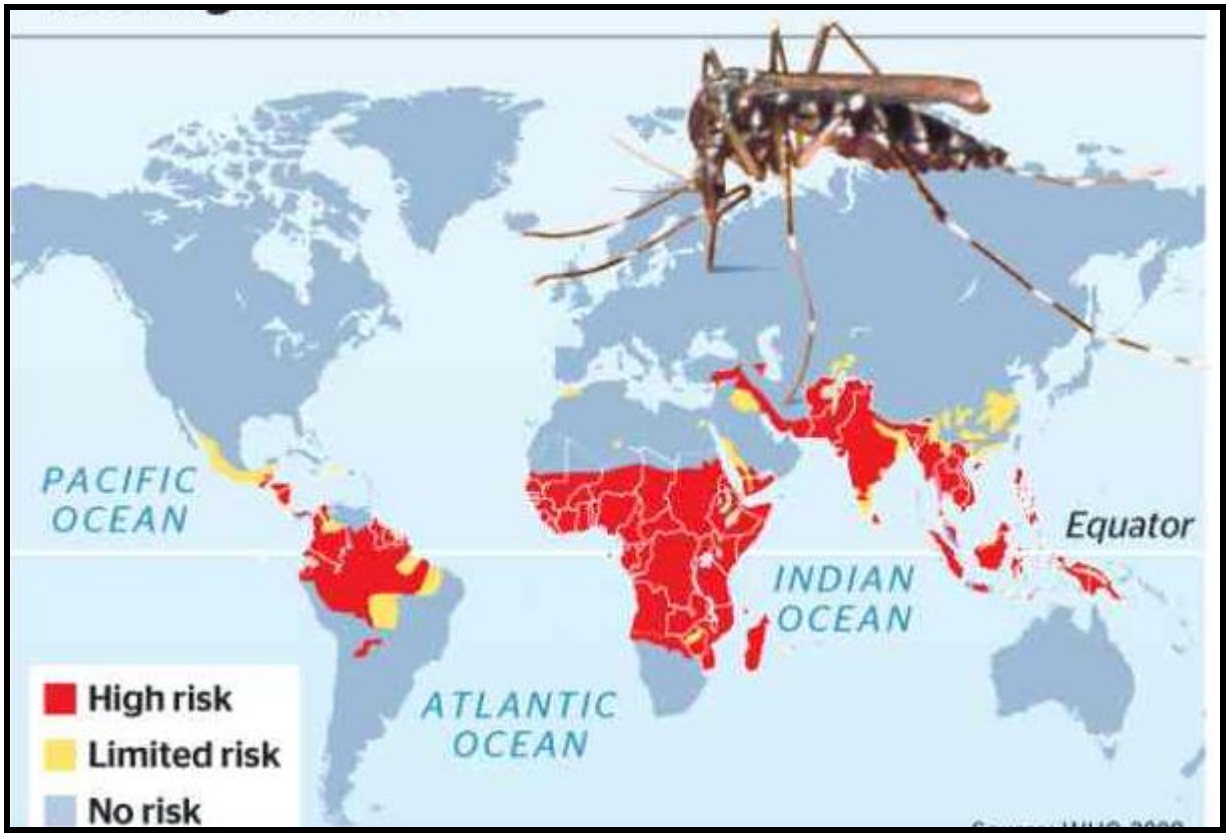

Figure 1: Global distribution of malaria (Source: WHO, 2009)

The worst hit regions of the world include countries of the Sub-saharan Africa, Latin America and some parts of Asia (Inoue *et al*., 2013; Sosovele *et al*., 2013). Malaria is preventable and treatable. However, in the resource-poor countries, it causes significant morbidity and mortality especially in children less than 5 years of age and also in pregnant women. This is attributed to reduced immunity in the two groups of individuals (WHO, 2013).

A combination of factors explains why Africa is worst hit by the malaria epidemic. Firstly, the area is predominated by a very efficient vector, *Anopheles gambiae* which is responsible for high transmissions of the *Plasmodium* parasite. Secondly, *P. falciparum* parasite, the most probable cause of severe malaria, predominantly exists in the area.

Thirdly, the local weather conditions are conducive in allowing all year round transmissions of the parasites. Finally, the area is hit by scarcity of resources coupled with socio-economic instability which pose challenges to the control programmes (WHO, 2012 b).

Malaria imposes a considerable barrier to economic development especially in developing countries. It imposes economic costs cutting across individuals and governments. The costs at individual level include purchase of drugs for treating malaria at home, travel expenses to and from health facilities in search for medication, lost days of work, absence from school, expenses for preventive measures as well as burial expenses in case of deaths. At government level, the costs incurred include costs for maintenance of health facilities, purchase of drugs and supplies, public health interventions against malaria such as insecticide spraying and distribution of insecticide-treated bed nets, loss of income due to lost days of work and reduced tourism (Sachs *et al*., 2002; Desai *et al*., 2007).

1.3 Life cycle of *Plasmodium*

Plasmodium parasites exhibit a complex life cycle that involves an invertebrate vector (*Anopheles* mosquito) and a vertebrate host (Fig. 2).

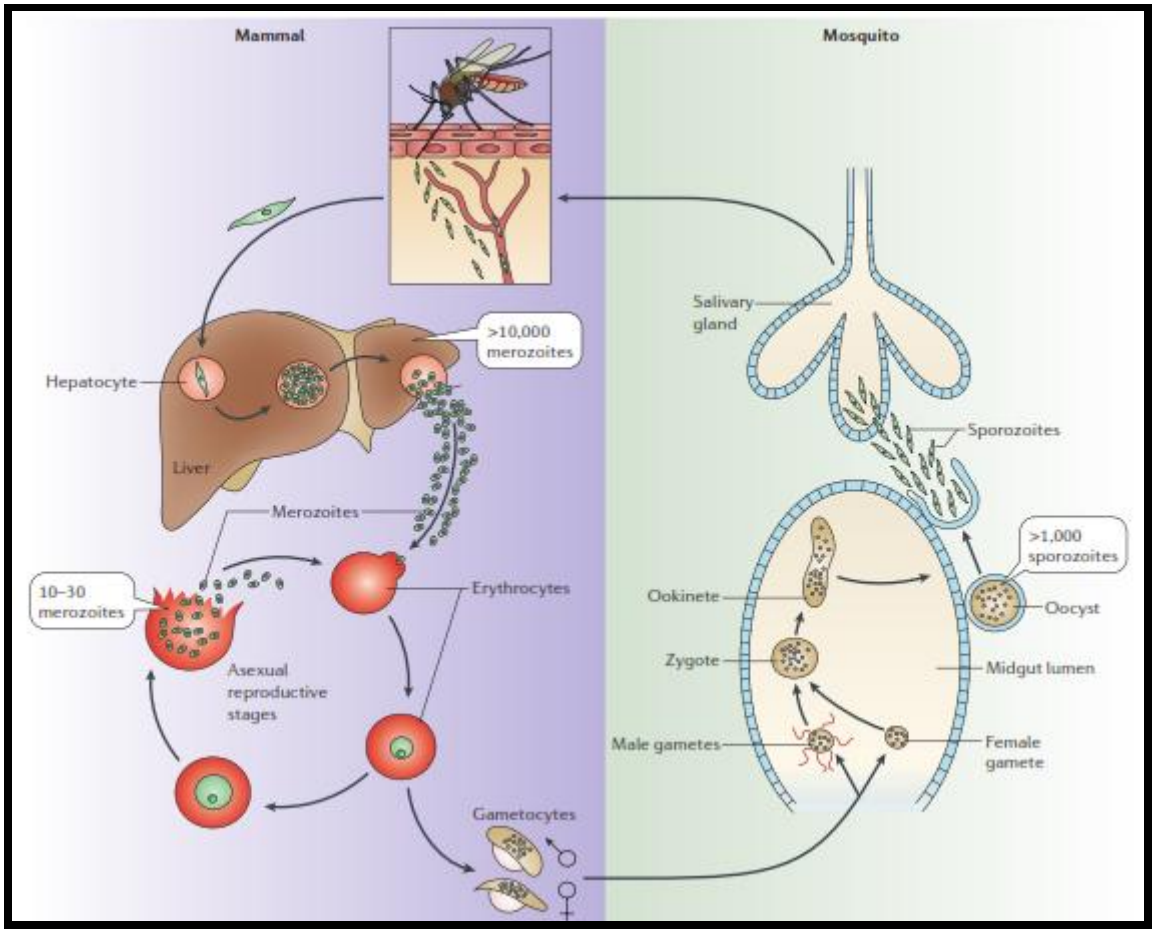

Figure 2: A diagramatic representation of the life cycle of *Plasmodium* species.

(Source: Robert *et al.*, 2013)

Malaria parasites undergo three distinct asexual replicative stages and one sexual stage. The asexual stages are the exoerythrocytic schizogony, blood stage schizogony and sporogony. The sexual stage is the gametogony. These replicative stages result to production of invasive forms of the parasite which are the merozoites and sporozoites. When sporozoites are injected into a host with saliva of a mosquito during a blood meal, they are carried by the circulatory system to the liver where they invade hepatocytes. Within the hepatocytes, they undergo exoerythrocytic schizogony, leading to production of merozoites. The merozoites are released into the bloodstream. Some of the merozoites,

from *P. vivax* and *P. ovale,* instead of immediately undergoing asexual replication, become dormant within the liver. These are termed as hypnozoites. They become reactive after several weeks, months or years following the primary infection and do account for malaria relapses (Good, 2005).

In the blood stream, the merozoites invade erythrocytes and go through a trophic period. In this period, the parasites enlarge developing into the 'ring form'. During trophozoite enlargement, active metabolism involving ingestion of host cytoplasm as well as proteolysis of hemoglobin into amino acids occurs. This is then followed by multiple rounds of nuclear division that is not coupled with cytokinesis. The resultant is a schizont (segmenter) that matures and produces merozoites as buds. Following rupture of the infected erythrocytes, the merozoites (buds), are released and invade other erythrocytes reinitiating another phase of the blood-stage replicative cycle (Good, 2005).

Some of the parasites in the red blood cells differentiate into sexual forms (macrogametocytes & microgametocytes). These sexual forms develop into gametocytes through a process called gametogenesis. However, it is not clear as to what induces this process (Drakenley *et al.,* 2006). It has been suggested that gametogenesis is stimulated by a drop in temperature, an increase in carbon dioxide concentration, innate immune factors as well as mosquito metabolites upon ingestion of the gametocytes by the mosquito (Rameo *et al*., 2011). These factors also induce escape of the gametocytes from the erythrocytes. During gametogenesis, the microgametocytes exflagellate producing eight flagellated microgametes while the macrogametocytes transform into a single macrogamete. In the mosquito gut, the microgametes fertilize the macrogamete leading to a zygote which develops into a motile ookinete. The ookinites penetrate the epithelial cells of the gut of the mosquito and develops into oocysts. The oocysts undergoe sporogony (an asexual replication stage) leading to production of sporozoites which upon rupture, releases the sporozoites into the haemocoel of the insect vector, the mosquito.

The sporozoites migrate to and invade the salivary glands, in readiness for inoculation into the new vertebrate host in the next blood meal and thus initiate another life cycle (Arama & Troye-Blomberg, 2014).

1.4. Immmunopathology of malaria

Host adaptive immune responses to *Plasmodium* infection lead to reduced clinical impacts of the disease to the hosts. However, several immunological processes have been implicated in malaria pathogenesis resulting to different malaria syndromes with distinct clinical signs and symptoms, some of which are severe or fatal (Schofield & Grau, 2005).

One of the resultant clinical syndromes is cerebral malaria which is normally characterized by sustained impaired consciousness, coma and long-term immunological disorders. Possibly, this condition results from effects of cerebral parasite sequestration, T-helper 1 and T-helper 2 (TH I/TH 2) cytokine imbalances, immune cells recruitment, platelet and fibrinogen deposition, neurologic metabolic derangement and hypoxia (Milner *et al.*, 2012). Second is placental malaria. Placental malaria is characterized by placenta insufficiency, low birth weight, loss of fetus and premature delivery. It could be as a result of the binding *of P. falciparum* to the placenta endothelium. This binding is erythrocyte membrane protein 1 (EMP-1) mediated and is facilitated through chondroitin sulphate A and hyaluronic acid as well as chemokine mediated recruitment and infiltration of monocytes (Schofield & Grau, 2005). Third is the severe malaria anaemia. This is characterized by haemoglobin levels of 4-6g per 10ml of blood, pallor and lethargy. This results from suppression of erythropoiesis by cytokines and toxins, increased parasitaemia leading to increased destruction of red blood cells, erythrophagocytosis and TH I/TH 2 cytokine imbalances (Miller *et al.*, 2002). Fourth is the metabolic acidosis and this is characterized by respiratory distress and kussmaul breathing (deep breathing). This is as a result of widespread parasite sequestration, increased vascular permeability, reduced tissue perfusion, pulmonary airways obstruction and increased host glycolysis. Fifth is the shock-like syndrome. This is characterized by

shock, impaired organ perfusion, haemodynamic changes as well as disseminated intravascular coagulation. This has been associated with bioactive toxins and TH 1 cytokines (Schofield & Grau, 2005).

1.5 Diagnosis of Malaria

Malaria diagnosis starts with clinical suspicion of the infection (WHO, 2006). This can then be followed by other tests such as microscopy, immunological tests and PCR (WHO, 2006). Immunologically, malaria is diagnosed by use of commercially available kits. Such kits distinguish *P. falciparum* from other malaria species but not the other malaria parasite species (*P. ovale, P. malarae and P. vivax*) from one another. The tests include chromatographic detection of *P. falciparum* antigens which include the histidin-rich protein-2 (PfHRP-2) or parasitic lactate dehydrogenase (pLDH), (WHO, 2006). Enzyme linked immunosorbent assay (ELISA) has also been developed for malaria diagnosis. The ELISA detects HRP-2 in peripheral blood of patients and its sensitivity is equivalent to the one of well prepared thin blood films in cases of low parasite load (WHO, 2006). Microscopy is facilitated through thick and thin blood films. The thick blood films are more sensitive than thin smears but make speciation more difficult. Thin films facilitate confirmation of the diagnosis and help in the identification of the parasite species in question, as well quantification of the parasite load. The different species can be distinguished by their morphology (WHO, 2006).

1.6 Malaria prevention and treatment

Successful malaria control is based on early diagnosis and prompt treatment accompanied with vector (mosquitoes) control (WHO, 2012a). Access to both prevention and treatment services have been on the increase over the past decade (WHO, 2012a). The preventive strategies include; sleeping under insecticide treated bed nets (INTs), indoor residual sprays (IRS) and intermittent preventive treatment in pregnancy (IPTp), (WHO, 2012a). However, gaps still exist and they include existence of resistance to both insecticides and antimalarial drugs in some areas such Latin America, South East Asia and Western

Pacific (WHO, 2012a). In addition, the preventive tools are of limited access in developing countries especially in Sub-Saharan Africa due to financial constraints (WHO, 2012a). The number of people applying IRS has also gone down because of a shift from usage of pyrethroids to the usage on non-pyrethroid insecticides which are relatively expensive. The shift is due to the emergency of pyrethroid resistance (WHO, 2013).

Treatment for malaria involves the usage of primaquine, chloroquine and Artemisinin combination therapy (ACT) with ACT as the drug of choice for *P. falciparum* which is the most deadly form of malaria (WHO, 2012a; Randall, 2014). It is also the drug of choice in areas with drug resistant strains of *Plasmodium* (WHO, 2012a). This is because ACT is a formulation of 3 different drugs that act on different parts of the parasite (WHO, 2006). The three drugs (artesunate, pyrimethamine and sulphadoxine) contained in ACT, have different mechanisms of action on the parasite. This forces the parasite to develop several simultaneous mutations in order to become resistant to the drug. However, it is rare for two or more genetic mutations to occur simultaneously by chance so as to enable development of resistance (WHO, 2012a; Randall, 2014).

1.7 Malaria and Drug resistance

Drug resistance is a phenomenon where the parasite strain is able to partly or full resist the effects of one or more antimalaria drugs (WHO, 2006). This is facilitated by genetic mutations when the parasites are exposed to insufficient amounts of the drugs as a result of low prescription dosage, less dispensing of the amount of the drug, incomplete treatment taken by the patient, vomiting of the drug or low drug absorption that could arise from, for instance, diarrhea (WHO, 2006).

Malaria control is based on early diagnosis and treatment on one hand, and vector control on the other hand (WHO, 2012a). However, antiplasmodial drug resistance and vector resistance to insecticides seems to pose a serious problem in the medical field (Randall,

2014). In the 1970s and 1980s, resistance of *P. falciparum* to chloroquine and sulfadoxine-pyrimethamine (SP), became very rampant resulting to a reversal in child survival gains. Artemisinin-based combination therapy (ACT) is the best treatment available as of now, particularly for *P. falciparum* malaria (WHO, 2012a; Randall, 2014).

It is difficult for the parasites to develop resistance against ACT because it is rare for two or more genetic mutations to occur simultaneously by chance so as to facilitate resistance. Artemisinin combination therapy contains artesunate, pyrimethamine and sulphadoxine and each drug acts on a different part of the parasite (WHO, 2006). However, in 2009, instances of *Plasmodium* resistance to artemisinins were reported on the Cambodia-Thailand border and have since been reported in Myanmar and Viet Nam. This has been attributed, mainly, to oral artemisinin monotherapy which rapidly clears the malaria symptoms leading to premature discontinuation of medication by the patients and thus development of persistent parasites in the blood. Also countries in sub-Saharan Africa and India have witnessed widespread reports of mosquitoes being insecticide resistant especially to pyrethroids, and yet vector control has always been the best approach for controlling malaria transmissions. Pyrethroids are the only class of insecticides currently recommended for insecticide treated nets (ITNs) usage (WHO, 2012a; Randall, 2014).

1.8 The malaria vaccine

Vaccination is effective in reducing disease burden and also cost-effective (Syaifudin *et al.*, 2011). There is no effective vaccine currently available for malaria control (Syaifudin *et al.*, 2011). However, there is evidence that development of a malaria vaccine is feasible (Pouniotis *et al.*, 2004; Otarigho, 2012). The challenges facing malaria vaccine development include the complexity of the life cycle of the parasite, antigenic variation of the parasite especially for *P. falciparum* as well as lack of complete natural immunity to the parasite. These challenges are compounded by inadequacy of funds to carry out malaria research (Syaifudin *et al.*, 2011; Otarigho, 2012). The desired malaria vaccine should be able to work in very minimal doses as much as possible, be affordable, simple

to administer, be able to work against multiple stages of the parasite, offer long life protection and be protective against all *Plasmodium* parasite strains in all ages. In addition, it should be stable enough in different temperatures since malaria is endemic in various developing countries with differing climatic conditions (Syaifudin *et al*., 2011; Otarigho, 2012).

At present, there are several malaria candidate vaccines in development which include pre-erythrocytic (both sporozoite stage and liver stage) vaccines, blood-stage vaccines, transmission blocking vaccines, vaccines for malaria during pregnancy as well as DNA vaccines (Otarigho, 2012). Pre-erythrocytic vaccines target sporozoites when they are in transit from the mosquito inoculation site to the liver. Such vaccines include RTS, S /AS02 (Valupadasu & Maleti, 2012). They are aimed at preventing infection and clinical disease (Good, 2005). Blood-stage vaccine targets the merozoites when leaving the infected hepatocytes and travelling between ruptured liver cells and RBCs or the merozoites in transit between lysed erythrocytes (Arama & Troye-Blomberg, 2014).

Asexual blood stages of *Plasmodium* parasites are the cause of classic symptoms and pathology of malaria (Good, 2005). Blood-stage vaccines are aimed at lessening the disease by reducing the parasite density in blood after infection. Some blood stage-vaccines are also aimed at inducing production of antibodies that could neutralize parasite toxins (Good, 2005; Valupadasu & Maleti, 2012). Transmission blocking vaccines (Mosquito-stage vaccines) target whole infected RBCs or parasites ingested by the mosquito during a blood meal. The mosquito ingests immature gametocytes which later develop into infective stages inside the mosquito. Mosquito-stage vaccines are aimed at inhibiting the development of the gametocytes into the infective stages. An example of such a vaccine is the New York Vaccinia *P. falciparum* -7 (NYVAC - Pf.7) (Valupadasu & Maleti, 2012). Vaccines for malaria during pregnancy are aimed at reducing sequestration of infected *Plasmodium* parasite onto the placental endothelium membrane

proteins for this sequestration has been associated with placental malaria pathogenesis (Good, 2005). Deoxyribonucleic acid (DNA) vaccines constitute one of the most promising effective malaria candidate vaccines where sections of DNA are extracted from the genome of the parasite and inserted into a vector. The vector then enters the human host where it is incorporated into host DNA directing protein synthesis that label the host as infected inducing T cell responses. Thus, a population of memory T cells sensitive to the particular epitope is generated (Arama & Troye-Blomberg, 2014). An example of such a vaccine is the pf 155/RESA (Valupadasu & Maleti, 2012).

Nevertheless, none of the vaccine development strategies has achieved full potential of protection against malaria infection (Syaifudin *et al*., 2011; Chia *et al*., 2014). The most advanced vaccine candidate so far is the RTS, S/AS01, a sporozoite stage vaccine, developed against *P. falciparum* and is currently undergoing extensive Phase III clinical trials in Tanzania, Ghana, Gabon, Senegal and Sierra Leone. The vaccine has indicated potential of preventing clinical and severe disease in Phase II trials (Sauerwein *et al*., 2011). However, the vaccine has exhibited only about 30-50% prolonged protection from infection during trials (Casares *et al*., 2010; Sauerwein *et al*., 2011). The final clinical trials are ongoing and the WHO recommendations on the vaccine are expected in the year 2015 (WHO, 2012).

1.9 Transfection technology

Transfection is a eukaryotic cell mutating technology that involves introduction of exogenous genetic materials into the cell of interest. A particular gene is modified and the mutant cell assessed for the phenotype. This gene transfer technology helps in the study of the relationship between cell gene structure, function and protein expression (Carvalho & Menard, 2005).

Transfection may be transient or stable. In transient transfection, DNA is introduced into a recipient cell, retained and temporarily expressed. In stable transfection, the DNA

introduced into a recipient cell is maintained in a functional state for an extended period of time. This results into transfected cells with heritable changes that in return lead to a genetically modified organism. Stable transfections are used where the transformed cell has to be analyzed over several generations. The introduced DNA can be maintained as an extrachromosomal replicating plasmid (episomal) or integrated into host cell genome (integration dependent). During transfection, the transformed parasites are grown under drug pressures by use of selectable markers aimed at selecting and maintaining the transformed parasites foreign genes (Kocken *et al*., 1999). Biological studies require stable transformation which could involve a single homologous recombination event known as single cross over (SXO) integration or double cross over (DXO) integration. DXO is more significant because the targeted gene is physically deleted. In SXO, the targeted gene is only disrupted and it can reconstitute and reversing back to the wild type genotype (Duraisingh *et al*., 2002).

Transfection has provided an opportunity to identify genetic elements involved in the control of gene expression in *Plasmodium* parasites. It has facilitated the understanding of the basic processes that induce expression of genes in *Plasmodium* parasites. In *Plasmodium,* the process is accomplished by electroporation. *Plasmodium* electroporation involves generation of temporary pores in the cell membrane of the parasite. DNA enters the cell through these pores. Resistance genes conferring resistance to an antimalarial compound are used to select the transfected parasites (Carvalho & Menard, 2005; Balu & Adams, 2007).

Introduction of DNA into schizonts is more efficient than into ring and trophozoite stages. However, *P. berghei* cannot be readily cultured, *in vitro,* into mature schizonts for more than one cycle. Due to this reason, rats or mice are infected with *P. berghei* to propagate the blood-stage parasites for culturing and purification of mature schizonts. The most widely used DNA constructs contain the pyrimethamine resistant form of the *T.*

gondii DHFR/TS gene as a selectable marker. This facilitates selection of transfected parasites which are injected back into mice, to be selected by treating mice with pyrimethamine (Franke-Fayard *et al*., 2004).

1.10 *Plasmodium berghei* as a model parasite for malaria research

Murine malaria models have been developed for the studies and this give an insight into particular areas of interest in malaria research. Molecular genetics studies involving *P. falciparum*, are more difficult and time consuming relative to similar studies involving *P. berghei*. Therefore it is economical that important gene function analyses be done in *P. berghei* and then subsequently transferred to *P. falciparum*. Though there exists many differences between *P. berghei* and *P. falciparum*, there are also many conserved genes. For instance, data on IFN-γ gathered using rodent *Plasmodium* species correlate with data obtained with *P. falciparum* despite the diversity of protective mechanisms involved (Perlaza *et al*., 2011). *Plasmodium berghei* causes a fatal disease in BALB/c mice with pathology similar to the one observed in human due to *P. falciparum*. The BALB/c mice limit chances of variation of results of the variables under investigation (Jambou *et al*., 2011). *Plasmodium berghei* form a very efficient transfection model for human malaria (Tipsuwan *et al*., 2011). In addition, transfection involving *P. berghei* allows the parasites to be temporarily maintained *in vitro* at the mature schizont stage and transfection done directly targeting merozoites. By targeting the merozoite stages for transfection, the only barriers would be the parasite plasma membrane and the nuclear envelope (Franke-Fayard *et al*., 2004).

1.11 *In vitro* culturing of *P. berghei* parasites

Plasmodium berghei is an African murine malaria parasite. During a natural infection, the blood stages of the parasite undergo asynchronous development with a haploid cycle of 22 hours. *In vitro* cultivation of the parasites is done to collect and purify different developmental stages of the parasite for studies. These parasites have preference for immature red blood cells (reticulocytes). Therefore, continuous culture of these parasites

requires addition of blood with a high percentage of reticulocytes to the culture medium (RPMI 1640). Studies have shown the possibility of long-term *in vitro* cultivation of *P. berghei* at 15°C and at 27°C. However, there has been shown a decrease in parasite density for cultivation at 37°C probably due to instability of the red blood cells (Jambou *et al*., 2011).

1.12 Immunity to malaria

The innate and adaptive immune responses have an active role in *Plasmodium* immunity (Stevenson and Riley, 2004; Chia *et al*., 2014). Malaria parasites have a complex, multi-stage life cycle, during which they expresses a great variety of proteins at different stages that also keep changing often. This leads to natural immunity to malaria parasites being partial and short lived which is insufficient in protecting individuals against new infections. Adaptive immunity has been categorized as anti-disease immunity, anti-parasite immunity, and sterilizing immunity that is also called premonition. Anti-disease immunity protects against clinical disease, anti-parasite immunity protects against high parasitaemia and sterilizing immunity protects against new infections by maintaining a low-grade, asymptomatic parasitaemia (Doolan *et al*., 2009).

Cell-mediated immunity (CMI) to malaria is poorly understood (Good *et al.*, 2005). Dendritic cells (DCs) which are the professional antigen presenting cells present plasmodium antigens to T cells activating the naïve immune responses (Stevenson and Riley, 2004). B cells present the malaria antigens and also activate the production of plasma cells which are the antibody producing cells (Donati *et al*., 2004). Macrophages phagocytose infected red blood cells, present antigens in the context of MHC class II to T cells activating them and facilitate antibody production. This way, the macrophages link innate and adaptive host responses (Ayi *et al*., 2005). The T cell subsets of immunity play an important role in malaria immunity. T helper 1 (Th1) subset of CD4+ T cells secrete interferon-gamma (IFN-γ), Interleukin-2 (IL-2), Interleukin-8(IL-8) and tumor necrosis factor-1beta (TNF-1β) cytokines which are associated with strong cell mediated immune

responses as well as CD8+ T cell cytotoxicity. T helper 2 (Th2) subset of CD4+ T cells secrete Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-9(IL-9), Interleukin-10 (IL-10) and Interleukin-13 (IL-13) cytokines which are associated with antibody production (Funda *et al*., 2009). Dysregulation of the cytokines and the balance between the pro-inflammatory and anti-inflammatory cytokines (IL-4, IL-10 and TGFβ) has been shown to be associated with malaria pathology (On'gecha *et al*., 2008).

Antibody responses play an important role to *Plasmodium* infection (Julia *et al*., 2008; Chia *et al*., 2014). Malaria infection induces both polyclonal and specific immunoglobulin production; predominantly IgM and IgG (Doolan *et al*., 2009). Such antibodies are important in clearing parasites in peripheral blood (Langhorne *et al*., 2008). The antibodies facilitate phagocytosis of infected red blood cells (Marsh and Kinyanjui, 2006). Those antibodies that are specific to meroziotes protect red blood cells from being infected by the merozoites (Jiang *et al*., 2011). B cells secrete pathogen-specific antibodies through T cell help in later stages of plasmodium infection and such antibodies are important in clearing parasitaemia (Langhorne *et al*., 2008).

1.13 Role of interferon gamma (IFN-γ) in host immune responses to malaria

Interferon gamma is produced by $CD4^+$ cells (Th1 lymphocytes), $CD8^+$ cytotoxic lymphocytes, NK cells, NKT cells, B cells and dendritic cells (Schoenborn & Wilson, 2007; Meyer, 2009; Pollard *et al*., 2013). Interferon gamma production by dendritic cells acting locally may be involved in activation of self-cells or activation of adjacent cells (Schroder *et al*., 2004). Interferon gamma secretion by NK cells and or professional APCs could be involved in early host defense against infection. Interferon gamma secreted by T lymphocytes takes part in adaptive immune responses (Schroder *et al*., 2004). The IFN-γ produced by $CD8^+$ T cells in infection control and also by $CD4^+$ T cells. The IFN-γ produced by the $CD4^+$ T cells promotes inflammation, clearance of intracellular pathogens as well as immunoglobulin class switching to IgG2a, IgG2b and IgG3 (Pollard *et al*., 2013).

Interferon gamma production is positively regulated by cytokines secreted by antigen presenting cells (APCs), especially interleukin (IL)-12 and IL-18. Macrophage recognition of many pathogens induces secretion of IL-12 and chemokines such as macrophage-inflammatory protein-1α (MIP-1α). Natural killer (NK) cells are attracted to the inflammation sites by the chemokines where IL-12 promotes IFN-γ synthesis by the NK cells. In combination, the IL-12 and IL-18 stimulation further stimulates macrophages, NK and T cells leading to increased secretion of IFN-γ. Interferon gamma production is negatively regulated by IL-4, IL-10, TGF-β and glucocorticoids (Schroder *et al*., 2004; Perlaza *et al*., 2011).

Interferon gamma has been associated with mediation of protection and pathogenesis in *Plasmodium* infection (Inoue *et al*., 2013). It has been suggested that the ability of IFN-γ to mediate either protection or pathogenesis in malaria is dependent on where and when it is produced (Mitchell *et al*., 2013).

A strong effect of IFN-γ upon liver stage schizogony has been documented in many rodent *Plasmodium* species employed in various strains of mice (Tsuji, 2009; Perlaza *et al*., 2011). Use of IFN-γ receptor knockout animals has indicated failed protection of the host against irradiated plasmodium sporozoites. Involvement of NO synthase, downstream of the IFN-γ signaling, as well as the direct effect of NO- radicals has been documented, and it has been suggested that, for the liver stage, IFN-γ induces the infected hepatocytes to produce nitric oxide that kills the *Plasmodium* parasites (Ozwara *et al*., 2003). Immunization schemes that have induced better protective immunity have been associated with very strong local T cell responses that produce IFN-γ (Perlaza *et al*., 2011).

In human beings, production of IFN-γ by peripheral blood mononuclear cells (PBMC) in response to liver-stage antigens has been associated with resistance to re-infection with *P. falciparum* in young African children (Perlaza *et al*., 2011). Indeed, it has been suggested that the strategies and formulations towards development of a successful liver-stage malaria vaccine should aim at inducing host CD4+ cell responses involving high levels of IFN-γ production (Perlaza *et al*., 2011). Therefore, IFN-γ has been highly associated with protection against plasmodium parasite infection, both in animal models and human subjects.

1.14 Host IFN-γ expressed by transgenic pathogenic microorganisms

Transfection technology has been used to generate transgenic organisms that are able to express host cytokines such as IFN-γ, a key effector cytokine in protection against malaria, especially during the liver stages of the *Plasmodium* parasites (Ozwara *et al*., 2003). IFN-γ has a central role in host immune response to infection by liver stage *Plasmodium* parasites (Perera *et al*., 2001; Ozwara *et al*., 2003).

Intracellular transfected pathogenic microorganisms (Vaccinia virus, Simian immunodeficiency virus and *Leishmania* major) expressing host cytokines such as interferon gamma (IFN-γ), have been shown to induce immunomodulatory and protective effects to the pathogens (Perera *et al*., 2001; Ozwara *et al*., 2003). Studies in rodent and human models of malaria have indicated that IFN-γ has an important role in protection against malaria infection by targeting either the liver stage or the blood stage parasites. It has been suggested that, for the liver stage, IFN-γ induces the infected hepatocytes to produce nitric oxide that kills the *Plasmodium* parasites **(**Ozwara *et al*., 2003**)**. It has also been shown that malaria parasites express bioactive recombinant host cytokines that induce immunomodulatory and protective effects to the host: an indication that may provide an approach to development of attenuated and immunopotentiated malaria vaccines (Ozwara *et al*., 2003).

1.15 Statement of the problem

Malaria is a major cause of mortality especially in the tropical and subtropical regions of the world. It is also imposes a considerable barrier to economic development both at individual level and at government level especially in developing countries. Vector control coupled with early diagnosis and treatment has always been very effective in malaria control. However, parasite and vector resistance to drugs and insecticides respectively, as well as unavailability of sufficient funds act as strong drawbacks in the fight against the disease. Vaccination could help ease the impact of this infectious disease but currently, no registered vaccine is available for its control. Most malaria vaccine development approaches have faced challenges such as the choice of appropriate adjuvant and inconsistent efficacy results during trials. Search for a feasible malaria vaccine continues. Use of whole-organism vaccines such as live attenuated or immunopotentiated parasites could resolve this challenge. Host IFN-γ immunopotentiation of *Plasmodium* parasites may enhance host immune response to active infection. The role of host interferon gamma transgenically expressed by *Plasmodium* as a potential immunomodulator in malaria infection remains a research concern. This study aimed at determining the immunomodulatory potential of the parasite expressed host IFN-γ in a mouse malaria model.

1.16 Justification of the study

Immunization is often extremely effective in reducing disease burden and is also cost-effective. Most of the vaccines in use today are whole organisms and there is renewed interest in use of parasites that are genetically attenuated in malaria vaccine development strategies. Although whole organism vaccines raise interesting questions such as ability for mass production and possibility of generating a live infection, they present a nearly total repertoire of antigens to the immune system. In addition, antigen is delivered in native conformation and no precise antigen or epitope determination is required.

Increase in genomic and biological knowledge especially the genetic manipulation of *Plasmodium* parasites by transfection has facilitated the possibility of developing genetically immunopotentiated malaria parasites through expression of host cytokines. One such cytokine is interferon gamma, which apart from its attenuating activity, is a key effector cytokine in protection against malaria. This study sought to enhance our understanding on the role of parasite produced host IFN-γ in *Plasmodium* immunomodulation in view of contributing towards designing an effective malaria vaccine.

1.17 Hypothesis

Plasmodium berghei expressed mouse IFN-γ culture supernatant does not mediate protection of mice against murine malaria.

1.18 Objectives

1.18.1 General objective

To determine the immunomodulatory effects of *P. berghei* expressed mouse IFN-γ culture supernatant in mice challenged with homologous wild-type parasites.

1.18.2 Specific objectives

1. To determine the expression of interferon gamma by transgenic *P. berghei*.
2. To determine parasitaemia of wild-type *P. berghei* in mice immunized with the culture supernatant.
3. To determine survival rates of mice challenged with wild-type *P. berghei* following immunization with the culture supernatant.
4. To evaluate antibody and cytokine responses in mice immunized with *P. berghei* expressed interferon gamma culture supernatant and challenged with homologous wild-type parasites.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study site

This study was carried out at the Department of Tropical and Infectious diseases laboratories in the Institute of Primate Research (IPR), Kenya.

2.2 Study Design

A randomized controlled study design was adopted with replicates.

2.2.1 Experimental model and parasites

Inbred male BALB/c mice were used in this study. These mice were bred at the rodent facility of the Institute of Primate Research (IPR), fed on standard pellet diet and water provided *ad libitum*. Care, use and disposal of animals were done according to the IPR guidelines on handling of experimental animals. The study involved use of *P. berghei* ANKA parasites that were donated by Kenya Medical Research Institute (KEMRI). These parasites cause a lethal disease in BALB/c mice with pathology similar to the one observed in human due to *P. falciparum.* Also, *P. berghei* give very efficient transfection results (Janse *et al*., 2006a).

2.2.2 Sampling and sample size determination

The experimental mice were assigned to the experimental groups through simple random sampling technique. Only male BALB/c mice, 6-8 weeks old and weighing above 20g were used in this study. The sample size of the study subjects was estimated based on a previous study that yielded statistically analyzable (conclusive) results (Cao *et al*., 2009). The study involved 4 experimental groups with 3 sampling points. Each treatment group constituted 5 mice at each sampling point. Therefore, a total of 60 mice were used in this study.

2.2.3 Experimental design

Wild-type *P. berghei* parasites were transfected with a pUC plasmid expressing mouse IFN-γ gene. The success of the transfection was confirmed by a plasmid rescue experiment. The transfected parasites were cultured and the IFN-γ secreted in the culture supernatants was quantified using enzyme linked immunosorbent assay (ELISA). The IFN-γ culture supernatant was inoculated into naïve mice. Four experimental groups of mice were involved in this study; one test group and 3 control groups (Table 1). The test group mice were inoculated with 37.5pg of parasite expressed IFN-γ in 200µl of transfected *P. berghei* derived culture supernatant. The control groups comprised of a group of mice administered with supernatants from wild type *P. berghei*; culture supernatants from naïve red blood cells (RBCs); as well as a group of mice administered with RPMI culture medium as a blank control. Fourteen days later, sampling was done for antibody and cytokine determination. All the mice were then challenged by intraperitoneal injection of wild type *P. berghei* suspension containing 1×10^6 parasites. Parasitaemia was monitored daily as described by Munyao *et al*. (2008) and survival time (days) recorded. Seven days post-challenge, sampling was done once again. The experimental design was as shown in Figure 3.

Table 1: Experimental groups

Experimental group	Treatment
A	Mice immunized with IFN-γ culture supernatants and challenged with wild-type *P. berghei* (Test group)
B	Mice immunized with wild-type *P. beghei* culture supernatants and challenged with wild-type *P. berghei* (control 1)
C	Mice immunized with naïve red blood cells culture supernatants and challenged with wild-type *P. berghei*(control 2)
D	Non-immunized group but challenged with wild-type *P. berghei* (control 3)

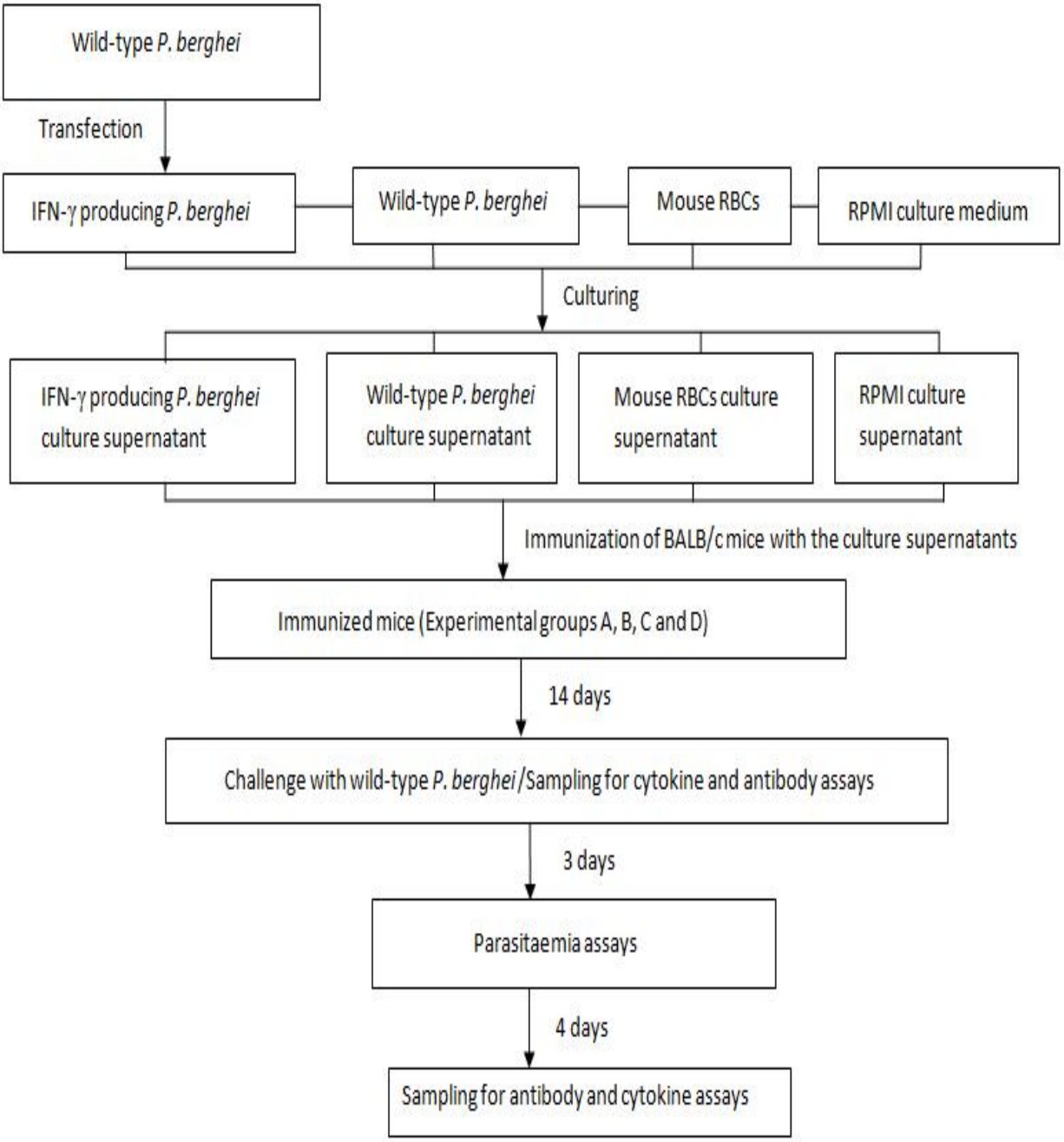

Figure 3: Experimental design (Source: Adapted from Diana *et al*., 2006)

2.3 Transgenic expression of mouse interferon gamma in *P. berghei*

Plasmodium berghei parasites were transfected with a pUC plasmid expressing mouse IFN-γ and DHFR-TS genes, meant to facilitate IFN-γ expression and selection of the transformed parasites respectively (Fig. 4). The wild-type *P. berghei* are pyrimethamine sensitive and would be cleared by the drug while the transfected parasites are pyrimethamine resistant and would survive through the drug pressure.

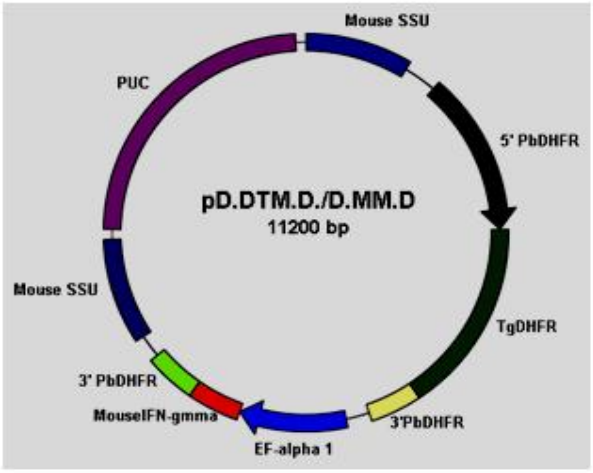

Figure 4: Map of plasmid used to transform *P. berghei*: the relative position of the genes and their relative sizes: Mouse SSU - 1 Kb, 5'PbDHFR - 1.5 Kb, TgDHFR - 1.8 Kb, 3'PbDHFR - 0.5 Kb, EF-alpha 1 - 1Kb, Mouse IFN-γ - 0.5Kb, 3'PbDHFR - 0.5Kb and pUC - 2.7Kb.

2.3.1 Preparation of the transfection constructs

The mouse IFN-γ expression vector transfection construct was prepared by isolating the open reading frame (ORF) of murine IFN-γ by restriction digestion of the murine IFN-γ cloning vector with the help of *Xba*I and *Spe*I restriction enzymes. Klenow polymerase was used to extend it and purification done using Qiagen gel extraction kit. The IFN-γ gene was cloned into the blunted *Bam*HI site of the plasmid (pD.D_{TM}.D./D.-.D.) to generate (pD.D_{TM}.D./D.$_{\gamma MM}$.D). Multiplication of the transfection constructs copies was

achieved by transformation of *E. coli* cells (DH5-α) using the transfection constructs followed by isolation of the transformed cells. The competent DH5-α cells were transformed using heat shock method as described by Panja (2008). Competent DH5-α cells (Invitrogen sub-cloning efficiency) were transformed using heat shock method.

Five microlitre of plasmid sample was transferred into 15ml centrifuge tubes (Corning®, USA) and 100µl of the competent DH5α cells added to the plasmids. After 30 minutes of incubation at room temperature, the plasmid/bacterial cells mixture was put in a water bath (Precision scientific, CAT NO. 66798) set at 42°C for 20 seconds. The tubes were immediately returned to ice and allowed to incubate for 2 minutes. One milliliter of non-selective LB broth was added to the mixture and incubated for 1 hour at 37°C, with shaking in an incubator shaker. The cell suspensions were plated on agar plates containing 50µg/ml of ampicilin. These were then incubated at 37°C overnight. After the overnight incubation, growth of white single colonies was checked for (an indicator of transformation). Single bacterial colonies were picked, and each colony transferred into a different culture tube containing 3ml selective LB broth with 50µg/ml ampicilin. These were placed on an incubator shaker at 37°C, shaking at 15g for 18 hours to allow multiplication of the cells.

2.3.2 Extraction of the Plasmid DNA from DH 5-α Cells

The plasmid DNA extraction was done by the alkaline lysis method as described by Birnboim and Doly (1979). The bacterial cultures were transferred into microfuge tubes (Eppendorf), centrifuged at maximum speed in microfuge (Eppendorf Centrifuge Model 5415) for 1 minute and then the supernatant carefully poured off. One hundred microliter of ice cold laboratory prepared alkaline lysis solution I, P1 (50mM glucose, 25mM Tris, 0.5M EDTA) was added to each pellet. These were resuspended by vortexing (Wilten and Co. Ltd, Holland) and left to stand at room temperature for two minutes.

Two hundred microliter (µl) of alkaline solution II, P2 (0.2M NaOH and 1% SDS in 250ml distilled water) was added and the tube inverted severally and rapidly to thoroughly mix the solutions. This was followed by an addition of about150µl of alkaline lysis solution III,P3 (5M KAC and 5.75ml glacial acetic acid in 14.25ml double distilled water) and then the tubes inverted severally and incubated in ice for 5 minutes. The mixture was centrifuged at maximum speed in the microfuge (Eppendorf Centrifuge Model 5415) for 5 minutes. The supernatant was transferred to tubes corresponding in labels to the original ones. Then, 270µl of isopropanol at room temperature was added. The mixture was vortexed and allowed to stand for 2 minutes at room temperature.

The tubes were centrifuged at maximum speed for 5 minutes in a microfuge (Eppendorf Centrifuge Model 5415) and the supernatant carefully poured off. Approximately 1 ml of 70% ethanol was added and the tubes inverted several times. The plasmid DNA was recovered by centrifuging at maximum speed for 3 minutes (Eppendorf Centrifuge Model 5415). Carefully, the supernatant was removed and excess ethanol allowed to drain off, on paper towels. Tris EDTA (30µl) with RNase was added to each pellet and the tubes gently tapped to enhance pellets dissolution.

2.3.3 Confirmation of correct plasmid extraction

To confirm correct plasmid extraction, 1.0% agarose gel (Gibco BRL, CAT NO.540-551013) was prepared by dissolving 1.0g of agarose into 100ml of 1 × TBE buffer, stirred and let to boil in a microwave for 3 minutes so as to allow for complete dissolution of the powder. Ethidium bromide (3µl) was added to the gel solution to facilitated DNA visualization under UV. The solution was put into a casting tray. Randomly picked samples (5µl each) were mixed with 3µl bromophenol blue (loading dye) run on agarose gel and DNA bands visualized under ultraviolet light. Restriction digestion of the extracted DNA was done using two enzymes (EcoRI and EcoRV), separately. One µl of 10x buffer and 1µl of restriction enzyme was added to about 3µl of the extracted sample and then the mixture topped up with 5µl of triple distilled water. This mixture was incubated in a water bath at 37°C (Precision scientific, CAT NO.

66798) for 1 hour to allow for the restriction digestion after which it was loaded onto agarose gel and visualization and photography of the bands done (Doolan, 2002).

2.3.4 Concentration of the plasmid DNA for transfection

This was achieved by ethanol precipitation (Doolan, 2002). The isolated plasmid DNA samples were pooled into microfuge tubes (Eppendorf). Each tube contained 300µl of the sample. Potassium acetate (30µl,) at a concentration of 5M was added to each tube. Then, isopropanol (180µl) was added and the tubes kept overnight at -20°C. The tubes were then be spun at 1600g (Beckman GPR centrifuge) for 15 min at 4°C, the supernatant drained off on paper towels and then 70% ethanol (600µl) added. The tubes were incubated at room temperature for 10 min and then spun at maximum speed for 5 minutes. Carefully, the supernatant was drained off on paper towels and the pellet left to dry. The DNA construct was then resuspended in 300µl sterile cytomix, for each DNA construct.

2.3.5 Propagation of *P. berghei* parasites for transfection

Propagation was done in accordance with the protocols described by Doolan (2002). The aim was to obtain enough parasite stock for transfection. *Plasmodium berghei* ANKA parasites donated by KEMRI were obtained from the Institute of Primate Research cryopreserved isolates. The parasites were quickly thawed in a 37°C water bath (Precision scientific CAT No. 66798) and an equal volume of 1 x PBS added. This PBS/parasite mixture was then injected into 6 mice (100µl of parasite/PBS mixture per mouse). From day 3 post-infection, thin blood smears were made on microscope slides, air dried, fixed with methanol, air dried and then flooded with 10% Giemsa stain (Loba Chemie, Lot No. G57181104, India) for 15 minutes. The slides were washed, air dried and then examined under a microscope using immersion oil. Percentage parasitaemia was determined by counting the number of parasitized blood cells out of at least 2000 red blood cells in random fields of the microscope (CARL ZEISS, Standard 20, West Germany) and expressed as described by Munyao *et al*, (2008).

At parasitaemia of between 4% and 5%, the mice were euthanized by carbon dioxide asphyxiation and bled via cardiac puncture. Heparinized blood from all the mice was pooled into a 50ml centrifuge tube (Corning, USA, Product No. 430290) containing complete RPMI 1640 culture medium. The complete culture medium was prepared by supplementing 150ml RPMI 1640 medium (Gibco, Lot No.RNBC0616) with 7.6ml of 25mM HEPES (Sigma, Lot No. BCBG2879B), 2ml of 200 mM L-glutamine, and 25% fetal bovine serum and 100µl Neomycin (Sigma, Lot No. 097K2326) and topping up to 200ml by adding the RPMI 1640. This blood was centrifuged at 200g for 10 minutes (Beckman GPR centrifuge) and then the parasites resuspended in the complete culture medium at 2.5% hematocrit. The cultures were then transferred into a T-75 culture flask (corning flask, USA) and gassed for 2 minutes using 5% O_2, 5% CO_2 and 90% N_2. It was then incubated overnight at 37^{o}C (CO-150, New Brunswick Scientific) for development into mature schizont stage.

2.3.6 Purification of mature *P. berghei* schizonts

Purification of mature schizonts was done as described by Janse *et al*, (2006b) with some slight modifications as follows. Parasite development to healthy mature schizont stages in culture was confirmed by making thin smears from cultures and observing under light microscope (CARL ZEISS, Standard 20, West Germany). The culture suspension was then transferred to a 50ml centrifuge tube (Corning, USA, Product No. 430290). Then, 55% Nycodenz solution was gently layered underneath the suspension using a 10ml serological pipette (Eppendorf). The cultures were then centrifuged at 200g in a swing out rotor (Beckman GPR centrifuge) for 30 min at 24ºC. The brown layer (schizonts) at the interface was carefully collected with a pasteur pipette into a clean 50 ml centrifuge tube. Complete RPMI 1640 was added from the top of the nycodenz density gradient into the 50ml tube to help wash away the nycodenz by spinning the tube at 200g for 15 min at 24ºC (Beckman GPR centrifuge). The supernatant was gently pipetted out and the pellet (schizonts) resuspended in 500µl of plain RPMI 1640 culture medium (Gibco, Lot No.RNBC0616) ready for each transfection.

2.3.7 Electroporation of *P. berghei*

Plasmodium berghei electroporation involved generation of temporary pores in the cell membrane of the parasite so that transfection plasmid DNA would enter the parasite through these pores. Into about 300 µl sterile cytomix containing the transfection plasmid DNA, 100µl of schizont suspension was added. Aseptically, the mixture was transferred into a 0.4 cm electroporation cuvette and immediately transferred into the electroporation machine (Bio Rad gene Pulse^{TM}). This suspension was subjected to a single pulse (1000 V, 25 F) of the Bio-Rad genepulser I electroporation machine and immediately transferred into ice for 5 minutes. About $5x10^9$ merozoites were intravenously inoculated into the tail vein of each of five BALB/c mice.

2.3.8 Selection and storage of transfected *P. berghei*

This was achieved through pyrimethamine drug (Sigma EEC NO.200-364-2, USA) treatment in drinking water in accordance with the protocols described by Doolan (2002). Parasites not harboring transfected DNA would be cleared by the drug while those harboring the transfected DNA would continue growing. The mice that were intravenously inoculated with the electroporated parasites were treated 0.007mg/ml pyrimethamine in drinking water 25 hours after injection of parasites. The drug treatment was done for 4 consecutive days, stopped for the next 4 days and then continued when parasites were detectable in the thin blood smears once again. This constituted the transfected *P. berghei* parasites (Munyao *et al*., 2008).

At parasitaemia of 1%, the mice were euthanized by carbon dioxide asphyxiation and the blood containing the transfected parasites inoculated into four donor mice to expand parasites in numbers. Each mouse was inoculated with 100µl of the blood. The excess blood was subjected to cryopreservation upon addition of an equal volume of freezing solution (30% glycerol in sterile PBS). Parasitaemia was monitored daily till parasitaemia of between 4% and 5%. Blood was collected from the mice through cardiac puncture into 23-G needles (Ken Healthcare, China) attached to 1ml syringes (BD) flashed with

heparin solution (Rotexmedica, Batch NO.80216, Germany). This infected blood was mixed with an equal volume of the freezing solution, aliquoted into cryovials (500µl per cryovial) and left to stand at 4°C for 5 minutes. This was then transferred to liquid nitrogen for storage till use.

2.3.9 Confirming the success of *P. berghei* transfection

This was achieved by plasmid rescue of circular transfection vectors from the transfected parasites followed by restriction digestion of the plasmid DNA and PCR. Total parasite DNA was isolated as described by Birnboim and Doly (1979) and used to transform competent *E. coli* by electroporation. The DH 5-α cells and total parasite DNA were mixed and the mixture incubated in ice for 30 minutes. The DNA/cell mixture was transferred into an electroporator set at 2.4Kv, 25µF and 200 Ω (Bio Rad gene Pulse^{TM}). After pulsing, the mixture was immediately transferred to a culture tube, LB broth added and incubation done at 37°C for 1 hr with shaking at15g. The cell suspensions was plated on LB agar plates containing 50µl/ml ampicilin and cultured overnight at 37°C. Singled bacterial colonies were picked and cultured in LB broth containing 50µg/ml ampicilin for 18hr after which plasmid isolation from the DH 5-α cells was carried out. The isolated plasmid DNA was subjected to restriction digestion and then diluted to a concentration of 50ng/µl.

Then, polymerase chain reaction (PCR) was performed to confirm the integrity of the mouse IFN-γ gene and the *T. gondii* DHFR gene. Each sample tube contained a total or 25µl of reaction mixture (2.5µl of 10x PCR buffer, 1.5µl of 25mM $MgCl_2$, 0.5µl of Taq, 2.5 µl of forward primer, 2.5µl of reverse primer, 1µl of dNTPs, 1µl of sample DNA and 13.5µl of triple distilled water). The PCR programme was set as follows; Initial denaturation temperature (94°C for 3 minutes), 30 cycles of denaturation (94°C for 1 minute), annealing (54°C for 30 seconds), elongation (72°C, 1 min) and a final elongation (72°C for 4 minutes).

2.3.11 Culturing of the transfected *P. berghei*

This was done to facilitate development of the transfected *P. berghei* to schizont stage and subsequent release of interferon gamma into culture media. The parasites were retrieved from cryopreservation, passaged in mice thorough intraperitoneal injection and parasitaemia monitored daily as from day 3 post-infection. At parasitaemia of between 3% and 5%, the mice were euthanized by carbon dioxide asphyxiation and the parasitized cells collected, mixed with an equal volume of 1×PBS and injected into 6 mice for *in vivo* propagation. Parasitaemia was monitored daily from day 3 post-infection. At parasitaemia of between 4% and 6%, the mice were bled via cardiac puncture and blood from all the mice pooled. The blood was washed thrice with an equal volume of plain RPMI 1640 (Gibco, Lot No.RNBC0616) at 200g (Beckman GPR centrifuge) for 10 minutes at 24°C. The supernatant and top layer of blood cells and other blood factors were discarded. The pellet (1ml at 5% parasitaemia) constituting of about 4.5 $\times 10^8$ parasites was diluted at 2.5% hematocrit in complete culture medium. The complete culture medium was prepared by supplementing 150ml RPMI 1640 medium (Gibco, Lot No.RNBC0616) with 7.6ml of 25mM HEPES (Sigma, Lot No. BCBG2879B), 2ml of 200 mM L-glutamine, and 25% fetal bovine serum and 100µl Neomycin (Sigma, Lot No. 097K2326) and topping up to 200ml by adding the RPMI 1640.

The culture was aseptically transferred into a T-75 culture flask (corning flask, USA), gassed (90% N_2, 5% O_2 and 5% CO_2) and incubated overnight at 37^0C for 12 hours, with agitation at regular intervals, for development into schizont stage. After the 12 hours, the culture was washed once at 200g (Beckman GPR centrifuge) for 10 minutes at room temperature. The pellet (1ml) was resuspended at 20% hematocrit in complete RPMI culture medium, transferred into a T-25 culture flask (corning flask, USA), gassed and incubated for 6 hours for interferon gamma expression. The culture was then spun at 200g (Beckman GPR centrifuge) for 10 minutes at 24°C and the supernatant collected (about 4ml). The supernatant was then filter-sterilized using 0.45um-pore membrane

syringe filters (Millipore Co., USA) and aliquots taken for interferon gamma expression confirmation and quantification by ELISA (Mabtech, Sweden) as per the manufacturer's instructions. The same protocol was adopted for culture of wild-type *P. berghei* (about 1ml pellet at 5.3%) and red blood cells (about 1ml pellet) from uninfected mice for control experiments (Munyao *et al*., 2008).

2.3.12 Confirmation of Interferon gamma expression by the transfected *P. berghei* parasites

This was to confirm if the transformed parasites would produce interferon gamma. Mouse interferon gamma ELISA was performed on the collected parasites culture supernatant using a kit (Mabtech AB, Sweden, code: 3321-1A-6) according to the manufacturer's instructions. A high protein binding ELISA plate (Dynatech, USA, Cat NO: 0110103455) was coated with MAb AN18, diluted to 1µg/ml in PBS, pH 7.4, by adding 50µl/well. It was incubated overnight at 4^{o}C. The following day, the plate was washed twice with 100µl of PBS per well. The plate was then blocked by adding 100µl/well PBS with 0.05% Tween containing 0.1% BSA (incubation buffer). It was incubated for 1 hour at room temperature and washed 5 times with PBS containing 0.05% Tween. The mouse IFN-γ standard was prepared by reconstituting recombinant mouse IFN-γ in 1ml PBS with 1% BSA to a concentration of 1µg/ml. The tube was left at room temperature for 15 minutes and then vortexed. This stock was put into aliquots of 20µl and kept at -20°C for future use. For immediate use, dilutions of the stock were prepared using the given standard range (4-400pg/ml) as a guideline.

Approximately, 50µl/well of the sample and standard diluted in incubation buffer were added and incubated at room temperature for 2 hours after which the plate was washed 5 times with PBS containing 0.05% Tween. Then, 50µl/well of mAbR4-6A2 biotin at 0.5µg/ml in incubation buffer was added and incubated for 1 hour at room temperature. The plate was washed 5 times as above, 50µl/well of streptavidin-ALP diluted 1:1000 in incubation buffer added and incubated at room temperature for 1 hour. The plate was

washed 5 times as above and 50µl/well of p-nitrophenyl-phosphate (Sigma, USA, product code: 1001231716) added. Optical density was read, after 30 minutes, at 405nm in an ELISA reader (Dynatech MRX) and a standard curve used to determine the concentration of IFN-γ in the samples.

2.5 Determination of Parasitaemia and Survival rates of mice

This was to determine if immunizing mice with interferon gamma culture supernatants would protect mice against a challenge infection by wild-type *P. berghei.* Clean mice were each inoculated with 37.5pg of parasite expressed mouse interferon gamma in 200µl of transfected *P. berghei* derived culture supernatant. Fourteen days later, they were challenged by intraperitoneal injection of wild-type *P. berghei* suspension containing 1×10^6 parasites. From day 4 post-challenge onwards daily, thin smear blood films were taken from the peripheral blood of the tail of each mouse in the test and control groups. Thin blood smears were made on microscope slides, air dried, fixed with methanol, air dried and then flooded with 10% Giemsa stain for 15 minutes. The slides were washed, air dried and then examined under a microscope (CARL ZEISS, Standard 20, West Germany) using immersion oil. Percentage parasitaemia was determined by counting the number of parasitized blood cells out of at least 2000 red blood cells in random fields of the microscope and expressed as described by Munyao *et al*. (2008). Survival rate was determined by measuring the survival time (days) after challenge. The same protocol was adopted for determination of parasitaemia and survivorship of the control groups which included a group of mice pre-treated with culture supernatants from wild-type *P. berghei* and another group that was pre-treated with red blood cells from uninfected mice. Each treatment group constituted 5 mice at each sampling point.

2.6 Determination of Antibody and Cytokine levels

This aimed at determining whether the *P. berghei* expressed interferon gamma affected the host antibody and cytokine responses to challenge infection by wild-type *P. berghei.*

The levels of total IgG in serum and, IFN-γ and IL-4 in splenocyte culture supernatant were determined.

2.6.1 Serum preparation for antibody ELISA

For antibody assays, five mice from each treatment group were euthanized by carbon dioxide asphyxiation on day 14 after inoculation with the *P. berghei* expressed IFN-γ culture supernatants. The mice were bled via cardiac puncture and their blood kept in separate microfuge tubes for each mouse, on the bench till the blood formed a clot, and then transferred to +4°C overnight. The clots were then disturbed using wooden splints and centrifuged in a microfuge (Eppendorf Centrifuge Model 5415) at room temperature for 3 minutes at 200g. The supernatant (serum) was transferred into other appropriately labeled tubes and stored at -20°C to be used later for antibody ELISA, (Mabtech, Sweden) according to the manufacturer's instructions.

2.6.2 Splenocyte preparation and culture for cytokine ELISA

Cytokine production by the cells was determined by measuring the amount of the cytokines secreted into culture media during splenocyte culture. Splenocyte preparation and culture was done as described by Yole *et al*. (2006). After drawing blood for serum preparation, spleens were asceptically removed from the mice of each treatment group. Each spleen was homogenized using a sterile syringe plunger and fine wire mesh in a sterile petri-dish (Greiner, Germany) containing 2ml incomplete RPMI 1640 medium (Gibco, Lot No.RNBC0616) and cells centrifuged at 4°Cfor 10 min at 200g (Beckman GPR centrifuge). The cell-containing pellets was washed three times with RPMI 1640 supplemented with 5% FBS (Gibco). The supernatant was poured off and the cells suspended in 50ml centrifuge tubes (corning) containing laboratory prepared erythrocytes lysing solution (4.15g Ammonium chloride (NH_4Cl), 50ml 0.1 Tris HCl, made up to 500ml with H_2O distilled, pH 7.5 and filtered with 0.22µm filter). The cells were then washed by centrifuging at 10 min at 200g at 4°C (Beckman GPR centrifuge). The washing was repeated till the cells were clear (freed of erythrocytes). The splenocytes

were resuspended in RPMI 1640 culture medium (Gibco, Lot No.RNBC0616) supplemented with 10% FBS (Gibco,, Auckland, New Zealand), gentamycin (50µg/ml), and 5 x 10^{-5} Beta-mercaptoethanol (Sigma, USA, Product No. M3148), to make a cell suspension. Splenocyte viability was determined by trypan blue exclusion test (Sigma, Germany, Product No. T8154) and a haemocytometer (Hausser Scientific, USA, Cat. No. 1492).

Single cell suspension of isolated splenocytes were resuspended to a concentration of 2 x 10^5 cells per ml and plated in 48-well flat-bottom culture plates (Corning). Cultures in duplicate were stimulated with 0.05 pg/ml of the lysate antigen prepared from the IFN-γ expressing *P. berghei* parasites. The antigen was prepared as described by Cemalettin, (1997). Concanavalin A (Sigma, St. Louis, Mo.) and RPMI were used as positive and unstimulated controls, respectively. After 48 hours in a humidified 5% CO_2 incubator at 37°C (CO-150, New Brunswick Scientific), supernatants were carefully aspirated into labeled serum tubes and stored at -80°C to be used later for IL-4 and IFN-γ ELISA.

2.6.3 Splenocytes storage

The surplus cells suspended in culture medium as described in section 2.6.2 were spun down at 4°C for 10 minutes at 200g (Beckman GPR centrifuge). The supernatant was poured off and the pellet dislodged. These remaining cells were then stored at a concentration of 20 x 10^6 cells/ml in a freezing solution of 90% FBS (Gibco) in DMSO (Sigma, Lot No. 097K2326) for 15 minutes in a freezing chamber. The cells were then kept overnight at -80°C and finally stored in liquid nitrogen for further use (Yole *et al.*, 2006).

2.6.4 Mouse interferon gamma ELISA

Enzyme Linked Immunosorbent Assay for mouse interferon gamma was performed using a kit (Mabtech AB, Sweden, code: 3321-1A-6) according to the manufacturer's instructions. A high protein binding ELISA plate (Dynatech, USA, Cat NO: 0110103455)

was coated with MAb AN18, diluted to 1µg/ml in PBS, pH 7.4, by adding 50µl/well. It was incubated overnight at 4°C. The following day, the plate was washed twice with 100µl of PBS per well. The plate was then blocked by adding 100µl/well PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer). It was incubated for 1 hour at room temperature and washed 5 times with PBS containing 0.05% Tween 20. The mouse IFN-γ standard was prepared by reconstituting recombinant mouse IFN-γ in 1ml PBS with 1% BSA to a concentration of 1µg/ml. The tube was left at room temperature for 15 minutes and then vortexed. This stock was put into aliquots of 20µl and kept at -20°C for future use. For immediate use, dilutions of the stock were prepared using the given standard range (4-400pg/ml) as a guideline. Approximately, 50µl/well of the sample and standard diluted in incubation buffer were added and incubated at room temperature for 2 hours after which the plate was washed 5 times with PBS containing 0.05% Tween 20. Then, 50µl/well of mAbR4-6A2 biotin at 0.5µg/ml in incubation buffer was added and incubated for 1 hour at room temperature. The plate was washed 5 times as above, 50µl/well of streptavidin-ALP diluted 1:1000 inincubation buffer added and incubated at room temperature for 1 hour. The plate was washed 5 times as above and 50µl/well of p-nitrophenyl-phosphate (Sigma, USA, product code: 1001231716) added. Optical density was read after 30 minutes at 405nm in an ELISA reader (Dynatech MRX) and a standard curve used to determine the concentration of the samples.

2.6.5 Mouse Interleukin-4 ELISA

Mouse Interleukin-4 ELISA was performed using a kit (Mabtech AB, Sweden, code: 3311-1A-6) according to the manufacturer's instructions. A high protein binding ELISA plate (Dynatech, USA, Cat NO: 0110103455) was coated with mAb 11B11, diluted to 2µg/ml in PBS, pH 7.4 and filtered through 0.2µm syringe filter (Millipore Co., USA), by adding 50µl/well. Then, it was incubated overnight at 4°C. The following day, the plate was washed twice with 100µl of PBS per well. The plate was then blocked by adding 100µl/well PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer). It was incubated for 1 hour at room temperature and washed 5 times with PBS containing 0.05%

Tween 20. The mouse Interleukin-4 standard was prepared by reconstituting recombinant IL-4 in 1ml PBS to a concentration of 1µg/ml. The tube was left to stand at room temperature for 5 minutes and mixed thoroughly. This stock was put into aliquots of 20µl and kept at -20°C for future use. For immediate use, dilutions of the stock were prepared using a given standard range (4-400pg/ml) as a guideline. Approximately, 50µl/well of the sample and standard diluted in incubation buffer were added and incubated at room temperature for 2 hours after which the plate was washed 5 times with PBS containing 0.05% Tween 20. Then, 50µl/well of mAb BVD6-24G2 biotin at 0.1µg/ml in incubation buffer was added and incubated for 1 hour at room temperature. The plate was washed 5 times as above, 50µl/well of streptavidin-ALP diluted 1:1000 in incubation buffer added and incubated at room temperature for 1 hour. The plate was washed 5 times once again as above and 50µl/well of p-nitrophenyl-phosphate (Sigma, USA, product code: 1001231716) added. Optical density was read after 30 minutes at 405nm in an ELISA reader (Dynatech MRX) and a standard curve used to determine the concentration of the samples.

2.6.6 Total IgG ELISA

This was performed using mouse IgG kit instructions (Mabtech AB, Sweden, code: 3825-1AD-6) according to the manufacturer's. A high protein binding ELISA plate (Dynatech, USA, Cat NO: 0110103455) was coated with anti-IgG antibody, diluted to 1µg/ml in PBS, pH 7.4, by adding 50µl/well. It was incubated overnight at 4^{o}C. The following day, the plate was washed twice with 100µl of PBS per well. The plate was then blocked by adding 100µl/well PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer). It was incubated for 1 hour at room temperature and washed 5 times with PBS containing 0.05% Tween 20. The mouse IgG standard was prepared by reconstituting the recombinant IgG in 500µl PBS to make a stock solution of 50µg/ml. This stock was put into aliquots of 20µl and kept at -20°C for future use. For immediate use, dilutions of the stock were prepared using the given standard range (4-400pg/ml) as a guideline. Approximately, 50µl/well of the sample and standard diluted in incubation buffer were

added and incubated at room temperature for 2 hours after which the plate was washed 5 times with PBS containing 0.05% Tween 20. Then, 50µl/well of anti-IgG-ALP diluted at1:1000 in incubation buffer was added and incubated for 1 hour at room temperature. The plate was washed 5 times as above and 50µl/well of p-nitrophenyl-phosphate (Sigma, USA, product code: 1001231716) added. Optical density was read after 45 minutes at 405nm in an ELISA reader (Dynatech MRX) and a standard curve used to determine the concentration of the samples.

2.7 Ethical considerations

Approval to conduct the study was obtained from the Institute of Primate Research (IPR) scientific steering committee and ethical review committee. The proposal was reviewed based on the scientific merit of the study and ethical handling of the experimental animals.

2.8 Data management and analysis

Data collected was recorded in a laboratory workbook and MS excel worksheets as Means ± SD of the test variables per mouse per group. Statistical analysis of the differences between mean values obtained for the treatment groups was done by analysis of variance (ANOVA) using graphpad instat software (GraphPad software, inc. USA). All the significantly different means were further analyzed using the Student's Least significant Difference (LSD). *P* values of < 0.05 were considered significant.

CHAPTER THREE

RESULTS

3.1 Determination of the Expression of mouse IFN-γ by transgenic *P. berghei*

3.1.1 Deoxyribonucleic analysis of the transfected parasites

This was done to confirm whether the wild-type *P. berghei* had been successfully transfected. The success of the transfection was confirmed by plasmid rescue of circular transfection vectors from the transfected parasites followed by DNA analysis. A plasmid rescue recovered plasmids from the IFN-γ expressing *P. berghei* parasites (plate1). The IFN-γ gene used in the transfection was of molecular size, 0.5Kb and was confirmed to be present in the DNA isolated from the transfected parasites (plate 2). The DHFR gene was of molecular size, 1.8Kb. It was confirmed to be present in the DNA samples isolated from the transfected parasites (Plate 3). This showed that both the mouse IFN-γ gene and the mutagenised DHFR gene were intact in the transformed parasites.

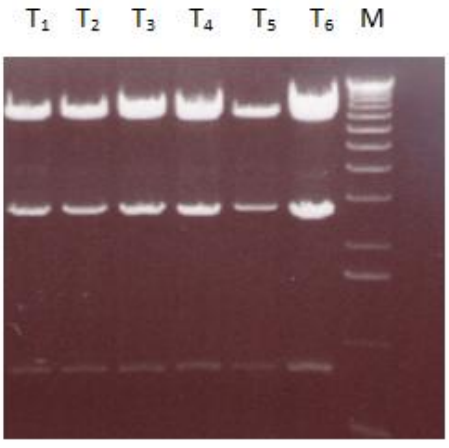

Plate 1: Gel electrophoresis of the products of the plasmid rescue experiment: M - molecular marker; T_{1-6} - DNA samples isolated from IFN-γ expressing *P. berghei*.

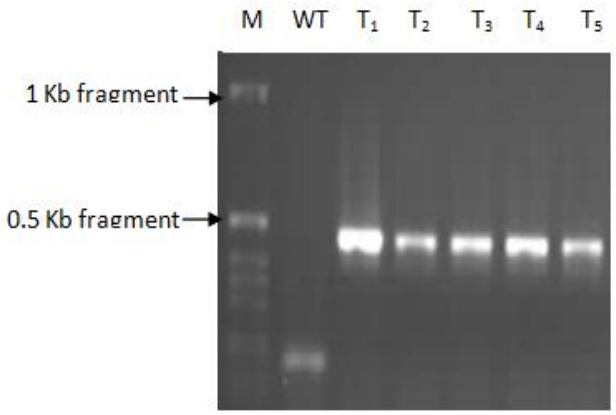

Plate 2: Gel electrophoresis of PCR product to show the integrity of mouse IFN-γ gene in the transformed *P. berghei*: M - molecular marker; T_{1-5} - DNA samples isolated from the IFN-γ expressing *P. berghei*; WT - DNA sample isolated from wild-type *P. berghei.*

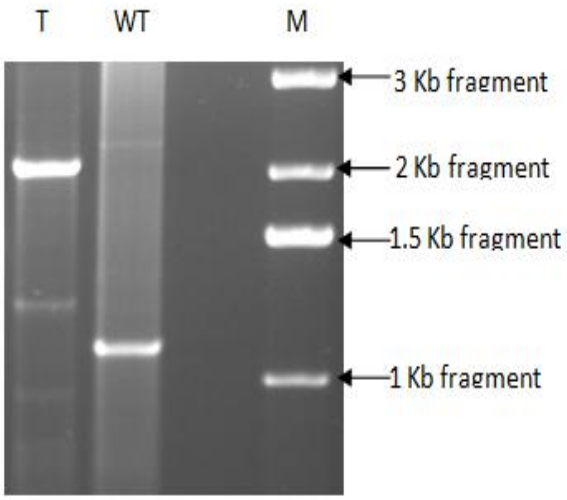

Plate 3: Gel electrophoresis of PCR product to show the integrity of *Tg*DHFR-TS gene in the IFN-γ expressing *P. berghei*: M - molecular marker; T - DNA samples isolated from the IFN-γ expressing *P. berghei*; WT - DNA sample isolated from wild-type *P. berghei*.

3.1.2. Quantification of interferon gamma in culture supernatant

This was done to determine if the transfected parasites would produce interferon gamma. The culture supernatants obtained upon culture of the IFN-γ expressing *P. berghei*, the wild-type *P. berghei* and the uninfected red blood cells were subjected to sandwich ELISA to determine the concentration of mouse IFN-γ released in culture. Interferon gamma production in the culture set up containing the IFN-γ expressing *P. berghei* was 9 fold higher than in the control culture set ups (Table 2).

Table 2: Quantities of interferon gamma in the parasites culture supernatants

Culture type	IFN-γ concentration (pg/ml)
Transfected *P. berghei* culture supernatant	187.52
Wild type *P. berghei* culture supernatant	20
Red blood cells culture supernatant	< 20

3.2 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on parasitaemia in mice challenged with wild-type *P. beghei*

The results were as shown in Fig 5. The group immunized with IFN-γ expressing *P. berghei* culture supernatant exhibited a 4 day delay in patent parasitaemia in thin blood smears, compared to other treatment groups**.** In this group, patent parasitaemia was first noted on day 7 post-infection. The parasite growth rate in this group was highly reduced with parasitaemia suppression on day 11 being at 94.15% relative to the non-immunized control. The parasite growth rate in the other treatment groups was steady with an early patent parasitaemia (day 4 post-infection). Parasitaemia suppression on day 11 post-infection was only 8.81% in the group immunized with wild type *P. berghei* culture supernatant.

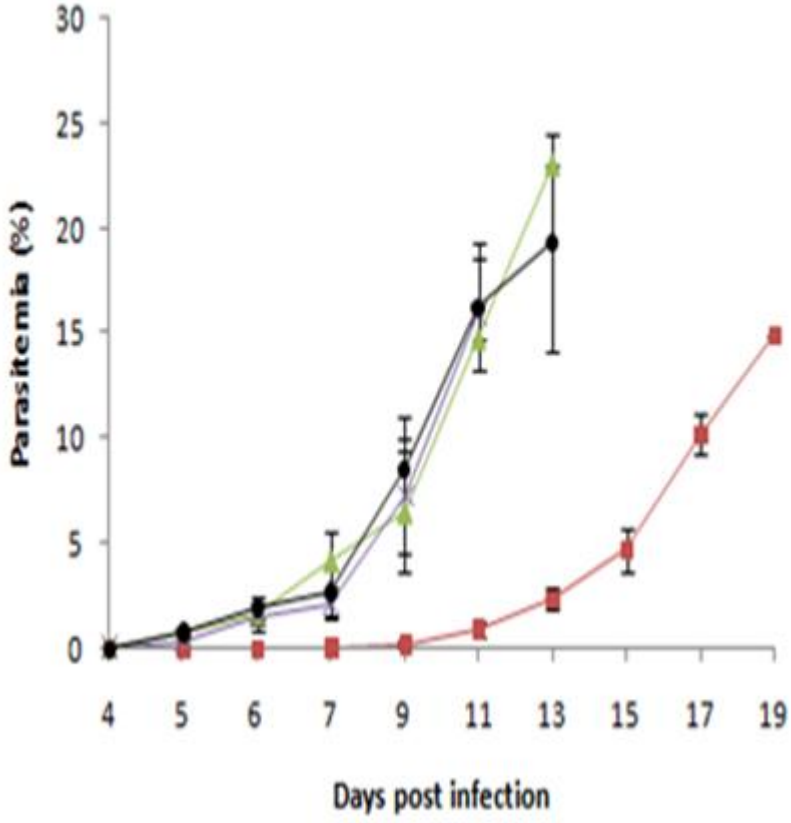

Figure 5: Comparative mean parasitaemia curves for the different treatment groups of mice upon challenge with wild-type *P. berghei* (Number of mice per group is 5) :—■— Immunized with interferon gamma expressing *P. berghei* culture supernatants; —▲— Immunized with wild-type *P. berghei* culture supernatants; —×— Immunized with naïve RBCs culture supernatants; —●— Non-immunized

3.3 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on the survival of mice challenge with wild-type *P. berghei*

This was done to show the protective potential of the culture supernatants in mice challenged with wild-type *P. berghei*. The group immunized with IFN-γ expressing *P. berghei* culture supernatant had a longer mean survival time. This group survived 5 days longer than the other treatment groups before the first deaths occurred. However, the mice in all the treatment groups succumbed to the infection by day 19 post-infection (Fig. 6).

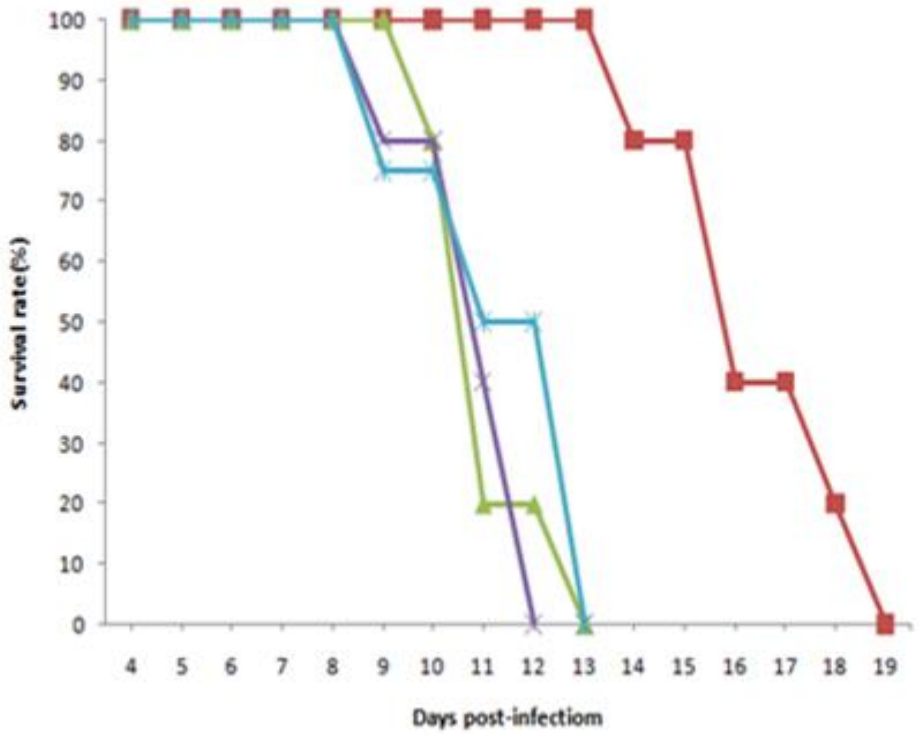

Figure 6: Survivorship curves for the different treatment groups of mice immunized with the culture supernatants and challenged with wild-type *P. berghei* (Number of mice per group is 5): —■— Immunized with interferon gamma expressing *P. berghei*

culture supernatants; Immunized with wild-type *P. berghei* culture supernatants; Immunized with naïve RBCs culture supernatants; Non-immunized.

3.4 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on cytokine and antibody responses in mice

This aimed at determining whether IFN-γ culture supernatants would immunomodulate the host response to wild-type *P. berghei* in a challenge infection.

3.4.1 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on IFN-γ production

Comparison of the different treatment groups before challenge showed no significant difference in the levels of IFN-γ ($p = 0.61$). However, after challenge, the group immunized with IFN-γ *P. berghei* culture supernatant exhibited significantly higher levels of IFN-γ ($p = 0.001$), (Fig. 7).

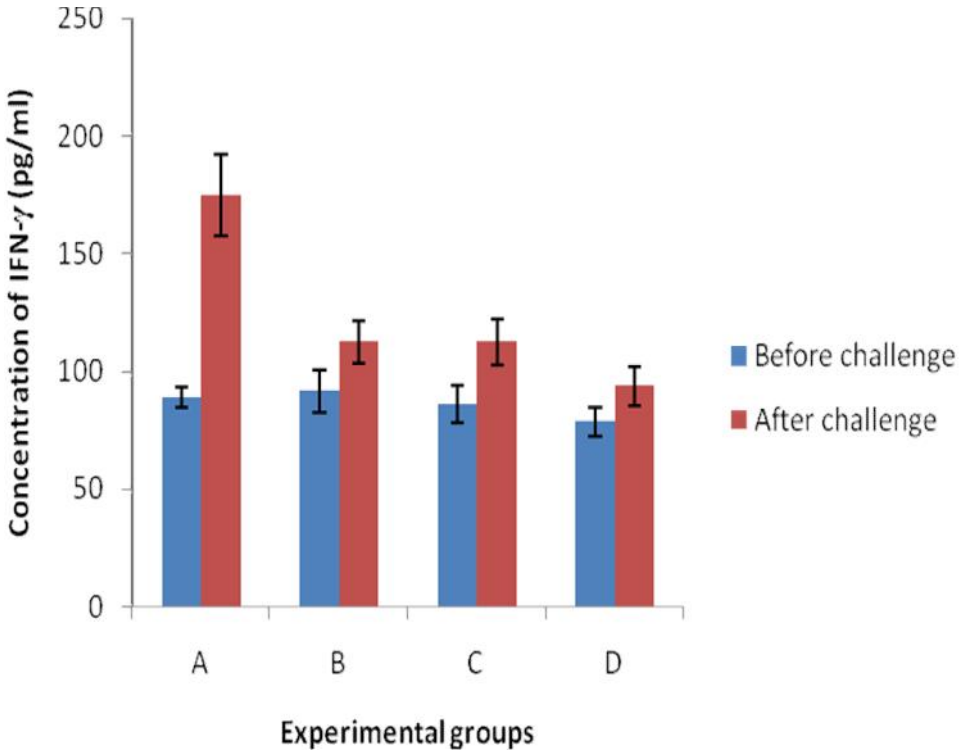

Figure 7: Concentrations (pg/ml) of interferon gamma before and after challenge with wild-type *P. berghei* (Number of mice per group is 10): A - Immunized with IFN-γ expressing *P. berghei* culture supernatant; B- Immunized with wild-type *P. berghei* culture supernatant; C- Immunized with red blood cells culture supernatant; D- Non-immunized.

3.4.2 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on IL-4 production

Comparison of the different treatment groups before challenge showed no significant difference in the levels of IL-4 ($p = 0.78$). However, after challenge, the group immunized with IFN-γ expressing *P. berghei* culture supernatant had a significantly low level of IL-4 ($p = 0.036$), (Fig. 8) compared to the other treatment groups.

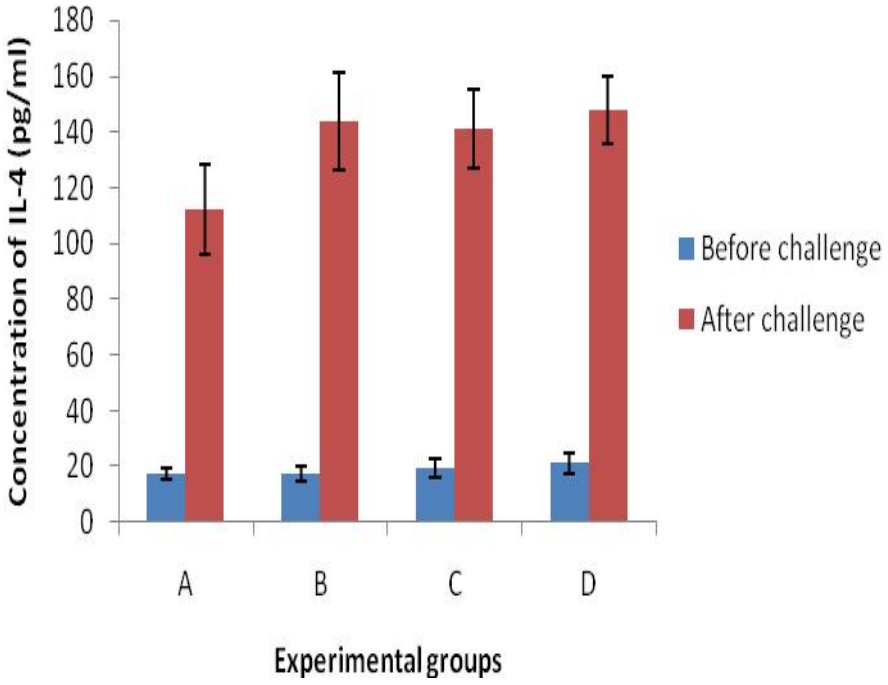

Figure 8: Concentrations (pg/ml) of interleukin-4 before and after challenge with wild-type *P. berghei* (Number of mice per group is 10): A- Immunized with IFN-γ expressing *P. berghei* culture supernatant; B- Immunized with wild type *P. berghei* culture supernatant; C- Immunized with red blood cells culture supernatant; D-Non-immunized.

3.4.3 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on IgG production

There was no significant difference in the levels of total IgG ($p = 0.09$) amongst all the treatment groups after challenge (Fig. 9). However, the group immunized with IFN-γ expressing *P. berghei* culture supernatant had a slightly lower level of IgG though statistically insignificant.

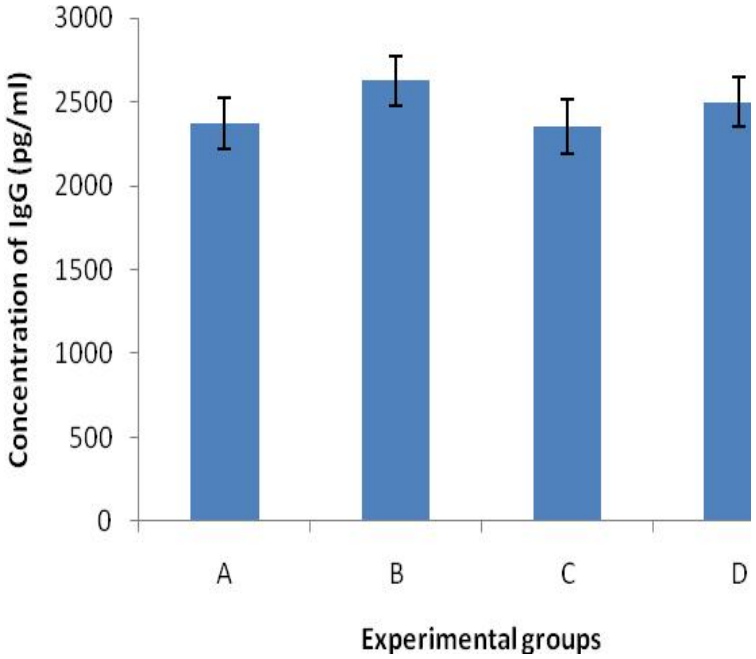

Figure 9: Concentrations (pg/ml) of immunoglobulin G after challenge with wild-type *P. berghei* (Number of mice per group is 5): A-immunized with IFN-γ expressing *P. berghei* culture supernatant; B - immunized with wild type *P. berghei* culture supernatant; C - immunized with red blood cells culture supernatant; D – non-immunized.

CHAPTER FOUR

DISCUSSION,CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

4.1 Overview

This study aimed at determining the immunomodulatory potential of the parasite expressed cytokine in a mouse malaria model. This would provide more insight into the possibility of utilizing immunopotentiated malaria parasites in malaria vaccine development strategies. The findings of this study have demonstrated the potential for culture supernatants of IFN-γ expressing *P. berghei* in protecting mice against virulent infection. This forms an important initial step in understanding the use of cytokines to immunopotentiate malaria parasites, as a component of molecularly attenuated parasite vaccines.

4.1.1 Determination of Interferon gamma expression by the transfected parasites

Plasmodium berghei transfected with mouse IFN-γ expressing plasmid produced significant amounts of mouse IFN-γ. This shows that the parasite transcribed the IFN-γ gene, assembled the protein in the right conformation and expresssed IFN-γ. This is in agreement with a previous study by Ozwara *et al*. (2003). However, it is not yet clear whether parasite expressed IFN-γ is secreted by the erythrocyte, or released upon schizont rupture. To maximize on interferon gamma release, the parasites in the current study were thus cultured to schizont stage. Schizont stage of the parasite was identified by presence of 12-16 ‘free’ merozoites within one red blood cell and one cluster of haemozoin pigment. A similar approach to identifying schizont stage of the *Plasmodium* parasites is described elsewhere (Menard, 2013). The IFN-γ detected in the control cultures, though in minimal concentrations (9 fold lower), may probably have originated from mouse lymphocyte cells. In the current study, the lymphocytes were not removed

before culturing. Lymphocytes such as natural killer cells, T cells, and dendritic cells are a potential source of IFN-γ (Inoue *et al*., 2013).

4.1.2 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on parasitaemia and survivorship of mice

Plasmodium berghei IFN-γ culture supernatants delayed parasitaemia patency and reduced mean parasitaemia during challenge infection with wild type *P. berghei*. Similar findings have been documented by Sally *et al*. (1990). The delay in parasitaemia patency and increased parasitaemia suppression coupled with prolonged survival of the mice with high levels of IFN-γ production upon challenge observed in this study is an indication of immunomodulation. Similarly, other studies have previously reported of an association between levels of IFN-γ and reduced parasitaemia in blood stage *Plasmodium* infection (Inoue *et al*., 2013; Rosette *et al*., 2013). It is probable that the cytokine enhances the ability of macrophages to destroy the *Plasmodium* parasites thereby limiting parasite progression and thus prolonging the survival of the mice. Interferon gamma is a potent pro-inflammatory cytokine, offering protection against intracellular bacteria, some viruses as well as parasites (Menard, 2013).

Interferon gamma up-regulates the activity of phagocytes leading to elimination of bacteria and parasitic protozoans (Inoue *et al*., 2013). It has also been reported that IFN-γ up-regulates IL-12β2 expression and thus priming the IFN-γ-producing Th1 cells (Das and Janeway, 2001). Therefore, it is possible that upon challenge, the primed Th1 cells produce cellular IFN-γ that acts on macrophages enhancing their ability to destroy the *Plasmodium* parasites.

4.1.3 Effects of *P. berghei* expressed mouse IFN-γ culture supernatant on Antibody and Cytokine responses

4.1.3.1 Effects of *P. berghei* expressed mouse IFN-γ culture supernatants on IFN-γ production

There was increased production of IFN-γ in the group immunized with the *P. berghei* expressed IFN-γ culture supernatants. The findings of this study concur with data obtained in previous studies (Sally *et al*., 1990). Exogenously inoculated IFN-γ primes IFN-γ-producing Th1 cells (Yoshifumi *et al*., 2002). This explains the increased production of IFN-γ in the group pre-treated with the *P. berghei* expressed IFN-γ culture supernatants. The involvement of IFN-γ in Th 1 priming has also been reported by Das and Janeway, (2001). The increased IFN-γ production, as observed in the other treatment groups in the current study is as a result of the strategy by the host immunity mechanism to limit parasite progression and thus evade succumbing to the infection. It has been reported elsewhere that survival of BALB/c mice from severe *P. berghei* infection involves up-regulation of the levels some pro-inflammatory cytokines and anti-inflammatory cytokines (Das & Janeway, 2001; Schofield & Grau, 2005; Funda *et al*., 2009).

4.1.3.2 Effects of *P. berghei* expressed mouse IFN-γ culture supernatant on IL-4 production

The group of mice that was immunized with IFN-γ expressing *P. berghei* culture supernatant exhibited a significantly low level of IL-4 production. In addition, this group of mice had significantly high levels of IFN-γ production. These results tally with the findings by Jungi *et al*. (1997). The reduced level of IL-4 production in the group of mice that was immunized with the parasite expressed IFN-γ culture supernatants was as a result of enhanced differentiation of naïve CD4 T cells into Th1 cells. Consequently, the Th1 cells, through the elevated production of IFN-γ, down-modulate Th2 cell function. IFN-γ is known to enhance differentiation of naïve T cells (Th0) into Th1 cells which in

turn down-regulate Th2 cell function through production of elevated levels IFN-γ (Jankovic *et al*., 2007; Sun *et al*., 2012).

4.1.3.3 Effects of Effects of *P. berghei* expressed mouse IFN-γ culture supernatant on IgG production

There was no significant difference in the levels of IgG produced amongst all the treatment groups after challenge with wild-type *P. berghei*. Other studies have demonstrated that IFN-γ is important in inducing B cells to produce $IgG2_a$ isotype, which is protective against *Plasmodium* infection (Waki *et al*., 2007). In addition, it has been reported that IFN-γ secreted by T-cells may help in induction of cytophilic IgG that may in turn take part in antibody-dependent protective mechanisms against *Plasmodium falciparum* (Malaguarnera & Musumeci, 2002). In the current study, it is evident that the IFN-γ culture supernatants had no marked impact on total IgG response in the mice. This could be explained by the fact that IFN-γ enhances differentiation of naïve CD4 T cells into Th1 cells. The elevated levels of Th1 cells enhance production of pro-inflammatory cytokines. The resultant Th1 cytokines down-modulate the production of the Th2 responses that are associated with antibody responses, given that Th1/Th2 responses are antagonistic. Th1 cells mediate antibody independent protection during *Plasmodium* infection (Sun *et al*., 2012).

4.2 Conclusions

1. Mouse interferon gamma gene transfected *P. berghei* expressed interferon gamma.
2. Parasite culture supernatants containing *P. berghei* expressed IFN-γ reduced parasite burden in mice challenged with virulent *P. berghei*.
3. Parasite culture supernatants containing *P. berghei* expressed mouse IFN-γ prolonged the survival of mice challenged with virulent *P. berghei*.

4. Parasite culture supernatants containing *P. berghei* expressed IFN-γ immunomodulated IFN-γ and IL-4 responses in mice challenged with virulent *P. berghei*.

   In general, *P. berghei* expressed mouse IFN-γ culure supernatants mediated protection of mice against challenge with wild-type *P. berghei*.

4.3 Limitations

It was not possible to purify transgenic mouse IFN-γ from the *P. berghei* culture supernatants and determine its effects on rodent malaria due to limited resources. In addition, the immunological responses were determined for only one time point after challenge. Yet, the immunological profiles in BALB/c - *P. berghei* model are known to change as the infection progresses.

4.4 Recommendations

From the results of the present study, the following recommendations were made;

i. IFN-γ immunopotentiation of *Plasmodium* should be adopted as an approach to malaria vaccine development by the researchers.
ii. *Plasmodium berghei* expressed mouse IFN-γ should be purified and its effects on murine malaria determined at different time points and in synergy with whole parasite antigen.
iii. The immunological profiles should be determined at several time points after challenge as the infection progresses.

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APPENDICES

Appendix 1: *Plasmodium berghei* parasitaemia for the different treatment groups

(A-Pre-treated with IFN-γ expressing *P. berghei* culture supernatant; B-Pre-treated with

wild-type *P. berghei* culture supernatant; C-Pre-treated with red blood cells culture supernatant; D-Untreated).

Days post-infection	4	5	6	7	9	11	13	15	17	19
A	0	0	0	0.04± 0.04	0.22± 0.06	0.95± 0.087	2.39± 0.091	4.68± 0.07	10.24± 0.38	14.94± 1.98
B	0.1± 0.01	0.81± 0.03	1.67± 0.91	4.19± 0.23	6.53± 0.79	14.81± 0.069	23.08± 3.01			
C	0.03± 0.02	0.37± 0.02	1.54± 0.53	2.04± 40.18	7.24± 0.33	15.94± 1.02				
D	0.04± 0.01	0.84± 0.05	1.99± 0.57	2.76± 0.09	8.6± 0.0.67	16.24± 0.71	19.33± 2.97			

Appendix 2: Concentrations (pg/ml) of interferon gamma, interleukin-4 and immunoglobulin G (A-Pre-treated with transfected *P. berghei* culture supernatant; B-Pre-treated with wild type *P. berghei* culture supernatant; C-Pre-treated with red blood cells culture supernatant;D-Untreated).

	Before challenge		After challenge		
Treatment groups	IFN-γ (pg/ml)	IL-4(pg/ml)	IFN-γ (pg/ml)	IL-4 (pg/ml)	IgG (pg/ml)
A	88.89 ± 4.42	17.5 ± 2.24	175 ± 17.42	112.5 ± 16.12	2375 ± 152.77
B	91.75 ± 8.48	17.5 ± 2.84	112.5 ± 8.84	143.75 ± 17.42	2625 ± 148.49
C	86.07 ± 8.12	19 ± 3.72	112.5 ± 9.77	141.25 ± 14.21	2350 ± 163.18
D	78.69 ± 6.34	21.25 ± 3.49	93.75 ± 8.41	147.5 ± 12.1	2500 ± 145.66