SAFETY AND EFFICACY OF *PLASMODIUM BERGHEI* EXPRESSING MOUSE INTERFERON GAMMA IN INDUCING PROTECTION AGAINST VIRULENT WILD TYPE *P. BERGHEI* IN A RODENT MODEL

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Safety and Efficacy of *Plasmodium berghei* Expressing Mouse Interferon Gamma in Inducing Protection against Virulent Wild type *P. berghei* in a Rodent Model

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DECLARATION

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

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This research proposal has been submitted for our examination with our approval as the University supervisors

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DEDICATION

I dedicate the entire work to the Almighty God for His unfailing love and support throughout my studies. This work is also dedicated to my beloved grandmother, Madam Georgina Aggrey for all the love she showed me during my studies. I am highly indebted to you, grandmother.

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ABBREVIATIONS AND ACRONYMNS

Ab - Antibody

ADCI - antibody dependent cellular inhibition

ANOVA- Analysis of Variance

COARTEM™- Arteminisinin Lumefantrine combination therapy

CSP - Circumsporozoite Protein

ECM - Experimental cerebral malaria

ELISA - Enzyme-linked immunosorbent assay

HCM- Human cerebral malaria

HEPES - *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) x

IFN- γ - Interferon gamma

IgG - Immunoglobulin G

iNOS - inducible nitric oxide synthase

Interleukin-4 - IL-4

IPR - Institute of Primate Research

mAb - Monoclonal antibody

mIFN-γ - Mouse interferon gamma

MSP - Merozoite surface protein

NAI - Naturally acquired immunity

NHP - Non-human primate

NO - Nitric oxide

PbA - *Plasmodium berghei* ANKA

PBS -Phosphate buffer saline

PCR - Polymerase chain reaction

pRBC - parasitized red blood cell

PE - Pre-erythrocytic

PfEMP1 - *P. falciparum* erythrocyte membrane protein 1

RBC - Red blood cell

RESA - erythrocyte surface antigen

TNF-α - Tumor necrosis factor alpha

WHO - World Health Organization

ABSTRACT

To complement existing control strategies, malaria vaccine is of high priority. During *Plasmodium* infection, $CD4^+$ T-cells produce interferon gamma (IFN-γ) which in turn induces Th1 responses to up-regulate its production together with other cytokines leading to enhanced protection. With the advancement in transfection technology for malaria parasites, mouse IFN-γ expressing *P. berghei* ANKA has recently been generated. A single vaccination of this transgenic parasite has been shown to transiently immunomodulate wild-type *Plasmodium berghei* in mice. However, whether repeated vaccination could induce substantial immune responses is yet to be investigated. This study was therefore designed to evaluate the safety and efficacy of transgenic *P. berghei* (TfPbA) expressing mIFN-γ in inducing protection against virulent rodent malaria parasites. The parasites were propagated and soluble lysate antigen prepared from *in vitro* culture. The levels of mouse IFN-γ in lysate was determined by Enzyme-linked immunosorbent assay. To determine the safety and appropriate route of IFN-γ-containing lysate vaccination, two groups of five Balb/C mice each were vaccinated with 75pg/ml IFN-γ in soluble lysate by either intravenous or intraperitoneal route. Control groups were vaccinated or not with soluble lysate of uninfected red blood cells. Clinical parameters were monitored daily for 7 days. At day 7 post-vaccination mice were euthanized with CO_2 and blood collected. Haematological parameters, liver and kidney functioning parameters and plasma levels of tumor necrosis factor alpha (TNF-α) were determined. To determine efficacy, a total of 120 BALB/c mice was divided into six groups (three experimental and three controls) of 20 mice each. Two experimental

groups were primed using lysate with 75pg/ml IFN-γ or 150pg/ml IFN-γ. At day 14 post priming, the mice were boosted weekly for two weeks. A third group was primed with live TfPbA parasites and at 4% to 6% parasitemia, the mice were orally cured with COARTEM™ at a dose of 450mg/kg. At day 14 post treatment, the mice were boosted weekly for two weeks using lysate with 35pg/ml IFN-γ. The culturing media RPMI 1640, lysate from uninfected red blood cell, and wild-type *P. berghei* (WtPbA) were used in the control groups. After the last boost, five mice from each group were challenged with WtPbA virulent parasites one week. At the time of challenge, a naïve control was introduced. Parasitemia and survivorship were monitored for 21 days. Sampling was done at day 0 prior to priming for baseline measurement and at three other individual sampling points (day 14, day 21 and day 28). Blood and spleen were collected and prepared for serum and splenocyte cultures respectively. Total IgG in serum and cytokines (IFN-γ and TNF-α) in culture supernatant were measured by ELISA. Clinical, biochemical and haematological (except platelet count) parameters were not significantly different ($p>0.05$) among the groups. Compared to unvaccinated control group, TNF-α levels in TfPbA lysate (i.v) group was higher ($p=0.01$). There was significant effect of vaccination on IFN-γ production between the groups at day 21 ($p= 0.035$) and day 28 ($p< 0.001$). Production of TNF-α among the groups was only significant at day 21 post-vaccination ($p= 0.037$). There was a delay (1-4 days) in onset of parasitemia in IFN-γ vaccinated groups compared to the naive control. Mean patent parasitemia was lowest in mice vaccinated with 75g/ml IFN-γ lysate and highest in WtPbA control ($p= 0.633$). Mice vaccinated with 75pg/ml IFN-γ lysate survived wild type challenge infection 5 days longer

than naive control group (p=0.013). The study has demonstrated that, vaccination of mice with transgenic *P. berghei* lysate with 75pg/ml IFN-γ may be safe in mice and repeated vaccination has significant effect on IFN-γ and TNF-α production by Th 1 cells leading to enhanced malaria protection. The study recommends combination of cytokine expressing parasites with soluble lysate in a priming-boosting manner in future vaccination experiments.

Chapter One

INTRODUCTION

1.1 Background Information

Despite the great strides made over the last decade and a half in reducing the burden of malaria globally, malaria continues to affect millions of lives with thousands of lives lost every year. A recent report by leading to the World Health Organization (WHO) estimates about 200 million cases of malaria over 400, 000 deaths occurred in 2015 year alone (WHO, 2015) and a greater proportion of these cases (estimated at 88%) and deaths (estimated at 91%) occurred in the sub-Saharan African countries. The African region especially the sub-Saharan African countries bear the greatest global burden of the disease. Children younger than 5years and pregnant women are usually the major victims of severe malaria and deaths. (http://www.who.int/, 2015; WHO, 2014). Some emerging challenges such as resistance to anti-malarial drugs especially to gold standard drugs; artemisinin-based drugs are major threats to disease reduction interventions. A vaccine with high efficacy remains the last alternative.

Although intensive research towards development of vaccine against malaria has been ongoing for more than 40years now, a highly efficacious vaccine with long term protection still remains desirous (Corradin & Engers, 2014; Targett, 1991). The RTS,S/AS01vaccine which recently received approval can only offer protection against malaria in children between the ages of 5-17 months for only four years. The efficacy is however less than 40% and declines with exposure. Hence, in areas of

high transmission that efficacy may be achievable when the vaccine is used in combination with other effective control measures. (Crompton, Pierce, and Miller, 2010; Malaria Vaccine Initiative, 2015; RTS, 2015). Other candidate vaccines include subunit vaccines (e.g. AMA-1-C1 and Ad35 CS) that are being investigated and at various stages of clinical development have failed to induce substantial protection (more than 50%) (Crompton et al., 2010). This lack of adequate and long term protection with subunit vaccines has led to the resurrection of the use of whole-organism vaccine approach (McCarthy & Good, 2010; Pinzon-Charry & Good, 2008). Vaccination with whole parasites has demonstrated long lasting immunity in some human volunteers (Richie et al., 2015; Schwartz, Brown, Genton, & Moorthy, 2012). Nevertheless, the result of RTS,S/AS01Phase III clinical trials gives a strong indication that vaccine against malaria is possible. But the fact remains that the fight against the disease is still far from over as result, a vaccine with higher efficacy and long term protection is actively being sought for.

Stages of the *Plasmodium* life cycle have been the targets for most current vaccine development approaches with the pre-erythrocytic stage and the asexual blood stage being the targets of most leading vaccine candidates such as the recently approved RTS,S/AS01vaccine, a pre-erythrocytic, subunit vaccine with the Circumsporozoite Protein (CSP) component. Most of these vaccines in Phase I/II of clinical development (Thera and Plowe, 2012) are based on either antigenic component of the parasite stages, DNA or irradiated sporozoites (Anders, 2011; Chia, Goh, and Rénia, 2014). This approach dominated by subunit vaccines have some shortcomings which include low protection and requirement of a strong adjuvant (Thera and

Plowe, 2012). The revisitation and use of whole-organism vaccines such as live attenuated sporozoites (irradiated) which have achieved 100% protection in earliest studies or immunomodulated parasites have been proposed as the way forward to resolve these shortcomings (Hoffman *et al.*, 2002; Keitany, Vignali, & Wang, 2014; McCarthy & Good, 2010; Richie *et al*., 2015). Even though the potential of pre-erythrocytic whole-organism vaccine to yield highly efficacious vaccine is undoubtedly high, there are few challenges such as delivery methods, consequent breakthrough infection, requirement for potent adjuvant and large scale production which need to be addressed.

Both humoral (Antibodies) and cellular immune (T-cell) responses play a crucial role in immunity to malaria (Douradinha & Doolan, 2011). Cytophilic antibodies (Abs) of the IgG1 and IgG3 subclasses are the most important Abs offering protection against *P. falciparum* malaria in humans (Aucan, Traoré, Fumoux, & Rihet, 2001). Acting in collaboration with effector cells such as monocytes and macrophages, they mediate opsonization and Ab-dependent cellular inhibition.

Cellular immune responses are mainly induced against pre-erythrocytic stages with immunity against these stages of malaria. This is regarded as the most promising approach due to its strong and sterile immunity. However, there is strong evidence that point to cytokines as immune effectors which mediate protection against mainly pre-erythrocytic stage of malaria infection. One such cytokine which has been demonstrated as potent and critical immune mediator of protective immunity against *P. falciparum* pre-erythrocytic is interferon gamma (IFN- γ) (King and Lamb, 2015; Perlaza, Sauzet, Brahimi, BenMohamed, and Druilhe, 2011). Infection with *Plasmodium* parasite induces IFN- γ production by both innate and adaptive immune

cells. Studies in mice have demonstrated that the natural killer (NK) cells are the earliest contributors of IFN- γ during both liver stage and blood stage of infection(De Souza, Williamson, Otani, & Playfair, 1997; Miller, Sack, Baldwin, Vaughan, & Kappe, 2014). The induction of the adaptive immune response also contributes to IFN- γ production via $CD4^+$ and $CD8^+$ cells with activation of B cells and macrophages as well as $CD8^+$ cells optimally enhanced by IFN- γ produced by $CD4^+$ (Walther et al., 2009).

Genetic manipulation of malaria parasites also known as transfection technology for malaria parasites leading to the development of transgenic parasite offers a useful genetic tool in preclinical evaluation of malaria vaccines as well as in malaria drug development (de Koning-Ward, Janse, & Waters, 2000; Espinosa, Radtke, & Zavala, 2016; Waters, *et al.*, 1996). In preclinical vaccine evaluation, it is now possible to express either antigenic targets or an important host gene in rodent malaria parasite or a nonhuman primate malaria parasite (Mizutani *et al*., 2016; Mlambo & Kumar, 2008; Ozwara *et al*., 2003; Tewari *et al*., 2014). This has allowed immune responses against such antigens to be studied *in vivo* rather than relying on the traditional approach of *P. falciparum* cultures. One important host gene that has been expressed in nonhuman primate parasites as well as in rodent malaria parasite is IFN-γ (Mogaka *et al*., 2015; Ozwara *et al*., 2003). The immunomodulatory potential and the critical role of this cytokine during malaria infection are well documented. However, the vaccine potential of this transgenic interferon gamma to induce malaria protection in mice has not been fully exploited.

Despite the fact that not all aspects of human malaria can be investigated using experimental models, the use of rodent malaria models in malaria studies have provided significant insights into disease pathogenesis, parasite biology, and immune regulation that protect against disease. Such studies have contributed immensely to the development of new vaccine strategies. Rodent malaria models are mostly used in malaria studies due to obvious reasons such as issues of ethics, practicability and cost involved in the use of human volunteers (Sacci *et al.,* 1992; Vaughan, Kappe, Ploss, & Mikolajczak, 2012; Wykes & Good, 2009).

1.2. Statement of the Problem

Despite the successes achieved through the current available control measures, malaria still poses a greater burden to millions of lives globally. According to the latest reports by WHO, an estimated 214 million new cases of malaria were recorded globally in 2015. Of this number of cases, an estimated 438, 000 people were killed by the disease (WHO, 2015). The sub-Saharan Africa region bore the highest burden of the disease accounting for most cases (88%) and deaths (90%) worldwide (WHO, 2015). One major challenge associated with the use of current control measures against malaria is the growing concern of rapid development of drug and insecticide resistance. *Plasmodium* resistance to artemisinin based drugs have been reported in some countries (Ajayi & Ukwaja; Dondorp *et al.,* 2010; Global Malaria Programme, 2014). Similarly, resistance of mosquitoes to at least one class of WHO recommended insecticides used in bed nets and indoor spraying have also been reported in over 50 countries, mostly from Africa region (WHO, 2013). These

developments raise worrying concerns and hamper the many efforts implemented to reduce drastically the global burden of the disease.

In order to complement the existing control measures, development of malaria vaccine remains a high priority. However, till date no vaccine with full protection against malaria has been developed. Most candidates vaccines under investigations are based on subunit vaccines . However, studies have shown that, subunit vaccine approach lack strong immunogenicity to induce protection against malaria (Crompton *et al.,* 2010; Draper *et al.,* 2015). The use of whole-parasite vaccines is now being considered and revisited as the potential approach to induce high levels of protection due to evidence gathered from several historical studies (Keitany *et al.,* 2014; McCarthy & Good, 2010; Pinzon-Charry & Good, 2008; Stanisic & Good, 2015).

Evidence available indicates that, vaccines based on whole parasites are very promising (Epstein *et al.,* 2011; Jones *et al.,* 2001; Kublin *et al.,* 2017; Seder *et al.,* 2013). So far irradiated sporozoites and genetically attenuated sporozoites have been shown to confer over 90% protection in human subjects against challenged infection (Hoffman *et al.,* 2002; Kublin *et al.,* 2017). However, there are some concerns that need to be resolved about this vaccine approach (Arama & Troye-Blomberg, 2014; Butler, 2013). One such problem is the requirement of a potent adjuvant to enhance Th1 immune responses and which must be compatible with humans (Arama & Troye-Blomberg, 2014; Butler, 2013; Stanisic *et al.*, 2009).

Asexual blood stage vaccine is one such whole-parasite vaccine approach that is being investigated extensively for its potential as a malaria vaccine candidate

(McCarthy & Good, 2010) This vaccine approach involves inoculating parasitized erythrocytes in low doses to cause infection which is subsequently cleared with a chemotherapy. The rationale behind this approach is to induce protection against challenge infection in malaria naive individuals without intervening antibody-mediated responses (Targett, Moorthy, & Brown, 2013).

Many studies in both *in vitro* and *in vivo* have strongly indicated the critical role played by IFN-γ in immunomodulation and enhanced protection against malaria infection during both liver stage and blood stage of the parasites. As a result, Mogaka *et al*., (2015), using transfection technology for malaria parasites successfully transfected *Plasmodium berghei* ANKA (*Pb*A) to express bioactive mouse IFN- γ. However, the vaccine potential of this transgenic organism expressing mIFN-γ to immunomodulate wild type infection leading to enhanced malaria protection in mice requires further investigation.

1.3. Justification of the Study

There is substantial evidence that points to the central role of pre-erythrocytic stage induced cytokine responses in protection against *Plasmodium* infection. IFN-γ has been shown to be one of the potent effectors of cytokine responses offering protective immunity. Using transfection technology, *P. berghei* expressing mIFN-γ have been genetically engineered to produce bioactive mouse interferon. In this study the potential of this transgenic IFN-γ to induce malaria protection in mice will be investigated. This study will apply the prime-boost vaccination strategy to explore the potential of the transfected *P. berghei* expressing mIFN-γ to induce malaria protection in mice. In prime-boost approach, individuals immunized usually receive

first shot of the vaccine followed by another one or more shots at different periods (Nascimento and Leite, 2012; Woodland, 2004). The use of prime-boost approach has been shown to be efficient in eliciting strong immune responses in animal models. In this study the protection will be assessed by estimating the vaccination effects on parasitemia levels, survival time and quantities of immunological parameters such as immunomodulatory cytokines (IFN-γ, TNF-α, IL-4) and mouse IgG antibody. The outcome of the study will contribute to the revived whole-parasite vaccine development approach. The outcome of study will also contribute immensely to the current available body of knowledge with respect to the vaccine potential of transgenic IFN-γ.

1.4.0. Objective

1.4.1. General Objective

To evaluate the safety and efficacy of *P. berghei e*xpressing mIFN- γ in inducing protection against virulent rodent malaria parasites in Balb/C mice.

1.4.2. Specific Objectives

i. To determine safety and appropriate route of *P. berghei* expressing mIFN-γ vaccination
ii. To determine the effect of *P. berghei* expressing mIFN-γ vaccination on production of Total IgG) in serum and cytokines (IFN-γ and TNF-α) in splenocyte cultures of Balb/C mice
iii. To determine the effect of *P. berghei* expressing mouse IFN-γ immunization on parasitemia levels and survivorship in Balb/C mice.

1.5. Research question

a. Which is safest and best route of *P. berghei* expressing mIFN-γ vaccination in Balb/C mice?

b. What is the effect of *P. berghei* expressing mIFN-γ immunization on levels of IgG, IFN-γ and TNF-α in Balb/C mice?

c. What is the effect of *P. berghei* expressing mIFN-γ immunization on parasitemia levels and survivorship in Balb/C mice?

1.6. Research Hypothesis

1.6.1. Null Hypothesis

Prime-boost immunization with transfected *P. berghei* expressing does not induce protection against virulent rodent malaria parasites in Balb/C mice.

1.6.2. Alternative Hypothesis

P. berghei expressing mIFN-γ induces protection against virulent rodent malaria parasites in Balb/C mice.

Chapter Two

LITERATURE REVIEW

2.1.0. Malaria

2.1.1 Epidemiology of Malaria

Malaria is the most wide spread infectious disease caused by *Plasmodium* parasites and spread through the bite of infected female *Anopheles gambiae* mosquito (Carlton *et al.,* 2008; Cox, 2010). In humans, five species of *Plasmodium* parasites- *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi.* are known to cause malaria with *P. falciparum* considered as the most lethal (Wykes and Good, 2009). According to WHO, an estimated 212 million cases of malaria occurred worldwide in 2015 with an estimated 429 000 deaths. (WHO, 2016).

Globally, malaria is most endemic to WHO African Region (90%), followed by the WHO South-East Asia Region (7%) and the WHO Eastern Mediterranean Region (2%) (WHO, 2016). It imposes significant economic burden mainly due to employee absenteeism, increased health care spending, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism. A recent report by the WHO indicates that malaria mortality reductions cost about US$ 2040 billion, which is 3.6% of the total GDP of malaria affected countries(WHO, 2016).

2.1.1 The life cycle of the Malaria parasite

Generally, the life cycle of the malaria parasite in the vertebrate and invertebrate hosts passes through three distinct developmental stages: Pre-erythrocytic (PE) stage, Erythrocytic stage and Transmission or Gametocytic stage (Day, Hayward, & Dyer, 1998; Ménard, Heussler, Yuda, & Nussenzweig, 2008) The life cycle, shown in **Figure 2.1** is initiated by injection of sporozoites into the bloodstream from infected female Anopheline mosquito during a blood meal. The mosquito uses its proboscis to locate the position of the capillary and then inject sporozoites from its salivary gland directly into the blood for circulation. The released sporozoites are carried to the liver where they invade the hepatocytes and multiply. This stage of the life cycle is referred to as the PE stage. Depending on the malaria parasite species, the sporozoites take over 5 to 16 days to grow, divide and produce tens of thousands of haploid forms, called merozoites, per hepatocyte. In the case of *P. falciparum*, the sporozoite takes over 5-7 days to develop and give rise to up to 40,000 merozoites which are released from each infected hepatocytes. For *P. berghei*, 47-52 hours are required for sporozoites development in the hepatocytes into mature schizonts which burst to release between 1500-8000 merozoites each (Gazzinelli, Kalantari, Fitzgerald, & Golenbock, 2014; Soulard et al., 2015; Wiser, 2009).

The release of merozoites from the ruptured hepatocytes initiate the erythrocytic stage or asexual parasite multiplication stage. The merozoites rapidly infect erythrocytes where they evolve from ring-forms into trophozoites which undergo asexual multiplication into mature erythrocytic schizonts containing a few merozoites. The schizonts burst from the erythrocyte to release the new sets of

merozoites which reinvade new erythrocytes in a periodic pattern with each cycle lasting two days (three days in the case of *P. malariae*), until interrupted by host immunity, drug treatment, or death. It is this erythrocytic cycle which is responsible for the clinical manifestations of the disease, including parasitemia, fever, anaemia and spleenomegaly.

However, not all of the merozoites that invade the erythrocytes undergo asexual multiplication. A fraction of them develop into sexual gametocytes. These sexual forms get ingested by the mosquito during a blood meal. Immature gametocytes are sequestered in the bone marrow and only mature gametocytes circulate in peripheral blood. Following ingestion by mosquito, each individual gametocyte either forms one female macrogamete or up to eight male microgametes. In the mosquito midgut, microgamete fuses with macrogamete to form a zygote that develops into a motile ookinete, which can penetrate the midgut wall and form oocysts. The oocysts enlarge over time, usually 10-20 days and burst to release about 1000 infectious sporozoites that migrate to the mosquito salivary gland, from where they can infect vertebrate host during the next blood meal. (Bousema, Okell, Felger, and Drakeley, 2014; Thera and Plowe, 2012).

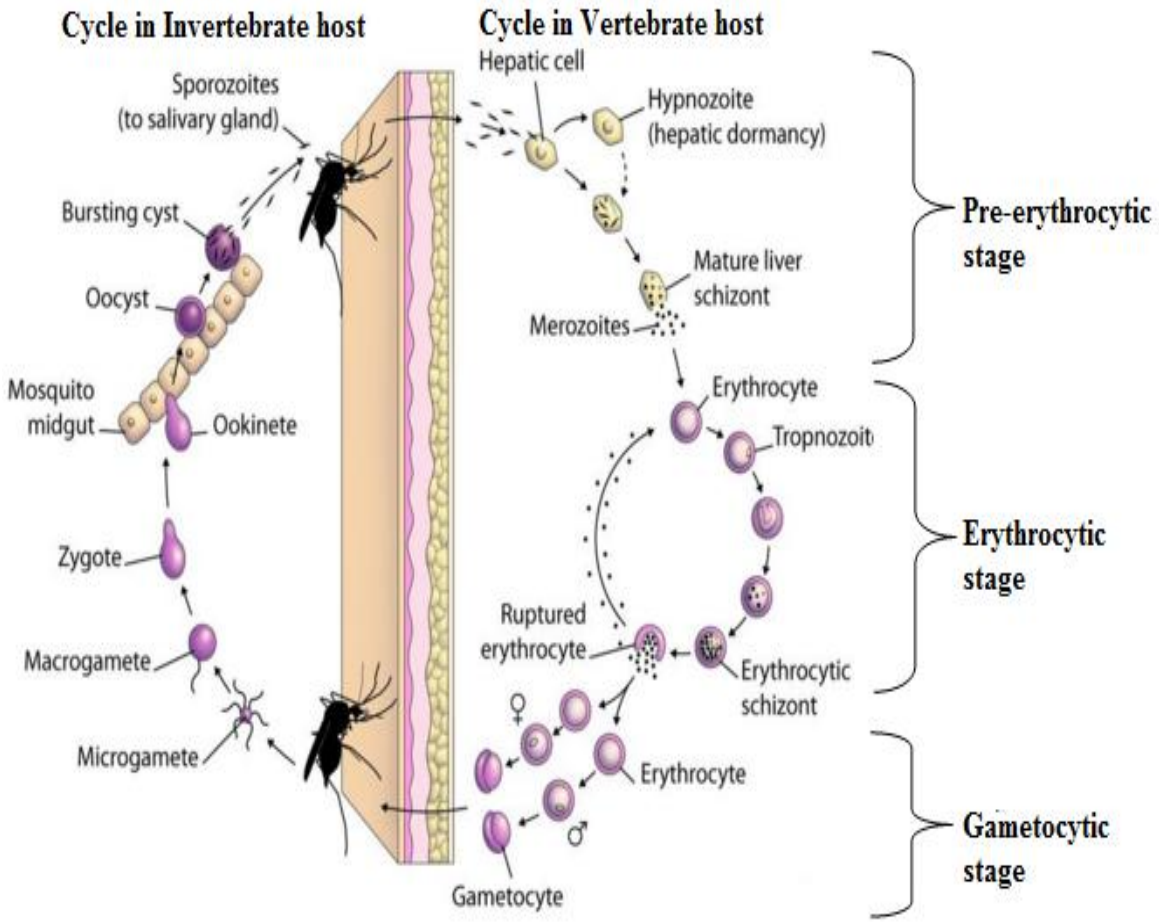

Figure 2.1: Life Cycle of *Plasmodium* spp. (Source: Open Course Ware; http://www.malwest.gr/en-us/malaria).

2.2.0. Animal Malaria Models and Malaria Research

2.2.1 The use of Animal Malaria models in malaria studies

The many limitations such as the lack of access to relevant organs and tissue samples, and the inability to manipulate the immune response for mechanistic studies associated with the use of humans in malaria studies have made the use of animal models an indispensible and proven tools for malariologists (Aikawa *et al.,* 1992; Langhorne *et al.,* 2011). Similarly, the use in vitro studies are also faced with some limitations, particularly on the nature and interpretation with regard to the behaviour

of host immune cells (Wykes and Good, 2009). Thus, the relevance of animal models malaria research worth the discussion. There are many reasons that favour the use of these models. Rodents and Non-human primates(NHP) are two groups of animals mostly used in malaria research (Craig *et al.*, 2012) and have contributed immensely to understanding host-parasite interactions.

Rodent malaria parasites (*Plasmodium chabaudi, Plasmodium vinckei, Plasmodium berghei and Plasmodium yoelii* and various strains) have been the most attractive models for studying different aspects of human malaria due to the existence of small differences in parasite biology and pathogenecity displayed by each parasite. (Sanni, Fonseca, and Langhorne, 2002). As such *P. berghei* is known to cause lethal infections in mice by invading reticulocytes. Infections caused by *P. vinckei, P. yoelii* and *P. chabaudi* have also been reported to be strain specific as some strains are able to produce lethal infections but infection with other strains are not lethal (Craig *et al.*, 2012; Sanni *et al.*, 2002; Schofield and Grau, 2005).

Rodent malaria models have served as useful tools for immunological studies and both lethal and non-lethal rodent malaria parasites have been used for such studies. Rodent models have also extended our understanding of disease pathogenesis and parasite biology have been obtained through the use of rodent malaria models (Lamb, Brown, Potocnik, and Langhorne, 2006). For instance, to investigate mechanisms of drug resistance and immune evasion, research has shown that *P. chabaudi* is an ideal model for such studies. Also, *P. berghei* used in combination with other mouse strains has been used as a model to study immunopathology, experimental cerebral malaria (ECM), pregnancy-associated malaria and lung pathology (Craig *et al.*, 2012;

Hee *et al.*, 2011). *P. yoelii* is also used as model to experimentally investigate the biology as well as the immune responses against the liver stages of malaria with the purpose of discovering potential targets of antimalarials and vaccines (Kappe, Vaughan, Boddey, and Cowman, 2010; Prudêncio, Mota, and Mendes, 2011).

Apart from rodent malaria models, particularly mouse models, NHP malaria models are also equally relevant to the study of human malaria. Malaria infection in NHPs are caused by several species of Plasmodium parasites with *P. knowlesi* and *P. cynomolgi* recently shown to naturally infect humans as well (Maeno *et al.*, 2015; Putaporntip *et al.*, 2009; Ta et al., 2014). These primate malaria models have contributed to the knowledge of human malaria. *Plasmodium coatneyi*, is a primate malaria parasite that infects simians and extensively used in rhesus macaques to study human cerebral malaria (HCM) and evaluation of vaccines against HCM (Aikawa *et al.*, 1992). Since *P. cynomolgi* is closely related *P. vivax* both in biology and pathogenesis, it has been suggested as a useful model for identifying drug and vaccine targets of *P.vivax* (Langhorne *et al.*, 2011). Though studies are not conclusive, *P. knowlesi* infection in olive baboon has been suggested as a useful models for human cerebral malaria (HCM).

2.2.2. The rodent malaria: *Plasmodium berghei*

A major characteristic of *P. berghei* is its exclusive preference for circulating reticulocytes during early infections (Ott, 1968). *P. berghei* reticulocytes tropism does not occur only during early stage of infection but also during late stage accounting for reduced levels of circulating reticulocytes (Cromer, Evans, Schofield, and Davenport, 2006). However, as the number of reticulocytes diminish due to cell

destruction, *P. berghei* merozoites tend to parasitize mature RBCs. This characteristic feature of *P. berghei* has been explored by (Baptista *et al.,* 2010) to investigate the pathogenesis of ECM in mouse models.

2.3. Malaria Vaccine Development Strategies

Vaccines are the most cost-effective way of averting the devastating effects of diseases on humans. Efforts to develop malaria vaccine have been ongoing for more than four decades, yet an effective and safe vaccine against the disease still remain elusive of us (Corradin & Engers, 2014). However, promising results from the recent Phase III clinical trials of the RTS,S/AS01 among infants and children in sub-Saharan African region shows development of effective and safe malaria vaccine is feasible (Gosling, Seidlein, Griffin, Asante, & Owusu-Agyei, 2016; RTS, 2015). Also, protection of human volunteers against challenged infection after immunization with irradiated sporozoites and genetically attenuated sporozoites in two independent studies further confirms the hope of discovering a malaria vaccine soon (Hoffman *et al.,* 2002; Kublin *et al.,* 2017; Richie *et al.,* 2015).

Traditionally, malaria vaccine strategies have been categorized into three mimicking the developmental stages of *Plasmodium* spp; Pre-erythrocytic vaccine, blood-stage and transmission blocking vaccines (Yimin Wu, Ellis, Miura, Narum, & Miller, 2009). The pre-erythrocytic vaccine targets sporozoites and liver-stage parasites with the aim to prevent infection and reduce disease. As a result, many sporozoite proteins such as Circumsporozoite Protein (CSP) and Thrombospondin related adhesive protein (TRAP) have been characterized. The most advanced malaria vaccine

candidate, RTS,S/AS01 and other many vaccine candidate under various stages of clinical trials are based on the CSP (Duffy, Sahu, Akue, Milman, & Anderson, 2012). Blood-stage vaccine is developed to elicit antibodies to neutralize antigenic epitopes of erythrocyte stage invading parasites thereby preventing clinical malaria and severe disease (Yimin Wu *et al.,* 2009). Most blood-stage malaria vaccine candidates are based on antigens that coat the surface of the invasive merozoites and/or are involved in the process of erythrocyte invasion (Miura, 2016). It is believed that immunization with these antigens will generate antibodies that block invasion and curtail parasite replication in the blood, reducing the risk or severity of clinical illness. The leading blood-stage vaccine candidates being pursued currently are all merozoite protein based. They include apical membrane antigen 1 (AMA1) (P12b), erythrocyte-binding antigen–175 (EBA-175) (El Sahly et al., 2010), glutamate-rich protein (GLURP) (Hermsen *et al.*, 2007; Mordmüller *et al.,* 2010), merozoite surface protein 1 (MSP1) (Ogutu *et al.,* 2009), MSP2 (Genton *et al.,* 2002), MSP3 (Mordmüller *et al.,* 2010), and serine repeat antigen 5 (SERA5) (Horii *et al.,* 2010), all of which are highly expressed on the surface of the merozoite. Unfortunately, recent phase II trials of the most advanced blood-stage candidates, AMA1 and MSP1, did not demonstrate efficacy in African children (Ogutu *et al.,* 2009; Sagara *et al.,* 2009). However, there are ongoing efforts to enhance AMA1 and MSP1 vaccine efficacies. These efforts include the use of novel adjuvant (Bouharoun-Tayoun, Oeuvray, Lunel, & Druilhe, 1995; Sagara *et al.,* 2009), viral vector prime boost strategies(Hill *et al.,* 2010), or by combining AMA1 and MSP1.

Finally, transmission blocking vaccines (TBVs) are intended to prevent development of parasites in mosquito by inducing protective immunity against sexual-stages of

parasites (Wu, Ellis, Miura, Narum, & Miller, 2009). Candidate TBV are based on surface proteins of gametocytes (Pfs 2400, Pfs 230, Pfs 48/45, Pfg 27), zygotes and ookinetes (Ps 25, Ps28) (Gonçalves & Hunziker, 2016).

2.4.0. Malaria and the Immune Responses

2.4.1 Immune responses to Malaria Infection

Both antibody-mediated and cell-mediated immune responses are involved in protective immunity against malaria infection. However, pre-erythrocytic stages of malaria are mainly mediated by cellular immune responses whereas humoral immune responses (Antibodies) mediate mostly blood-stage infection (Schwenk and Richie, 2011). The cellular immune responses involve both CD8 T cells and CD4 T cells as well as components of the innate immune system such as gamma-delta T cells (γδT), natural killer (NK) cells and natural killer T(NKT) cells. Many studies have established blood-stage infection correlate with increased levels of antibodies and antigen-specific antibodies are mainly of immunoglobulin G class. However, evidence shows that, whereas T cells are directed against parasite antigens expressed in the liver stage of the parasite life cycle, antibodies are also directed against sporozoite surface proteins (Doolan and Martinez-Alier, 2006).

2.4.2. Antibody-mediated Immune Responses

Infection with *Plasmodium* parasites elicit both humoral (Antibodies) and cellular immune (T-cell) responses and that both B cells and antibodies play a crucial role in immunity to malaria. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by about 60 vargenes per haploid genome is a variant antigen molecule

expressed on the surfaced of infected red blood cells (Baruch *et a*l., 1995; Gardner *et al*., 2002; Su *et al*., 1995). PfEMP1 allows the parasite to modify the antigenic and functional properties of infected red cells by switching the expression of its var gene at different times, thereby evading immunity and affecting infection outcome(Smith *et al.,* 1995; X. Z. Su *et al.,* 1995). A study by (Bull *et al.,* 1998) has shown that, the repertoire of specific, protective Ab directed against the PfEMP1 may be largely responsible for the development of naturally acquired immunity (NAI) in individuals exposed to prolonged infection in areas of endemicity. Purified malaria-IgGs transfered from immune African adults and acting in cooperation with monocytes have shown to reduce parasite load and clinical symptoms in some malaria non-immune infected patients in Thailand (Bouharoun-Tayoun, Attanath, Sabchareon, Chongsuphajaisiddhi, and Druilhe, 1990). Also, monoclonal Ab (mAb) raised against *Plasmodium yoelii* merozoite surface protein 1 (MSP-1) antigens suppresses parasitemia in naive mice (Spencer Valero *et al.,* 1998). Similarly, mice lacking B cells develop chronic parasitemia when infected with *P. chabaudi chabaudi* despite treatment with antimalarial drugs, thus the role of B cells in developing protective immunity is very crucial.

Plasmodium falciparum-mediated antibodies conferring protection in humans, monkeys and mice are of IgG class. Immunoglobulin G antibodies to asexual blood stage antigens increase with age and reduce both the prevalence and the density of asexual and transmission stages in erythrocytes (Chizzolini *et al*., 1988; Piper, Hayward, Cox, and Day, 1999). The IgG subclass responses against ring-infected erythrocyte surface antigen (RESA), merozoite surface protein (MSP) 1, MSP-2 and

crude P. falciparum antigen in people living in exposed areas are partly determined by host genetic factors and are age-dependent (Aucan, Traoré, Fumoux, and Rihet, 2001). Cytophilic Ab of the IgG1 and IgG3 subclasses are considered to be the most important Ab for protection against *P. falciparum* malaria in humans (Aucan et al., 2001). Acting in collaboration with effector cells such as monocytes and macrophages, they mediate opsonization and Ab-dependent cellular inhibition. A seroepidemiological study has shown that increased levels of P. falciparum-specific IgG1 and IgG3 in individuals living in endemic areas are associated with lower parasitaemia and reduced risk of malaria pathology (Aribot *et al.*, 1996; Bouharoun-Tayoun and Druilhe, 1992). Protection attributable to parasite-specific IgG3 is age-associated, with greater levels of protection seen in adults. In addition to IgG1 and IgG3, IgG2 may be involved in protection. High levels of IgG2 to RESA and to MSP2 are associated with resistance to P. falciparum at the end of the transmission season and levels tend to be higher in older individuals who are better protected against infection and disease(Aucan *et al.*, 2000). In contrast, levels of IgG4 to parasite extract, RESA, MSP1 and MSP2 are lower in individuals who do not develop malaria than in susceptible individuals and are positively correlated with risk of infection. It has been suggested that IgG4 competes with cytophilic Ab for antigen recognition and may therefore block cytotoxicity mediated by Ab-activated effector cells.

Antibodies against surface proteins of the merozoite has been found to function either by blocking RBC invasion or by making the merozoite susceptible to phagocytosis. Parasite antigen-specific IgG3 in particular may play an important role

in controlling parasitemia via an antibody dependent cellular inhibition (ADCI) mechanism involving monocyte- derived mediators whereby binding of antibodies to phagocytes via Fc receptors leads to inhibition of parasite growth(Aucan *et al*., 2000; Bouharoun-Tayoun *et al*., 1990; Bouharoun-Tayoun, Oeuvray, Lunel, and Druilhe, 1995; Tebo, Kremsner, and Luty, 2001). Antigen-specific Ab can also initiate parasite clearance by opsonization, thus enhancing the activity of phagocytic cells (Doolan, Dobaño, and Baird, 2009) or initiating complement-mediated damaging (Giribaldi, Ulliers, Mannu, Arese, and Turrini, 2001; Ramasamy and Rajakaruna, 1997).

2.4.3. Cell-mediated Immune responses: Cytokines

Cytokines play a critical role as mediators of the cellular arm of immune responses triggered by Plasmodium infections. Infection with Plasmodium leads to the differentiation of CD4 helper T(Th) cells into Th1 or Th2 effector subsets. (Taylor-Robinson, Phillips, Severn, Moncada, and Liew, 1993). These cells differ in the production of different kinds cytokines which mediate immune responses (Dong and Flavell, 2001). Whereas Th1 cells response lead to the of production interleukin (IL)-2, interferon (IFN)-γ, transforming growth factor (TGF)–β, tumor necrosis factor (TNF)-α, Th2 cells response lead to the secretion of IL-4, IL-5, IL-6 and IL-10 (Luckheeram, Zhou, Verma, and Xia, 2012; Zhu and Paul, 2015). The role of these cytokines as well as other cytokines such as IL-12 and IL-18 in malaria infection has been investigated and reported in various studies. However, evidence gathered shows that cytokines offer protection against malaria and in some instances promote susceptibility to infection. Thus, they play dual roles in malaria protection and

pathogenesis (Angulo and Fresno, 2002; Artavanis-Tsakonas, Tongren, and Riley, 2003).

The antimalarial activity of IFN- γ has been established already (Favre, Ryffel, Bordmann, and Rudin, 1997; Yoneto *et al.,* 1999) and mice deficient in interferon gamma receptor(IFN-γ R) exhibit high mortality rate when compared with the wild type mice. The early production of IFN-γ has also been suggested as necessary to give protection against blood-stage Plasmodium infection (Choudhury, Sheikh, Bancroft, Katz, and de Souza, 2000; De Souza *et al.,* 1997).

The production of nitric oxide(NO) has been sufficiently demonstrated as protective against *P. chabaudi chabaudi* infection (Taylor-Robinson *et al.,* 1993). Similarly, induction of nitric oxide synthase (iNOS) has been also shown to protect mice against irradiated Plasmodium berghei infection and series of signals from IFN- γ and Tumor necrosis factor alpha (TNF-α) lead to the induction of NOS to produce NO (Liew, 1996; Seguin *et al.,* 1994). Interestingly, cytokines such as IL-4, IL-10 and TGF-β inhibit the induction of NOS by IFN- γ. Excessive production of NO could also lead to pathological effect (Liew, 1996). Nevertheless, the protective role of NO against malaria infection in both humans and mice is well documented (Anstey, 1996; Jacobs, Radzioch, and Stevenson, 1995; Kremsner *et al.,* 1996). For instance, a study by Jacobs et al., (1995) showed a higher levels of iNOS mRNA from spleen during early infection in resistant C57BL/6 mice than susceptible A/J mice and that the NO expression in spleen accounted for the observed protection against blood-stage malaria in mice.

Pro-inflammatory cytokines such as IL-1β and IL-6 also induce parasite killing through activation of macrophages and neutrophils (Kumaratilake and Ferrante). IL-12 is another cytokine involved in early responses against malaria infection. Apart from its role in protection against primary infection, a study by (Su and Stevenson, 2002) has demonstrated that IL-12 is also required for antibody-mediated immunity. In that study, protection against *P. chabaudi* AS infection in wild type C57BL/6 mice correlated significantly with high levels of IgG2a and IgG3 antibodies. However, C57BL/6 mice whose IL-12 p40 gene was knocked out showed higher levels of primary parasitemia and this was alluded to the lower levels of IgG2a and IgG3 antibodies analyzed. IFN- γ production is however believed to be associated with the activity of IL-12 during this early IL-12 response (Su and Stevenson, 2002; Yoshimoto, Yoneto, Waki, and Nariuchi, 1998).

A recent study to investigate whether IL-18 plays any role in malaria protection has indeed indicated that IL-18 defends host against malaria infection (Singh *et al.*, 2002). The study suggested that IL-18 protect infected mice by enhancing IFN-γ production during blood-stage infection. IL-18 is a potent pro-inflammatory cytokine and homologous but different in function to IL-1. It stimulates in collaboration with IL-12 both Th1 and Th2 responses but can also stimulate Th2 cytokine production independent of IL-12 (Nakanishi, Yoshimoto, Tsutsui, and Okamura, 2001).

Despite the prominence of TNF-α in causing malaria pathology (Angulo and Fresno, 2002; Gimenez, Barraud de Lagerie, Fernandez, Pino, and Mazier, 2003), several studies have equally confirmed the involvement of TNF-α in Plasmodium clearance. It has however been suggested that, TNF-α is not directly involved in parasite killing

but acts by activation of other cells such as macrophages which leads to the release of NO (Langhorne, Quin, and Sanni, 2002). The protective function of NO has been discussed earlier. Similarly, IL-4 though implicated in the inhibition of iNOS by IFN-γ, it has been shown to be very important for parasite clearance through antibody-mediated response during late stage of infection (Troye-Blomberg, Berzins, and Perlmann, 1994).

Although cytokines are critical mediators of cellular immune responses protecting host against malaria infection, they also exacerbate infection. Malaria is characterized by pathologies such as cerebral malaria(CM), severe malaria, anaemia and hypoglycemia (Langhorne *et al.,* 2002) and pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-6 and IL-12 have been implicated in one or more of these malaria pathological complications both in humans and animals. With the exception of IL-6, whose non-involvement in experimental cerebral malaria induced by Plasmodium berghei ANKA (PbA) infection in mice has been established (Grau, 1990), there are several evidence that implicate especially TNF-α and other cytokines like IFN-γ and IL-12 involvement in CM. With regard to TNF-α, evidence suggest that excess TNF-α induces murine CM and anti-TNF-α therapies did not show any efficiency in protection from CM. In contrast, the involvement of IFN-γ, TNF-α, IL-6 and IL-12 in severe anaemia is poorly established as no universally accepted findings has been generated. Instead, a ratio of IL-10/TNF-α has been suggested as an indicative of severe anaemia(Angulo and Fresno, 2002). Similarly, there are many studies that suggest the involvement of IFN-γ in ECM but its role may not be as strong as that of TNF-α (McCall and Sauerwein, 2010).

2.5.0. Interferon gamma (IFN- γ):Protective Role and Production

2.5.1. Protective Role of Interferon gamma (IFN- γ) in Pathogenic Infection

IFN-γ is the only type II IFN and it is considered as the most potent pro-inflammatory cytokines with a pivotal role in controlling Plasmodium infection against pre-erythrocytic and blood stages of Plasmodium parasites (Doolan and Martinez-Alier, 2006; McCall and Sauerwein, 2010). In assessing the protective role of endogenous IFN-γ blood-stage *Plasmodium chabaudi* AS malaria, it was shown that knockout mice developed high parasitemia with severe mortality in acute infection than wild type mice when inoculated with infected RBCs (Su and Stevenson, 2000). Subsequent evaluation of cytokine profiles indicated an enhanced production of IL-12, TNF-α and NO in the wild type compared to the knockout mice suggesting the interrelated activity among these cytokines as well as the central role of endogenous IFN- γ in protecting against blood-stage infection.

The protective role of this cytokine against viral, bacterial and protozoan infections is well documented in literatures. Endogenous IFN-γ has been demonstrated to protect mice from intracellular Listeria infection (Buchmeier and Schreiber, 1985). Also, IFN-γ can complement type I IFN in host defence against viral infections (van den Broek, Müller, Huang, Zinkernagel, and Aguet, 1995). There are also evidence in mice models to demonstrate the protective role of IFN- γ in mediating immune responses against both intracellular and extracellular protozoan infections such as *Leishmania donovani*, *Toxoplasma gondii* and *Trypansoma cruzi*.

2.5.2. The cells involved in IFN- γ production

Both innate and adaptive immune responses have been demonstrated as sources of IFN- γ. Innate immune cells that secret IFN-γ include natural killer (NK) cells, natural killer T cells, gamma delta T cells(γδT) and professional antigen presenting cells (APCs) such as monocyte/macrophage, dendritic cells (DCs) production. The induction of the adaptive immune response also contributes to IFN- γ production via $CD4^+$ and $CD8^+$ cells with activation of B cells and macrophages as well as $CD8^+$ cells optimally enhanced by IFN- γ produced by $CD4^+$ (Walther *et al.,* 2009). However, majority of IFN-γ in the adaptive immune response are contributed by secretion from T lymphocytes. Studies in mice has demonstrated that natural killer (NK) cells are the earliest contributors of IFN- γ during both liver stage and blood stage of infection (De Souza et al., 1997; Miller et al., 2014). It has also been demonstrated in a study that a maximum secretion of IFN- γ occur when murine macrophages are stimulated with combined IL-12 and IL-18 (Munder, Mallo, Eichmann, and Modolell, 1998). A similar study also confirmed this earlier study when concomitant treatment of mice with IL-12 and IL-18 led to significant detectable levels of IFN- γ positive cells in the most lymphoid organs and tissues (Otani *et al.,* 1999). As a result, IL-12 and IL-18 have been described as effective IFN- γ inducing cytokines that link infection with IFN-γ production in the innate immune response.

2.5.3. The Immunomodulatory Potential of Recombinant IFN-γ

The advancement in recombinant DNA technological has enable scientists to develop various useful DNA constructs to express desirable product such as heterologous proteins (Jackwood, Hickle, Kapil, and Silva, 2008). One major application of this technology is in vaccine development, where immunogenic protein/gene is identified, isolated and cloned with corresponding expression vector to produce a recombinant DNA in an immunized individual upon injection or expressed in another host to obtain recombinant DNA for use in vaccines (Jackwood *et al.*, 2008; Nascimento and Leite, 2012).

In this regard, transgenic pathogenic microorganisms have been extensively exploited to express various host cytokines such as IFN-γ to immunomodulate with the aim of enhancing protection. The phenomenon has been demonstrated to be effective in attenuating transgenic pathogens in vivo as well as enhance immune protection against infections such as HIV-1 and colitis (Gherardi *et al*., 1999; Giavedoni *et al*., 1992; Steidler, 2000). For instance, IL-12 delivered from Recombinant Vaccinia Virus was shown to attenuate in mice leading to enhance immune protection against HIV-1 infection (Gherardi *et al.*, 1999). Similarly, infection of nude mice with IFN-γ expressing *Leismania major* showed slower progression of disease as compared to infection with wild type *L. major*

Replication of this transfection technology has been carried out recently in murine malaria (Mogaka *et al.*, 2015). The study showed that mouse IFN-γ expressing wild-type P. berghei can generate immunopotentiated transgenic *P. berghei*. Prior to this study, Ozwara *et al*.,(2003) have demonstrated the feasibility of generating malaria

parasites expressing bioactive host immunomodulatory cytokines. Data from both studies clearly suggest that *in vivo* expression of host IFN-γ by *Plasmodium sp*. has immunomodulatory potential leading to enhanced immune protection upon single exposure.

2.6. Transfection of Malaria Parasite

Transfection technology for malaria parasites offers scientists a useful genetic tool in preclinical evaluation of malaria vaccines (Tania, de Koning-Ward *et al.,* 2000; Espinosa *et al.,* 2016; Waters *et al.,* 1996). It has allowed immune responses against transgenes such as antigenic targets of human malaria parasites or an important host gene to be studied *in vivo* in rodent malaria parasite or nonhuman primate malaria parasite thereby eliminating the laborious and difficult traditional approach of *P. falciparum* culture assays (Mizutani *et al.,* 2016; Mlambo & Kumar, 2008; Ozwara *et al., 2003;* Tewari *et al.,* 2014*)*. In transfection of Plasmodium blood-stage parasites, transgenes are retained and expressed either transiently or stably (Tania, de Koning-Ward, Gilson, & Crabb, 2015). Stable transfection could be episomally based or integration dependent based (Philip, Orr, & Waters, 2013; Tomas *et al.,* 1998). Episomally based plasmids suffer from plasmid loss over several generations due insufficient replication as a result maintenance is done under drug pressure (Matz & Kooij, 2015; Tomas *et al.,* 1998; Wu, Kirkman, & Wellems, 1996).

2.7. The Prime-Boost Vaccination Strategy

Throughout history, the administration of more than a single dose of vaccine has proven to be effective in achieving the purpose of immunization. Individuals immunized usually receive first dose of the vaccine followed by another one or more doses at different periods in a prime-boosting manner (Nascimento and Leite, 2012; Woodland, 2004). Vaccination by this approach (i.e. prime-boost) has shown greater prospect for improving vaccination as substantial evidence gathered indicate its potential to provoke strong levels of memory T cells, particularly CD8 T cells (Estcourt, 2002; Ramshaw and Ramsay, 2000; Woodland, 2004). The approach has recently been used to assess the DNA replicon and protein antigen as potent vaccine candidates against Chikungunya Virus in C57BL/6 mice (Hallengärd *et al.*, 2014). Prime-boost strategy can either be homologous or heterologous (Lu, 2009).

Homologous prime-boost strategy is the traditional approach of repeated vaccination to induce strong immune responses. In this approach, the same vaccines given in the earlier priming immunizations are used for subsequent boost immunizations (Lu, 2009). However, evidence shows that heterologous prime-boost approach is currently the preferred approach in many vaccine development research (Lu, 2009; Woodland, 2004). In heterologous prime–boost strategy, the immune system is primed with a target antigen delivered by one vector and then selectively boosted by re-administration of the same antigen in a second but distinct vector (Woodland, 2004). This has been applied in malaria vaccine development in both humans and animal models (Dunachie, 2003; Hill *et al.*, 2010). It is capable of inducing enhanced immunogenicity required for eliciting high levels of memory T-cells than the

homologous approach (Lu, 2009; McConkey *et al.*, 2003; Saade and Petrovsky, 2012). Nonetheless, homologous prime-boost strategy is still popularly and has been shown to be efficient in inducing CD8 T cell responses equally as heterologous prime-boost strategy (Schwarz *et al.*, 2005).

Chapter Three

MATERIALS AND METHOD

3.1. Study Site

All experimental work was undertaken at the laboratory of the Infectious disease department, Institute of Primate Research (IPR), Karen, Kenya.

3.2. Experimental Animals and Parasites

Male and female BALB/c mice, six to twelve weeks old were used. A total of 200 Balb/c mice were used in the whole experiments. The mice were maintained at the pathogen-free rodent facility of Institute of Primate Research (IPR). Mice obtained outside IPR were allowed to acclimatize for at least one week before using for any experimental work. The mice were fed on standard pellet diet and water ad libitum in a 12-h alternating light–dark cycle.

Transfected *P. berghei* ANKA strain expressing mouse IFN- γ (TfPbA) and wild type *P. berghei* ANKA (WtPbA) parasites were used in this study. Both parasites were obtained from the malaria programme, Institute of Primate Research. The TfPbA parasites were generated previously by Mogaka *et al.,* (2015). The parasites used contained recombinant mouse IFN-γ carried by Mgamma 10 plasmid and constitutively expressed under the *P. berghei* ef-α1 promoter. The plasmid contains a mutagenised *Toxoplasma gondii* Dihydrofolate Reductase (*Tg*DHFR) gene sequence conferring pyrimethamine resistance (**Figure 3.1**) Thus *in vivo* and *in vitro* propagation of this transfected parasites under pyrimethamine drug pressure allows maintenance of the plasmid in the parasites.

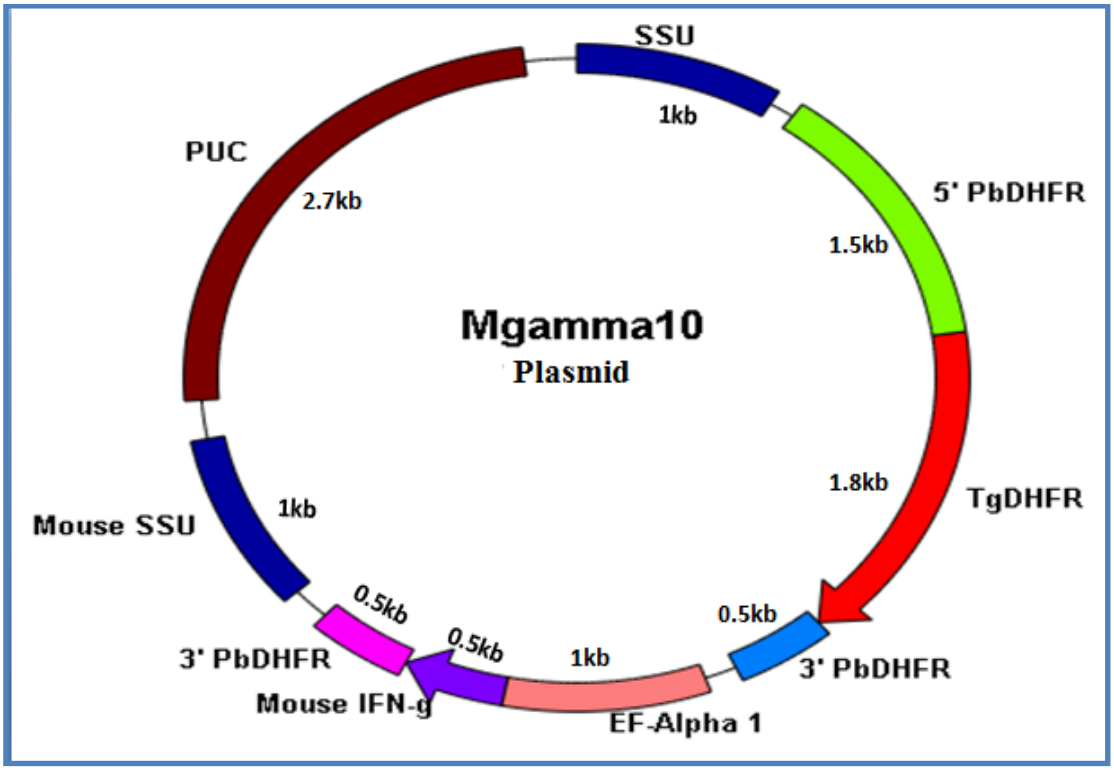

Figure 3.1: Map of the plasmid used to transform *P. berghei* parasites. Source: Mogaka *et al.*, (2015).

3.3. Sample Size Calculation of Animals

Sample size for time to an event approach was used to determine the number of mice per group (Dell, Holleran, & Ramakrishnan, 2002). Given the estimate for proportion of the control group exhibiting the event (p_c) and the desired proportion of the experimental group exhibiting the event (p_e), then sample size, $n = C\frac{Pcqc+Peqe}{d^2} + \frac{2}{d} + 2$, Where $q_c = 1-p_c$; $q_e = 1-p_e$; and $d=p_c-p_e$. d is the effect size. From previous study (Mogaka et al., 2015), the proportion of untreated control mice surviving challenge infection after an average period of 12days was 50%. That is $p_c = 0.5$. With a desire of 100% experimental group surviving after this period (i.e $p_e = 1$) at a power of 90% and a significance level of 5%, the effect size d= 0.5-1=0.5, an absolute figure. Using

the power of 90% and a significance of 5% the value of C (10.51), sample size of 16.5≈ 20 was calculated.

3.4. Culture Media for Parasites and Mouse splenocytes

In this study, complete RPMI 1640 media was used for culturing parasites and spleen cells. Complete RPMI media parasites was prepared by supplementing incomplete culture media [RPMI 1640 with L-glutamine (GIBCOBRL, USA, lot # 1015845), 25mM HEPES (Sigma, USA, Lot # 103H57255), 24mM Sodium Bicarbonate, and neomycin sulphate] with 25% fetal bovine serum (FBS) (GIBCO,USA). For splenocyte cultures, complete RPMI 1640 media was prepared by supplementing incomplete media [RPMI 1640 with L-glutamine (GIBCO, UK), 100µg/mL gentamicin (Sigma, UK), 0.05mM 2-mercaptoethanol (BDH, England) and 25mM HEPES (Sigma, England)] with 10% FBS.

3.5. Study Design

The flow chart of the study design is shown in **Figure 3.2.**

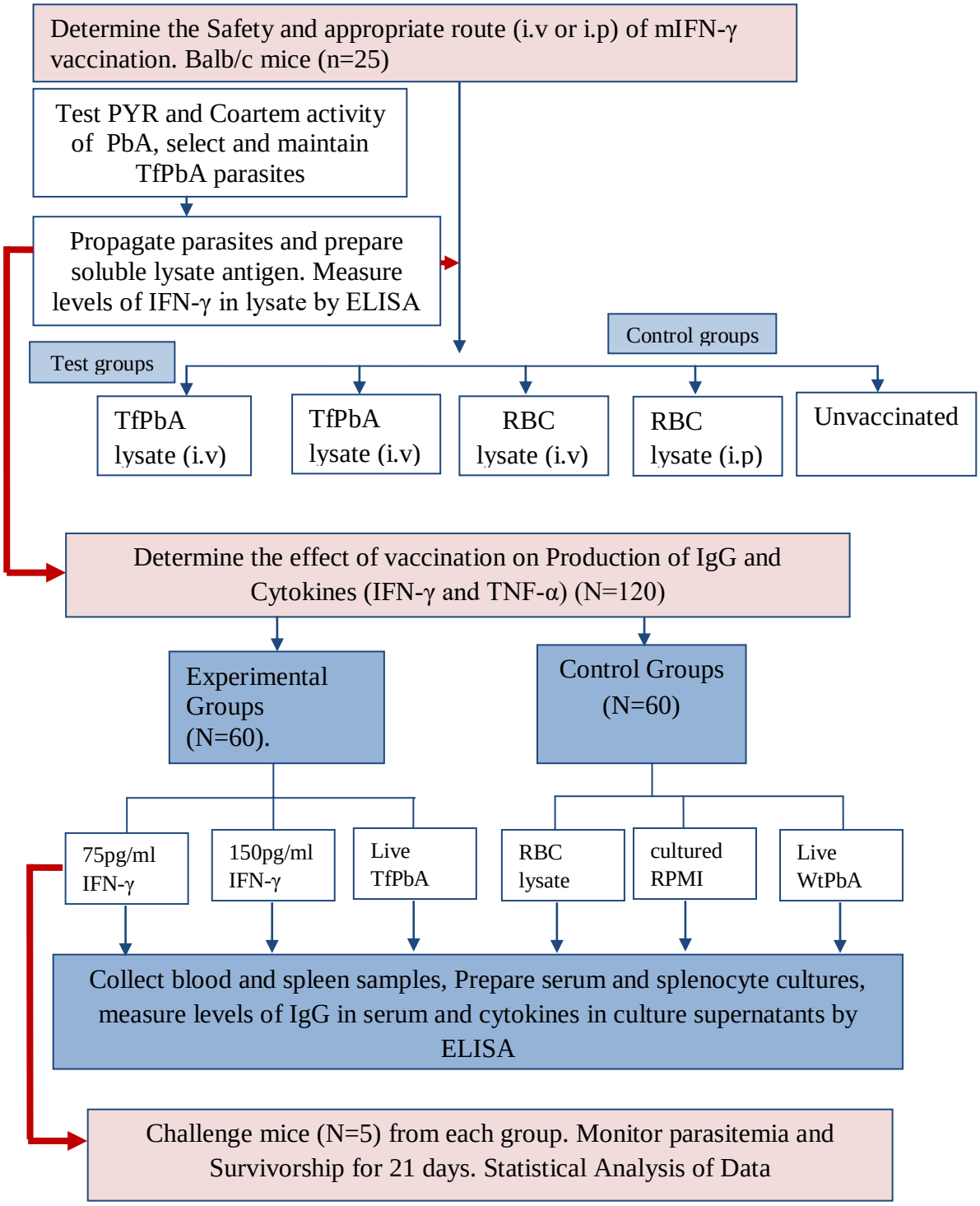

Figure 3.2: Flow Chart of Study Design

3.6. Determination of Safety and Appropriate Route of *P. berghei* expressing mIFN-γ vaccination

3.6.1. Antimalarial activity, Selection and Maintenance of Parasites

Pyrimethamine (Sigma, USA) and Arthemeter Lumefantrine Combination Therapy (Coartem) (Novartis-Pharma, Switzerland) were two antimalarials used in this study. Whereas pyrimethamine was used for maintaining plasmids in transfected *P. berghei* during propagation, COARTEM was used to cure mice "vaccinated" with parasites. In order to ensure these two drugs were effective, antimalarial activity of parasites was tested *in vivo*. Four groups (A, B, C and D) of three mice each were intraperitoneally infected with $2x10^6$ of either TfPbA or WtPbA and treated according to **Table 3.1**.

Table 3.1: Infection and Treatment of mice

Group (n=3)	Parasites (dose)	Treatment (Dosage)
A	Transfected PbA	Pyrimethamine (0.07mg/ml)
B	Wild type PbA	Pyrimethamine 0.07mg/ml
C	Wild type PbA	Coartem 450mg/kg
D	Wild type PbA	no treatment

Infections were monitored daily according to Doolan (2002). In brief, a snip at the tail of the infected mice was made with a pair scissors sterilized with 70% ethanol. A small drop of blood was placed on a microscope slide to prepare a thin smear. The smear was fixed with methanol for 30s and then stained with a 10% fresh Giemsa

working solution for 15 min. The slide was washed, air-dried and parasitemia determined using a light microscope (with immersion oil and objective at ×100) by counting 20 fields of 300 red blood cells (RBC) per field. The percentage of infected RBCs was then calculated. At day 3 post-infection (at 1.7% parasitemia), group A and B were subjected to 0.07mg/ml pyrimethamine in drinking water continuously for 4days. The pyrimethamine was reconstituted in DMSO to a final concentration of 7 mg/ml and dilute 100 times with normal tap water with an adjusted pH of 3.5–5.0 (with 1 M HCl) (Janse, Ramesar, & Waters, 2006). On day 4 post infection, group C mice (at about 7% parasitemia) were orally treated with 0.2mL COARTEM (preparing by crushing calculated number of tablets and dissolving in distilled water) (Kodippili, Ratnasooriya, Premakumara, & Udagama, 2011).

After testing the sensitivity of parasites to pyrimethamine, TfPbA parasites were selected under pyrimethamine pressure and maintained continuously by passages (at 4 to 6% parasitemia) in donor mice. Passages were done by intraperitoneal (i.p) injection with $2x10^6$ parasitized red blood cells (pRBCs) in phosphate buffer saline (PBS) and infections monitored by microscopy (Doolan, 2002).

3.6.2. Preparation of *P. berghei* soluble lysate antigen for Immunization

Four mice were inoculated intraperitoneally with $2x10^6$ TfPbA parasites (selected and maintained in **3.9**). After detection of parasites by microscopy on day 3 post infection, mice were introduced to pyrimethamine drinking water as described previously. On day four post-pyrimethamine treatment and at about 4% to 8% parasitemia, each mouse was euthanized with CO_2 and infected blood harvested via cardiac puncture. Blood was collected into 15-ml centrifuge tube containing 5mL of

incomplete media. The pRBCs were then washed thrice at 200g for 10min at 24^{o}C with equal volume of incomplete culture medium. The pellet was resuspended in complete culture medium at 5% hematocrit and aseptically transferred into T-25 culture flasks. Cultures were then inoculated with 0.1µM pyrimethamine and flushed with gas mixture (5% CO_2, 5% O_2, and 90% N_2), closed tightly and sealed with parafilm. The flasks were then incubated at 36.5^{o}C on an orbital shaker (Lab-line® Incubator-Shaker) at 30RPM overnight (18hrs). After 18 hrs of incubation, the cultures were span again at 200g for 10min at 24^{o}C and supernatant collected, filter-sterilized using 0.45µm-pore syringe filters and stored at -80^{o}C. The pellets were resuspended in complete culture medium at 20% hematocrit and incubated for 9hrs (i.e. making a total of 27 hours of culture). After 9hrs, the cultures were briefly sonicated on ice at 3 amplitude microns (Soniprep 150) for 5minutes using 30s pulse and 1min rest to cause rupture of schizonts. The lysate was centrifuged at 200g for 10min at 4^{o}C and supernatant filter-sterilized using 0.45µm-pore syringe filters and stored at -80^{o}C. Wild type *P. berghei* infected RBCs and uninfected RBCs were used as controls. To avoid experimental variations, all soluble lysates generated were pooled and aliquoted at 1mL in 1.5ml eppendorf tubes and stored at -80^{o}C for determining quantities of mIFN-γ by ELISA and prime-boost vaccination later.

3.6.3. Measurement of mIFN-γ concentration in Soluble Parasites lysate

The levels of interferon gamma concentration in soluble lysates prepared were determined using mIFN-γ ELISA development kit (Mabtech, Sweden). Following manufacturer's manual, high binding 96-well assay plate (Nunc, maxisorp) was coated with 100µl mAbAN18 diluted at 1µg/ml in PBS and incubate overnight at 4-

8°C. Plate was washed twice with 200µl/well PBS and blocked with 200µl/well of incubation buffer (PBS with 0.05% Tween 20 containing 0.1% BSA) followed by incubation at room temperature for 1hr. After 1hr, the plate was washed five times with washing buffer (PBS containing 0.05% Tween 20). Serial dilutions of the recombinant mIFN-γ standard was prepared in duplicates to generate the following concentrations; 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125pg/ml for the standard curve points. Hundred microlitre of soluble antigen lysate was also added into wells in duplicates. After adding the standard and diluted in 2x series and the sample, plate was then incubated overnight at 4°C. The plate was then washed five times and 100µl/well of mAb R4-6A2-biotin at 0.5µg/ml in incubation buffer added and incubated for 1hour at room temperature followed by washing five times. 100µl/well of Streptavidin-ALP diluted 1:1000 in incubation buffer was added and incubated for 1hr at room temperature followed by washing twice. After washing, 100µl/well of pNPP solution was added and incubated in the dark for 90min. The was then read at 405nm in an ELISA reader (Biotek). Concentrations of mouse IFN-γ in samples were interpolated from a standard curve drawn.

3.6.4. Safety and Appropriate Route of mIFN-γ Vaccination

A preliminary study was done to determine which route is effective in inducing significant immune responses (determined by measuring levels of tumor necrosis factor-alpha (TNF-α) in plasma) and to assess whether mIFN-γ vaccination is safe in mice. Two routes of administration; intra-peritoneal (i.p) and intravenous (i.v) were assessed. These routes were chosen over other routes such as intra-muscular and

subcutaneous routes of administration based on previous studies (Mogaka *et al.*, 2015; Ozwara, 2005). According to WHO guidelines on nonclinical evaluation of vaccines, standard parameters monitored during safety studies include daily clinical observations such as body weights and appetite levels (food consumption). Other recommended parameters include haematology and serum chemistry analyses, immunological investigations, gross and histopathological examinations (WHO, 2005). Two experimental groups and three control groups of 5mice each were used in this experiment. The experimental groups were i.v or i.p vaccinated with soluble lysate of TfPbA containing 75pg/ml IFN-γ (Mogaka *et al.*, 2015; Ozwara, 2005). The 75pg/ml IFN-γ dose was chosen based on previous study (Mogaka *et al.*, 2015). The control groups were either vaccinated with soluble lysate of uninfected RBC using the two routes (i.e. i.v and i.p) or not vaccinated with anything.

Table 3.2: Vaccination of mice by two routes

Group (n=5)	Route used	Material (Vol.)
Experimental		
TfPbA lysate (i.v)	intravenous	TfPbA lysate(200µl)
TfPbA lysate (i.p)	intraperitoneal	TfPbA lysate (200µl)
Control		
Uninfected RBC lysate (i.v)	intravenous	RBC lysate (200µl)
Uninfected RBC lysate (i.p)	intraperitoneal	RBC lysate (200µl)
Unvaccinated	none	none

After vaccination, the mice were monitored for 7 days during which the animals were observed daily by taking records on body weight and amount of pellet

consumed (WHO, 2005). At day 7 p.v, mice were euthanized using CO_2. Whole blood was collected via cardiac puncture and 100µL of whole blood transferred into 1.5mL K_2EDTA tubes. The remaining blood was transferred into a separate 1.5 eppendorf tube containing heparin and centrifuged at 5,000 RPM for 10min (Eppendorf centrifuge 1540C) to separate plasma. EDTA blood was analyzed for haematological parameters whereas plasma obtained was used for determining clinical chemistry parameters (AST, ALT, and urea) using Reflotron® Plus Clinical Chemistry Analyzer (Roche diagnostics, Germany) as well as measuring levels of tumor necrosis factor-alpha (TNF-α) responses by ELISA (Mabtech, Sweden).

3.7. Effect of *P. berghei* expressing mIFN-γ vaccination on Immunological Parameters

3.7.1. Vaccination of Mice and Challenge Infection

A total of 120 mice was divided into six groups of three experimental groups and three control groups with 20 mice each. The experimental groups were: TfPbA lysate (75pg/ml mIFN-γ);TfPbA (150pg/ml IFN-γ); and TfPbA and 35pg/ml vaccinated groups. Mice of TfPbA lysate (75pg/ml mIFN-γ), TfPbA (150pg/ml IFN-γ) groups were i.v primed using soluble lysate of TfPbA with 75pg/ml and 150pg/ml IFN-γ concentrations respectively. At two weeks (i.e. at day 14) post priming, mice were boosted once every week for two weeks. Mice of TfPbA and 35pg/ml group were i.v primed with $2x10^6$ of live TfPbA parasites (Ozwara, 2005). At 4% to 6% parasitemia, the mice were treated with COARTEM™ (Novartis Pharma, Switzerland) at a dose of 450mg/kg body weight (Kodippili et al., 2011). At day 14

post-treatment, the mice were boosted once every week for two weeks using soluble lysate of TfPbA with 35pg/ml IFN-γ. The control groups were RBC, RPMI 1640 and WtPbA. Mice of RBC and RPMI 1640 groups were i.v primed with soluble lysate of uninfected RBC and plain cultured RPMI 1640 respectively and boosted with the same material at one week interval after 14 days post priming. Mice of WtPbA control group were i.v inoculated with $2x10^6$ wild type *P. berghei* ANKA (WtPbA) and treated with COARTEM™ (at 4% to 6% parasitemia) (Kodippili et al., 2011). At day 14 post-treatment, the mice were boosted twice with soluble lysate of WtPbA at one week interval. One week after the second boost, mice (n=5) from each group were challenged with wild type virulent PbA parasites. The mice were then monitored from day 1 post-challenge for parasitemia and survivorship. The experimental design of the prime-boost vaccination is shown in **Figure 3.3.**

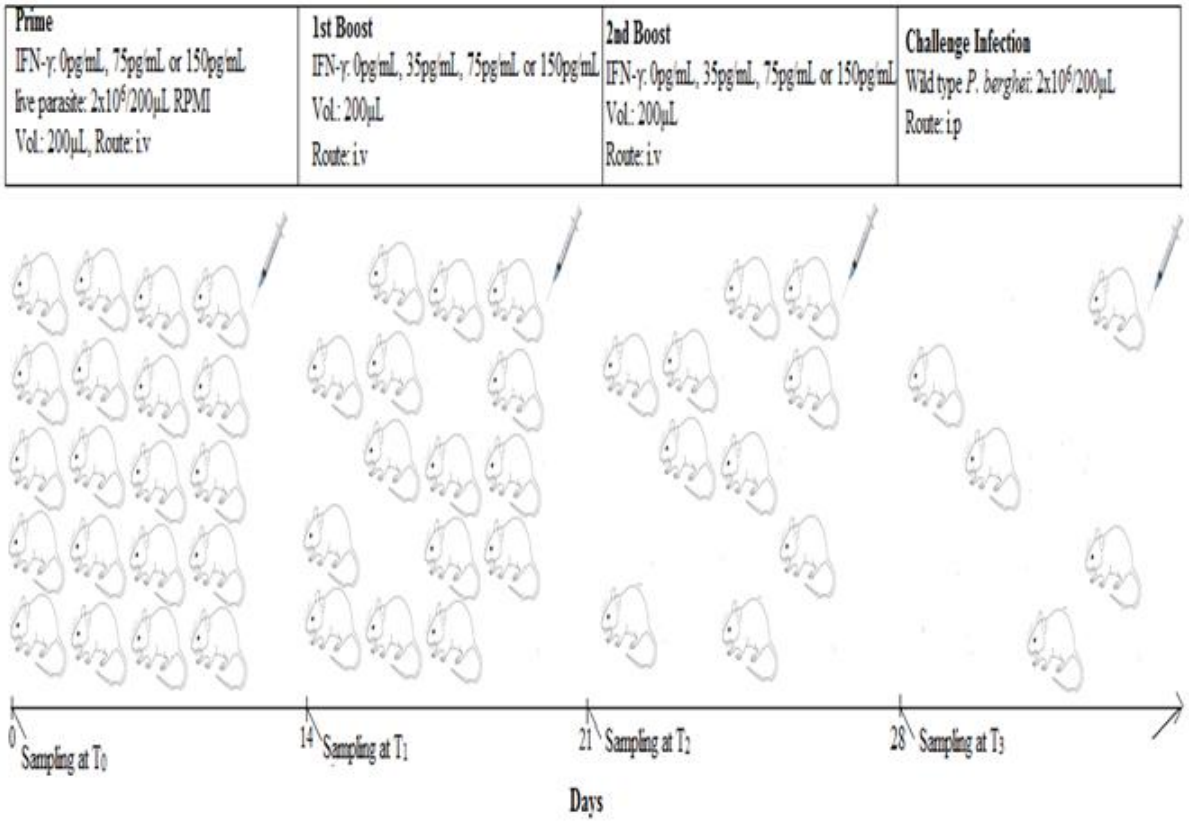

Figure 3.3: Experimental Design of Prime-Boost Immunization of Balb/C mice**.**

3.7.2. Sample Collection and Preparation of Serum

Prior to vaccination at week 0, mice (n=5) were sacrificed to collect blood and spleen for baseline analysis. At days 14, 21 and 28, mice (n=5) serially sacrificed for blood and spleen samples collection (**Figure 3.3)**. At each sampling point, mice were individually euthanized with CO_2. Euthanized mice were pinned down to a dissecting board using 23G needle and quickly rinsed (especially the abdominal region) with 70% ethanol. The thoracic region was carefully open to expose the heart using sterile forceps and a pair of scissors. The heart was then punctured to collect about 1mL of whole blood using 1ml syringe and needle (25G). The blood was transferred quickly into a 2ml eppendorf tube for processing of serum. The spleen located between the

kidney and the liver on the left side of the abdomen was exposed and aseptically removed using a sterile pair of dissecting scissors and dissecting toothed forceps into a 15mL centrifuge tube containing 3mL of pre-chilled sterile incomplete culture medium. The harvested spleen was then processed for single cell suspension and splenocyte culture. Whole blood collected was left on the bench at room temperature for 30min. After 30min, the blood was centrifuged in an eppendorf centrifuge for 10min at 2000 g (Eppendorf 5415C). The serum was carefully aspirated into clean 2mL eppendorf tube and stored at -20^{o}C for IgG ELISA later.

3.7.3. Preparation of Spleen Cells and Cultures

Spleen cells and cultures were prepared as described by (Yole, Shamala, Kithome, & Gicheru, 2007). Spleen from each mouse harvested above was placed on an autoclaved wire mesh (70µm) and squashed by pressing several times with the hard end of a 10mL syringe plunger into a Petri dish containing 3mL chilled incomplete culture. The wire mesh was rinsed with excess media into the Petri dish. Four millilitres of sterile laboratory prepared RBC lysing buffer (4.15g Ammonium chloride (NH4Cl), 50ml 0.1 Tris HCl, made up to 500ml with distilled H_2O, pH 7.5 and filtered with 0.22µm filter) was added drop-wise using sterile Pasteur pipette with shaking. The cell suspension was left on ice for 10min while RBC lyse. The cell suspension was transferred into a new 15mL centrifuge tube and spun down at 450g for 10min at 4^{o}C (Sigma Centrifuge). The pellet was resuspended in 2mL complete culture medium and washed thrice at 450g for 10min at 4^{o}C. The pellet was finally resuspended in 2mL complete culture medium and100µl aliquot taken for cell counting by trypan exclusion method using a haemocytometer. After enumeration,

the cell number was adjusted to $3x10^6$/mL with complete medium and plated at 3 x 10^5 cells/ 1mL complete medium in a 48-well culture plate (Costar) and stimulated with 1µg ConA. Cells in negative control wells had medium only (not stimulated). The plates were incubated for 72hours (Con A) at 37°C in a humidified incubator supplied with 5% CO_2. After incubation, culture supernatant was carefully aspirated into 2mL eppendorf tubes and stored at -80°C for TNF-α and IFN-γ measurement by ELISA later.

3.7.4. Measurement of IFN-γ Levels

IFN-γ levels in splenocyte culture supernatant were measured by ELISA according to manufacturer's manual (Mabtech, Sweden) and as previously in **sub-section 3.6.3** except that here, samples were 1:10 diluted in incubation buffer**.** The IFN-γ standard and culture supernatant from unstimulated cells were used as positive and negative controls respectively.

3.7.5. Measurement of TNF-α Levels

TNF-α levels in splenocyte culture supernatant were measured by ELISA according to manufacturer's manual (Mabtech, Sweden). Briefly, high protein binding 96-well ELISA plate was coated with monoclonal antibody MT1C8/23C9 at a concentration of 2µg/ml in PBS. The coated plate was incubated overnight at 4-8°C. The next day, the plate was washed twice with PBS at 200µl/well and blocked with 100µl incubation buffer. The plate was then incubated at room temperature for 1hour. After incubation, plate was washed five times with PBS-T and 100µl/well of samples diluted 10x and standard diluted according to the standard range (6-600pg/ml) in incubation buffer added. The TNF-α standard and culture supernatant from

unstimulated cells were used as positive and negative controls respectively. The plate was then incubated for 2hours at room temperature. After incubation, plate was washed five times and 100µl of Streptavidin-ALP diluted 1:1000 in incubation buffer added per well. The plate was incubated for another 1hour and washed five times. After incubation, 50µl/well of p-nitrophenyl-phosphate ELISA substrate (pNPP) (Mabtech, Sweden) was added and plate incubated in the dark for 30min. Plate was then read at 405nm in an ELx800 Absorbance Reader (Biotek, USA). The limit of detection of the assay was 6pg/ml.

3.7.6 Measurement of IgG Levels

Total serum IgG levels were determined by ELISA (Mabtech, Sweden). Following manufacturer's instructions, high protein binding 96-well ELISA plate (Nunc, maxisorp) was coated with anti-IgG antibody diluted to 1µg/ml in PBS and incubated overnight at 4-8^{o}C. Plate was then washed twice with 300µl PBS/well and blocked by adding 100µl/well of incubation buffer (PBS with 0.05% Tween 20 containing 0.1% BSA) followed by incubation at room temperature for 1hour. After incubation, plate was washed five times with PBS-Tween. 50µl of samples diluted 1:500 and standard diluted 2x series in incubation buffer was added to appropriate wells and incubated for 2hours at room temperature. The IgG standard and culture supernatant from unstimulated cells served as positive and negative controls respectively. After incubation, plate was washed five times and 50µl/well of anti-IgG-ALP diluted 1:1000 in incubation buffer was added. The plate was incubated for 1hour at room temperature. After incubation, 50µl/well of p-nitrophenyl-phosphate ELISA substrate (pNPP) (Mabtech, Sweden) was added and plate incubated in the dark for

15min. Plate was then read at 405nm in an ELx800 Absorbance Reader (Biotek, USA). The limit of detection of the assay was 0.1ng/ml.

3.8. Effect of *P. berghei* expressing mouse IFN-γ immunization on parasitemia and Survivorship

In order to determine whether vaccination protected mice from challenge infection, parasitemia levels were daily monitored from day 1 post infection for 21 days according procedures of Doolan, (2002). In brief, a small incision in the tip of the tail of the infected mice was made and a small drop of blood placed on a microscope slide to prepare a thin smear. The smear was fixed with methanol 20 for 30s and then stained with 10% fresh Giemsa solution for 20 min. The slide was washed, air-dried and parasitemia determined using a light microscope using immersion oil and X100 objective lens. Between 20–30 fields of 300–400 red blood cells (RBC) per field were counted and percentage of infected RBCs then calculated. The number of mice alive or dead were recorded every day.

3.9. Statistical Analysis

Data were first entered into Microsoft Excel and subsequently analyzed using GraphPad Prism®. Body weight data were expressed as mean ± standard error of mean, and pellet weight, haematology and clinical chemistry results were expressed as mean ± standard deviation and analyzed by one-way analysis of variance (ANOVA) followed by Turkey's *post* test. Means of the quantity of immunological parameters (total IgG, mIFN-γ, TNF-α), pre-patent parasitemia and survival times produced between treatment groups were also compared by ANOVA. Raw ELISA data were analyzed using a free online four parametric logistic curve software

(www.myassays.com). For statistical values, $p < 0.05$ was considered to be statically significant.

3.10. Ethical Statement

The study was carried out following strict adherence to the IPR guidelines on handling of experimental animals. Ethical approval on all animal procedures and protocols were obtained from the institutional ethics committee.

Chapter Four

RESULTS

4.1. Determination of Safety and Appropriate Route of *P. berghei* expressing mIFN-γ vaccination

4.1.2. Antimalarial Activity of *P. berghei* parasites

At day 3 post infection, pyrimethamine treatment was initiated for mice of groups A and B (at 1.81% and 1.68% parasitemia respectively). A day post treatment, parasitemia in group A dropped to 0.15% and remained undetectable by day 4 post treatment (i.e. day 7 post infection). Parasitemia in group B mice on the other hand increased to 9.44% day 2 post treatment and continued to increase after subsequent days until mice were euthanized and pRBCs passaged into donor mice for selection and maintenance. Treatment of mice in group C began at day 4 post infection (at 7.32% parasitemia) and by day 2 post treatment (i.e. day 6 post infection), parasitemia had dropped to 1.13%. Parasites were undetectable from day 3 post treatment by microscopy (**Figure 4.1**). The results show that wild-type *P. berghei* is sensitive to both pyrimethamine and coartem™ whereas transgenic *P. berghei* is resistant to pyrimethamine.

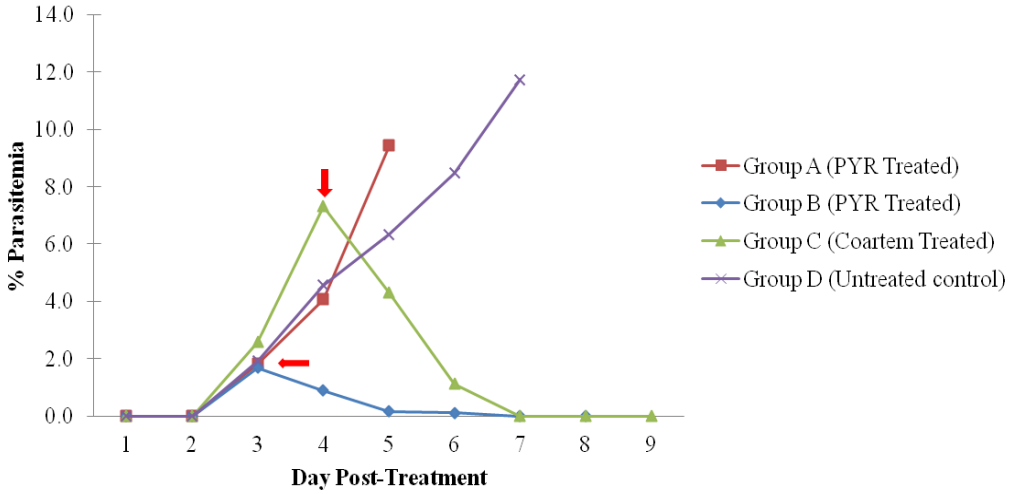

Figure 4.1: Sensitivity of transfected and wild type *P. berghei* to Pyrimethamine and COARTEM. Arrows show point of drug treatment.

4.1.3. Levels of mIFN-γ in Soluble Parasites lysate

Mouse IFN-γ was detectable in overnight cultures of both control (RBC and wild type *Pb*A) and experimental group (transfected *Pb*A). Levels of IFN-γ in wild type *Pb*A overnight culture was slightly higher (51.87pg/ml) than in transfected *Pb*A cultures (43.83pg/ml). However, after 9hours of culture with Sonication, IFN-γ levels in transfected *Pb*A soluble lysate was about 16folds higher than IFN-γ in wild type *Pb*A soluble lysate. IFN-γ level was below 20pg/ml in both overnight culture and soluble lysate for the RBC control (**Table 4.1**). This indicates that, transgenic *Pb*A is expressing mouse IFN-γ.

Table 4.1: Concentration of mIFN-γ in Soluble Lysate

Culture Type	18hrs	27hrs + sonication
TfPbA expressing mIFN-γ	43.83pg/ml	714.83pg/ml
WtPbA control	51.87pg/ml	63.32pg/ml
Uninfected RBC control	<20pg/ml	<20pg/ml

4.1.4. Body Weight Changes

Soluble lysate of TfPbA containing 75pg/ml mIFN-γ vaccination had no effect of the body weights of the animals (**Figure 4.2**). When compared to the unvaccinated control group, body weight gain was lower in i.v IFN-γ treated (9.4%) and i.p IFN-γ treated (1.7%) groups. However in all groups there was no significant change in body weight recorded at the beginning of experiment (day 0) and the last day of experiment (day 7) ($p > 0.05$). Thus, IFN-γ containing transgenic *P. berghei* lysate did not significantly affect body weights of vaccinated mice.

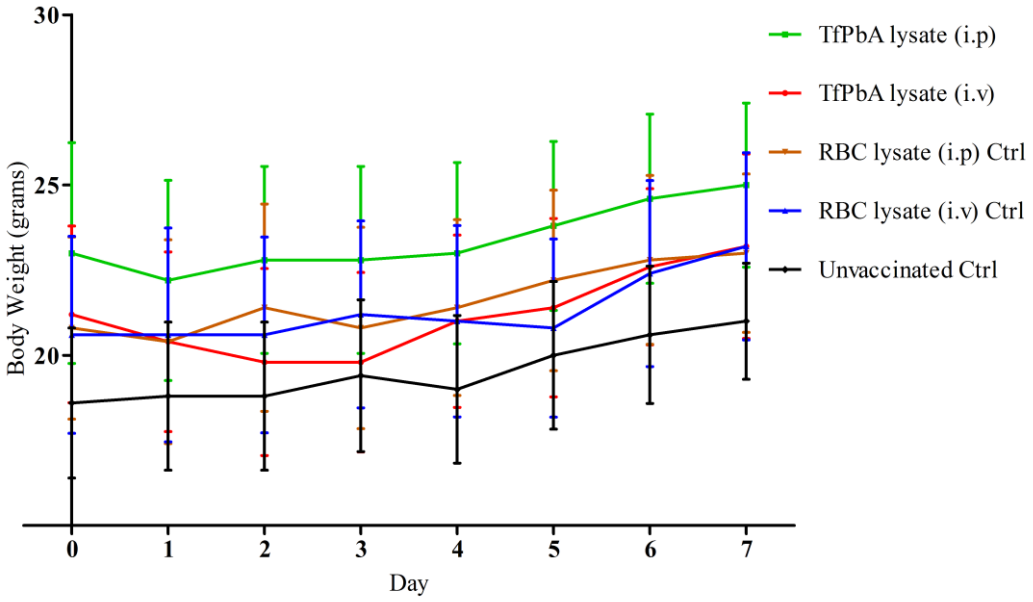

Figure 4.2: Variation of body weights before and during vaccination.

4.1.5. Food Consumption

Appetite levels of IFN-γ treated mice were also assessed. There was no significant difference in amount of pellet consumed between the groups (p value > 0.05) as shown in **Figure 4.3.** The mean weight of pellet consumed per week were 31.67g and 23.3g for RBC controls and the unvaccinated control respectively. The pellet consumed per week was 30.8g and 33.3g for i.v and i.p IFN-γ treated groups respectively. This implies that, vaccination with IFN-γ containing transgenic *Pb*A lysate has no significant effect on appetite levels of vaccinated mice.

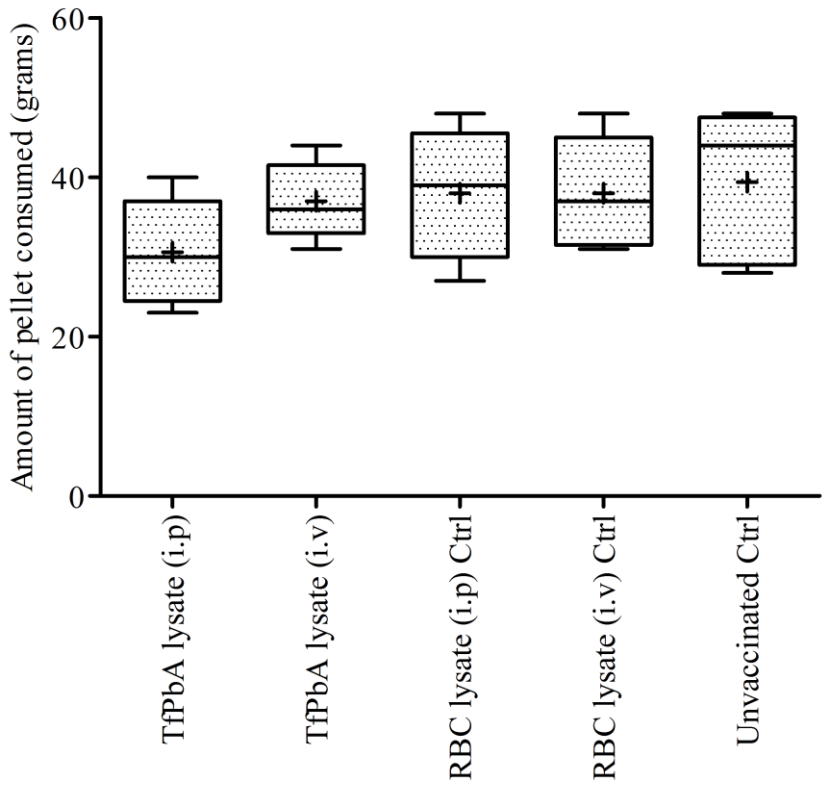

Figure 4.3: Amount of Pellet Consumed During Vaccination**.** Data represents mean ± standard deviation values for the 7day vaccination period.

4.1.6. Haematological Parameters

All the haematological parameters except platelet were within the normal values of the unvaccinated control group (**Table 4.2**). The mean ± SD platelet counts in unvaccinated control group was significantly lower (p=0.007) compared to TfPbA lysate (i.p) group. Some mean values were not within the normal range values (Araujo et al., 2015).

Table 4.2: Haematological Parameters of mice after 7days vaccination

Parameter		Group					Mice Normal values
	Unit	TfPbA lysate (i.v)	TfPbA lysate (i.p)	RBC lysate control (i.v)	RBC lysate control (i.p)	Unvaccinated control	
Haemoglobin	g/dL	14.60 ± 1.49	16.34 ± 0.73	15.40 ± 1.61	14.72 ± 1.75	15.42 ± 2.24	10.3 – 16.6
Leukocytes[b]	x 10^6/mm^3	7.55 ± 2.36	9.198 ± 1.13	8.412± 2.19	7.148 ± 1.04	9.278 ± 2.71	1.0 – 5.5
Erythrocytes	x 10^6/mm^3	9.37 ± 1.01	10.31 ± 0.43	9.97 ± 0.78	8.700 ± 1.69	9.946 ± 0.88	7.2 – 11.2
Hematocrit[b]	%	50.32 ± 5.52	54.98 ± 1.94	54.04 ± 4.32	51.20 ± 5.00	54.48 ± 6.12	33.1 – 52.0
MCV[b]	Fl	59.72 ± 13.53	53.28 ± 1.68	54.12 ± 2.00	54.16 ± 1.90	55.22 ± 1.69	45.0 – 47.0
MCH[b]	Pg	15.60 ± 0.33	15.84 ± 0.42	15.40 ± 0.75	15.44 ± 0.59	15.24 ± 1.09	13.9 – 15.5
MCHC	g/dL	29.04 ± 0.73	29.76 ± 1.37	28.48 ± 0.94	28.68 ± 0.80	27.90 ± 1.04	30.3 – 33.7
Platelet[b]	x 10^3/mm^3	963.8 ± 300.7	1207 ± 148.8[a]	886.8 ± 219	757.0 ± 174.4	840.4 ± 79.01	439.0 – 957.0

a: p value < 0.05 compared to unvaccinated group. Values represent mean ± standard deviation. b: Some mean values are not within the reference range values.

4.1.7. Biochemical Parameters in treated mice

Biochemical parameters of liver and kidney damages were assessed. The mean levels of ALT, AST and Urea in unvaccinated control were 22.33U/L, 14.24U/L and 58.8g/dL respectively. Comparison of values of among the groups showed that there was no significant difference ($p>0.05$). Levels of these parameters measured in the TfPbA vaccinated groups were within the normal range of the unvaccinated control group (**Figure 4.4**). Thus transgenic *Pb*A soluble lysate antigen containing 75pg/ml IFN-γ did not lead to liver and kidney damages.

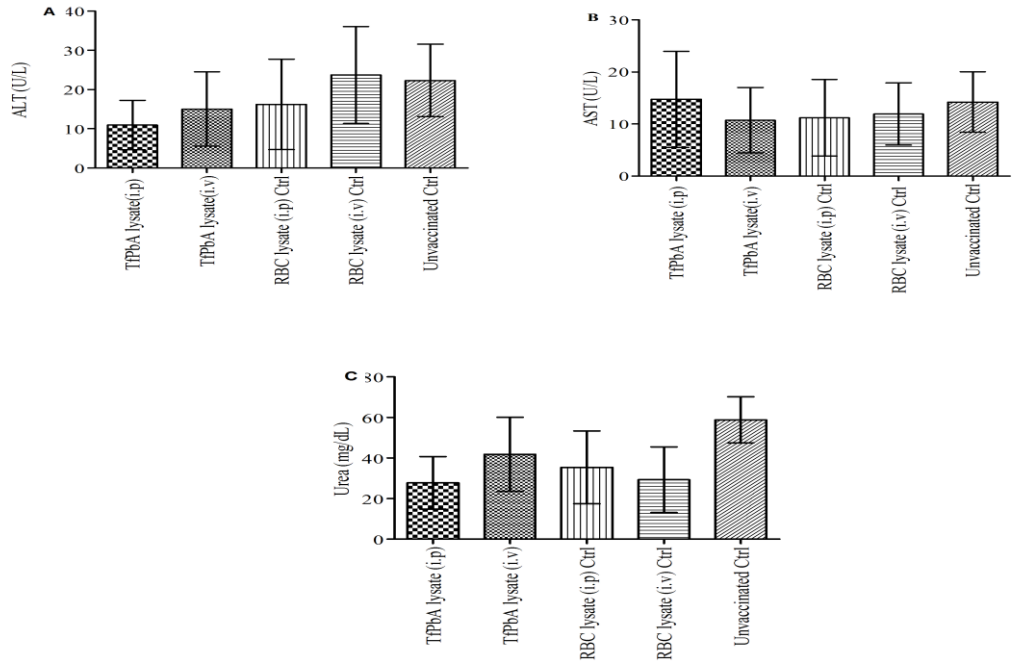

Figure 4.4: Levels of ALT (A), AST (B) and Urea (C) in Balb/c mice at the end of experiment on day 7. Error bars represent mean ± SD values.

4.1.8. Appropriate Route of mIFN-γ vaccination

Levels of TNF-α in plasma samples were used to determine the best route of vaccination. The plasma TNF-α of untreated control group was 113.6pg/ml. On the other hand, TNF-α levels in IFN-γ treated group was 168.1pg/ml and 143.3pg/ml in i.v treated group and i.p treated groups respectively. (**Figure 4.5**). The levels of TNF- α was significantly higher (*p=0.01*) in i.v treated group than in untreated control group. This indicates that vaccination via i.v route is better than vaccination via i.p route.

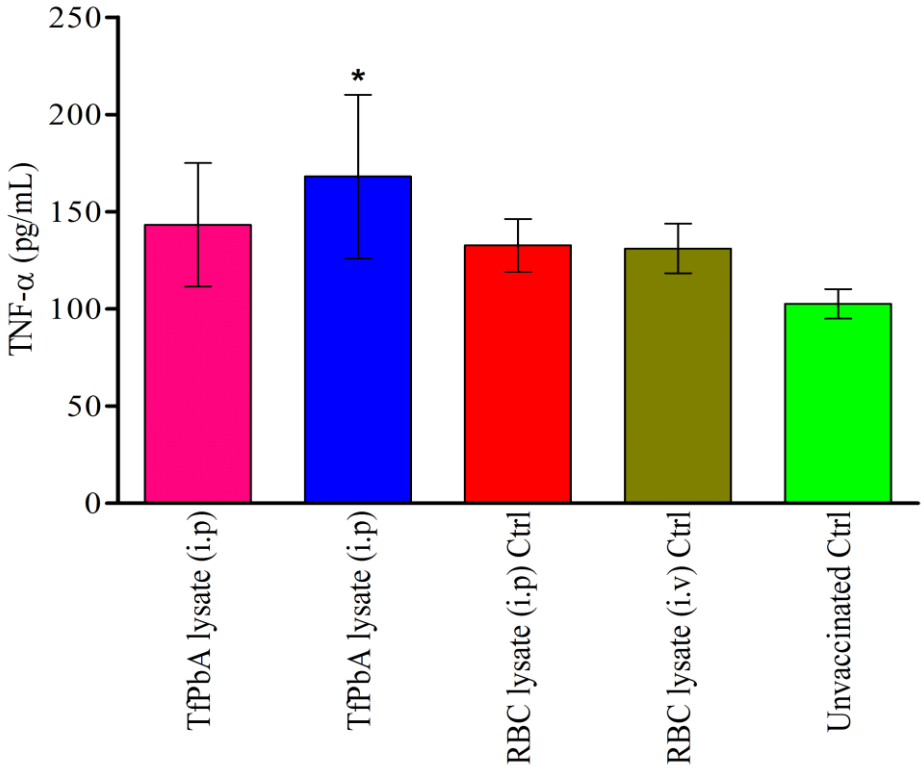

Figure 4.5: Levels of TNF-α in plasma samples of each group. *Error bars represent mean ± SD values. * p <0.05*

4.2.0. Effect of Vaccination on immunological parameters

4.2.1. Effect of vaccination on IFN-γ Production

Interferon gamma production levels in vaccinated mice were also measured from time point 0 to 28 (**Figure 4.6**). Generally, levels of IFN-γ produced were significant between the groups at day 21 (p= 0.035) and day 28 (p< *0.0001*). At day 14, IFN-γ production was highest in TfPbA plus 35pg/ml IFN-γ vaccinated group and lowest in WtPbA control (p= 0.113). One week after the first boost (at day 21), IFN-γ production was highest in 75pg/ml IFN-γ vaccinated group and lowest in RPMI control group. In the experimental groups, IFN-γ production was 1947pg/ml in 75pg/ml IFN-γ vaccinated group (p= 0.147), 739.7pg/ml in 150pg/ml IFN-γ vaccinated group (p= 0.013) and 190.4pg/ml IFN-γ vaccinated group (p= 0.077). At day 28 p.v, IFN-γ production was significantly higher in 75pg/ml IFN-γ vaccinated group compared to wild-type control group (p=0.006). Similarly, IFN-γ levels in 75pg/ml vaccinated group was significantly higher compared to the RPMI control group (p=0.005).

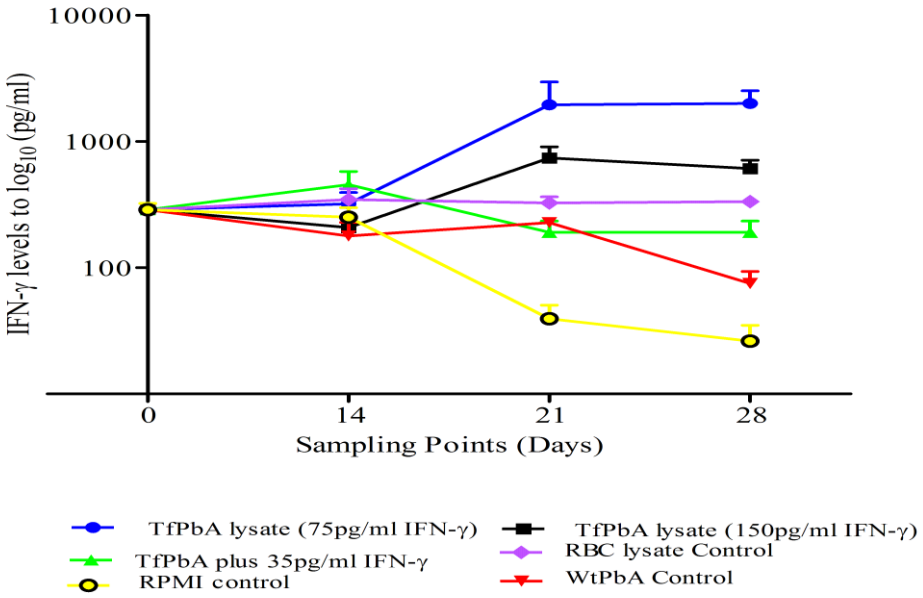

Figure 4.6: Levels of IFN-γ measured at different time points. Error bars represent Standard error mean.

4.2.2. Effect of immunization on TNF-α Production

Tumour necrosis factor alpha (TNF-α) production in vaccinated mice were also measured from time point 0 to 28 **(Error! Reference source not found.).** Generally, NF-α levels measured were not significant between the groups at day 14 and 28 post-vaccination but significant at day 21 post-vaccination (*p= 0.037*). Production was highest in TfPbA plus 35pg/ml IFN-γ group at days 14, 21 and 28 and lowest in RPMI control at day 14, and in RBC lysate control at day 21 and 28 p.v. Compared to RBC lysate control group, TNF-α production in TfPbA lysate (75pg/ml IFN-γ) was higher (p=0.017) at day 21.

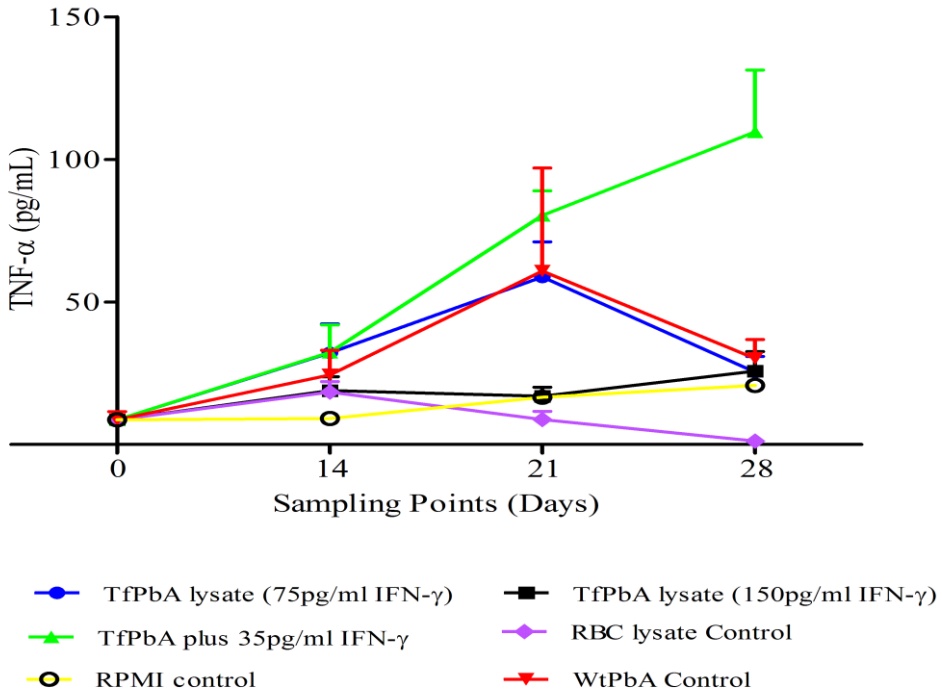

Figure 4.7: Levels of TNF-α measured at three time points. Error bars represent standard error of the mean.

4.2.3. Effect of vaccination on Total serum IgG Production

The effect of immunization on total serum IgG levels in mice was also analyzed (**Figure 4.8).** Generally, there was no significant variation in IgG levels among the groups at days 14, 21 and 28 (p= 0.388, p=0.205 and p=0.150 respectively). However, elevated levels were observed after priming at day 14 with highest levels observed in mice vaccinated with soluble lysate of TfPbA containing 150pg/ml, followed by WtPbA vaccinated group and lowest in TfPbA plus 35pg/ml IFN-γ group (p= 0.388). After the second boost, there was twofold decrease (p=0.2791) in IgG levels in mice vaccinated with soluble lysate of TfPbA containing 150pg/ml whereas there was no significant decrease also observed in WtPbA control group (p=0.185). In contrast, an increase in IgG levels (but no significant) from 62.94µg/ml

at day 14 to 73.71µg/ml after second boost at day 28 was in mice vaccinated with soluble lysate containing 75pg/ml IFN-γ. These findings suggest that, immunization of mice with soluble lysate had no significant effect on B-cell responses.

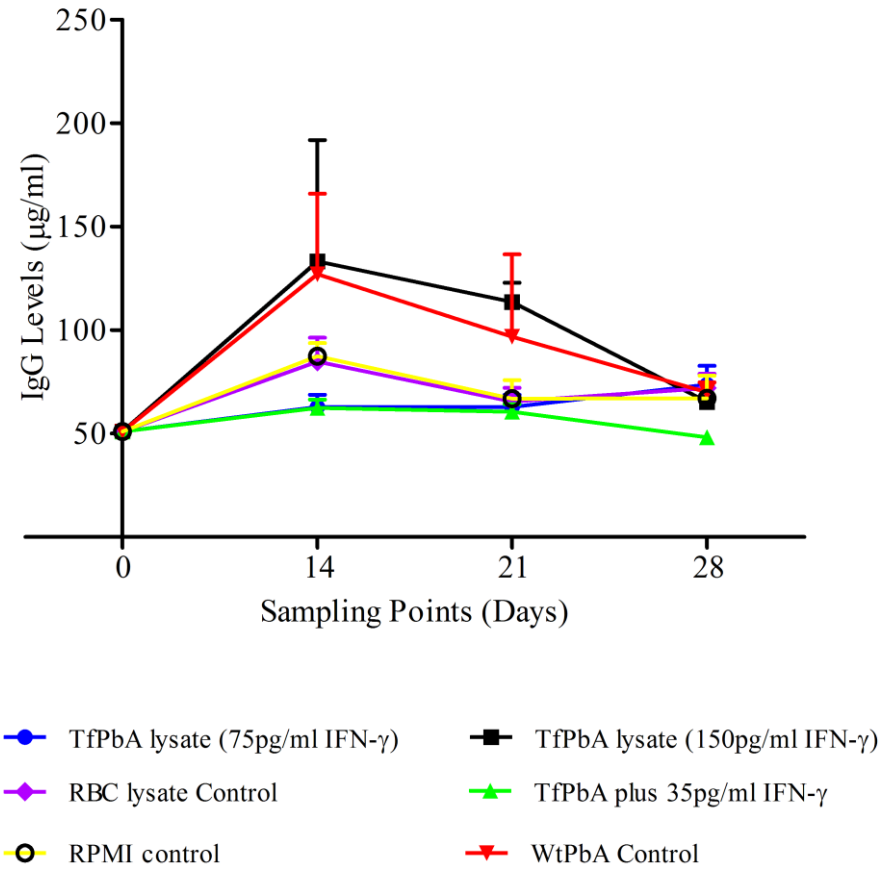

Figure 4.8: Total serum IgG levels measured at three time points. Error bars represents Standard Error of the mean.

4.3. Effect of Vaccination on Parasitemia and Survivorship

4.3.1 Effect of Vaccination on Parasitemia

Parasitemia of mice were monitored three days after challenge infection. Parasitemia profiles of the challenged mice were not statistically significant (*p=0.339)*. By day 3 post infection, mice vaccinated with RBC lysate and wild-type parasite lysate had developed mean parasitemia greater than 8 and 25parasites per 2000 erythrocytes respectively. In the naive control, mean parasitemia of about 18 parasites per 2000 erythrocytes was observed by day 3 post-challenged infection. By day 10 post-infection, the mean parasitemia had increased to more than 240 parasites per 2000 erythrocytes in these control groups. There was delayed onset of parasitemia for at least 1day in the vaccinated groups. The mean patent parasitemia was less than 100 parasites per 2000 erythrocytes for mice vaccinated with 75pg/ml IFN-γ and transfected *P. berghei* (**Figure 4.9**) and more than 300 parasites for mice vaccinated with 150pg/ml IFN-γ. Mice vaccinated with 75pg/ml IFN-γ lysate had the lowest mean patent parasitemia compared to the naive control group (p=0.633).

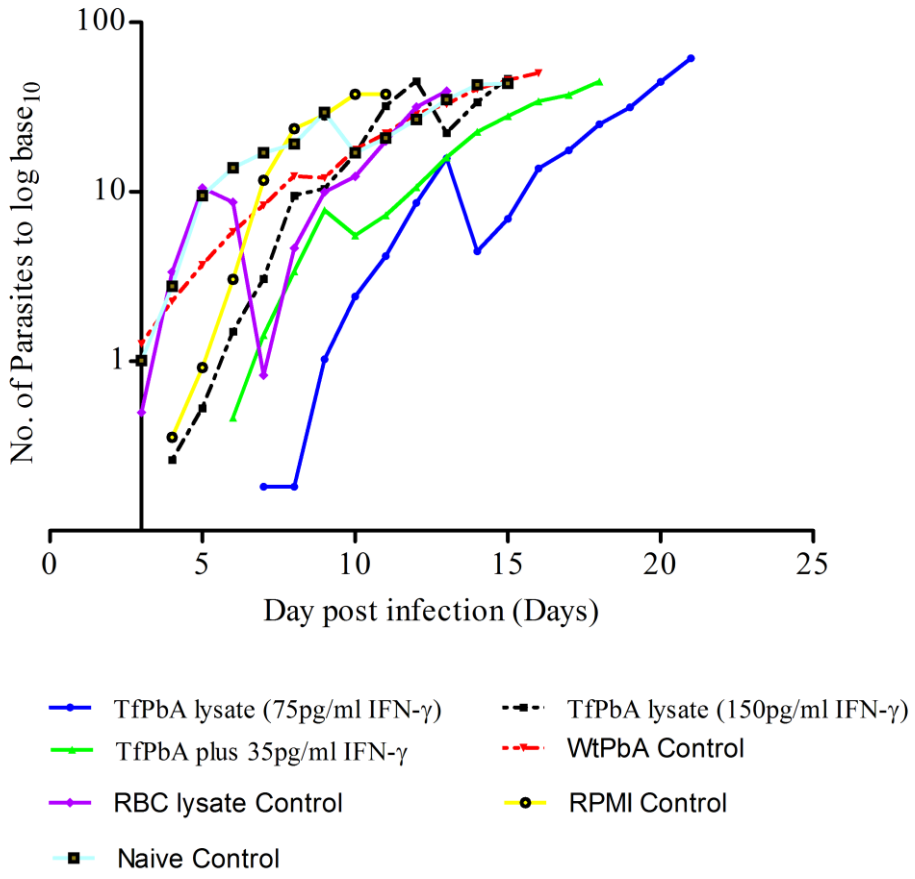

Figure 4.9: Parasitemia profile of mice after challenge infection.

4.3.2. Effect of Vaccination on Survivorship

Survival of mice was determined from day three post challenge infection (**Figure 13.4)**. By day 17, there was 0% survival (100% death) in the control groups. On the other hand, 80% and 60% respectively of the mice in the group vaccinated with 75pg/ml IFN-γ and transfected *P. berghei* survived infection by day 17. The remaining mice of the 75pg/ml IFN-γ and the transfected *P. berghei* groups survived infection until day 21 and 19 respectively. However, 100% death was recorded in the 150pg/ml IFN-γ vaccinated group by day 17. Compared to the naive control group, mice vaccinated with 75pg/ml IFN-γ lysate survived wild type challenge infection

5days longer (p=0.013). This result shows there is protective potential of IFN-γ expressing *P. berghei* in mice.

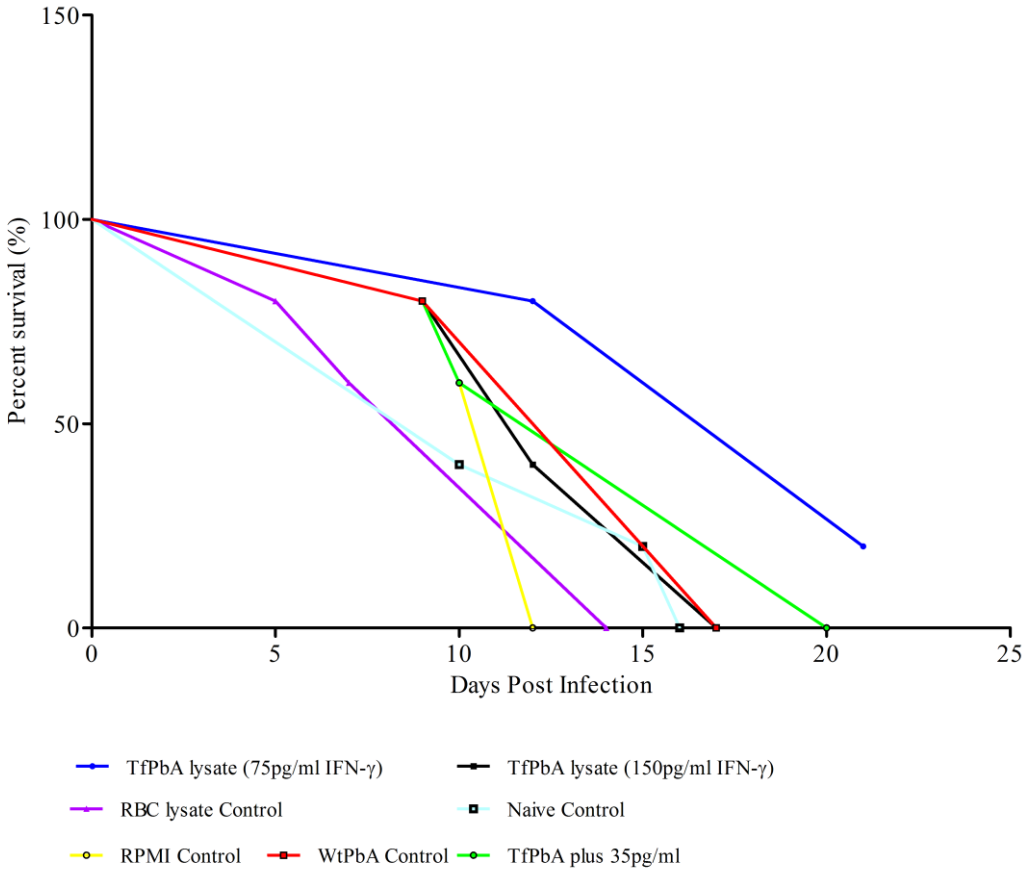

Figure 4.10: Survival of Balb/C mice after challenge infection.

Chapter Five

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1. Overview

In this thesis, prime-boost vaccination strategy with soluble lysate antigen of transgenic *P. berghei* expressing IFN-γ as well as the live transfected parasites was used to assess the potential of cytokine expressing *P. berghei* to induce moderate malaria protection balb/c mice. Several studies have demonstrated the protective role of IFN-γ cytokine either administered exogenously or produced endogenously against blood and liver-stages of *Plasmodium* infections in humans and animal malaria models (Okada *et al.*, 2015; Su & Stevenson, 2000). With the advancement in transfection technology for malaria parasites, IFN-γ expressing *P. berghei* ANKA has recently been generated (Mogaka *et al.*, 2015). In that study, IFN-γ expressing *PbA* was shown to expresses bioactive host IFN-γ which immunomodulate immune responses to malaria parasites. It was further demonstrated that, a single vaccination to this transgenic parasites delay slightly parasitemia patency and prolong parasitemia suppression in BALB/c mice. However, repeated exposure of mice to this transgenic parasites leading to moderate protection has not been investigated The aim of this study was therefore to evaluate the vaccine potential of transgenic *P. berghei* expressing mIFN-γ using prime-boost vaccination strategy.

5.2. Safety and Appropriate Route of Vaccination

5.2.1. Antimalarial Activity of *P. berghei* and Generation of soluble lysate

In this study, the transgenic *P. berghei* used was episomally transfected (Mogaka *et al.*, 2015). Earlier studies have shown that, in the absence of selective drug pressure, episomally based plasmids suffer from plasmid loss over several generations due insufficient replication (Matz & Kooij, 2015.; van Dijk *et al.*, 1997; Wu, Kirkman, & Wellems, 1996). Thus, in this study, plasmids contained in the transgenic parasites were maintained continuously under pyrimethamine pressure. Compared to wild-type form, growth of the transfected *P. berghei* was not inhibited by pyrimethamine treatment. This means that, the plasmid containing the TgDHFR is stable in the parasites.

To generate parasite lysate containing mIFN-γ, transgenic parasites were first propagated *in vivo* followed by *in vitro* into mature schizont stages. Measurement of IFN-γ levels in lysate reveals that, a significant amount of mIFN-γ was produced by transgenic parasites cultures compared to the wild type parasite cultures. The IFN-γ detected in the control cultures, though in minimal concentrations, may probably have originated from mouse lymphocytes such as natural killer cells, T cells, and dendritic cells (Inoue, Niikura, Mineo, & Kobayashi, 2013). In this study, parasites cultured for 18hours overnight had some levels of IFN-γ but in lower quantities. When parasites were cultured for additional 9hours followed by sonication, increased IFN-γ levels was detected. This was about 16folds higher than levels detected in overnight culture. The EF-α1 promoter activity has been shown to constitutively express transgenes throughout blood stage development (Tania, de Koning-Ward,

Sperança, Waters, & Janse, 1999; Horrocks, Pinches, Kriek, & Newbold, 2002). The difference in detected IFN-γ levels between 18hours and 9hours culture may be due to protein subcellular loacalization associated with *Plasmodium* spp. (Woodcroft *et al., 2012)*. Again, the level detected in this study was about 4 folds higher than levels reported earlier study (Mogaka *et al.*, 2015). In the previous study, IFN-γ levels were measured in culture supernatant after 6hours incubation without crushing mature schizonts by sonication. During intraerythrocytic stage infection, *Plasmodium* parasites secrete proteins which are transported into the cytoplasm of erythrocyte (MacKenzie, Gómez, Bhattacharjee, Mann, & Haldar, 2008). Thus, the high levels of IFN-γ detected in this study compared to the earlier study may be due to the rupture of the mature blood stage schizonts by Sonication resulting in the release of IFN-γ from the erythrocytes. Since this hypothesis was not extensively tested in this study, it is recommended a comparative study be performed to determine whether parasite expressed IFN-γ is secreted out by the erythrocyte, or released upon schizont rupture.

5.2.2. Safety and Appropriate Route of Cytokine Vaccination

Ideally, a vaccine should not lead to organ damage. Over production of IFN-γ during malaria infection leads to immunopathologies (Julius, Rebecca, Francis, Viviene, & Muregi, 2013; Lamb *et al.*, 2006). Also, cytokine administered exogenously could be toxic to the host (Curfs, van der Meide, Billiau, Meuwissen, & Eling, 1993).

In this study, preliminary studies were conducted to assess the safety as well as which route (intravaneous or intraperitoneal) is effective in inducing immune responses during acute conditions. Based on earlier studies conducted by Mogaka *et*

al., (2015), mice were vaccinated with soluble lysate of TfPbA containing 75pg/ml IFN-γ and then monitored for 7days. The dosage used was twice the dose used in previous studies.

Transgenic *P. berghei* soluble lysate containing IFN-γ had no effect the physical and health conditions such as body weight and appetite levels of the animals. Hematological analysis indicated a significant effect on platelets. Although the mechanisms involved in the increased levels of platelet count in this study remained to be elucidated. In an earlier study, similar findings were reported. Factors such as interleukin (IL)-6, IL-1, TNF-α and improper sampling techniques have been shown to increase platelets activation (Dan et al., 2009; Jirouskova, Shet, & Johnson, 2007). Whereas elevated ALT and AST levels reflect hepatocellular inflammation, damage and necrosis decreased urea levels signifies kidney damage in blood. The amount of AST in the blood is directly associated with the extent of tissue damage. In this study, there were no changes in ALT, AST and urea levels in vaccinated group compared to unvaccinated control groups indicating the absence of liver and kidney damages. Other tests which may be used to assess liver damage include γ-glutamyl transferase and serum blood urea nitrogen (BUN), serum creatinine for kidney damage. Gross pathology and histopathology are also good diagnostic tests for determining toxicity. However, they were not assessed in this study. Overall, IFN-γ vaccination may be safe as there were no significant indication of liver and kidney damages. This is in agreement with findings of Ozwara, (2005) where vaccination of IFN-γ expressing *P. knowlesi* via intravenous route was found to be safe in rhesus monkeys.

Vaccination via intravenous route was found to induce higher TNF-α responses than intraperitoneal route. This high TNF-α responses of i.v vaccinated mice compared to i.p vaccinated may be due to pharmacokinetic differences. IFN-γ in blood then leads to activation of blood cells to release TNF-α during acute conditions (Boehm, Klamp, Groot, & Howard, 1997; Sim, 2015).

5.3. Effect of Vaccination on IgG, IFN-γ and TNF-α Responses

In this study, effect of IFN-γ vaccination on T-cell responses (TNF-α and IFN-γ) and B-cell responses (total IgG) were assessed. Significant levels of TNF-α and IFN-γ were detected in spleen cells of mice vaccinated groups (75pg/ml IFN-γ containing parasite lysate, transfected *P. berghei* expressing mIFN-γ parasites) after first boost. It has been reported that, IFN-γ affects monocytes by shifting its differentiation from dendritic cells to macrophages (Delneste *et al.,* 2003). Similarly, exposure of macrophages to IFN-γ has been shown to enhance secretion of other cytokines such as TNF-α and IL-12 and promotion of Th1 cell development (Duque & Descoteaux, 2015). Thus, the high TNF-α and IFN-γ levels produced after first boost may be due to priming of Th 1 lymphocytes and activated macrophages (Inoue et al., 2013). It has also been reported that IFN-γ produced by $CD4^+$ can activates IL-12β2 expression leading to upregulation and production of other cytokines (De Souza et al., 1997). The IgG produced may be due to activation of B-cells by $CD4^+$ produced IFN-γ (Schindler, Lutz, Röllinghoff, & Bogdan, 2001; Zhu & Paul, 2015).

5.4. Effect of Vaccination on Parasitemia and Survivorship

The observed correlation between IFN-γ and TNF-α in these groups is in complete agreement with previous study by Megnekou *et al.,* (2013) The delayed parasitemia patency coupled with prolonged parasitemia suppression and survival time in

vaccinated mice (75pg/ml IFN-γ and transgenic parasites) could be due to the high IFN-γ and TNF-α levels present prior to challenge infection. This observation has been reported in previous studies where clearance of blood stage infection has been found to coincide with high IFN-γ levels (Inoue *et al.,* 2013; Megnekou *et al.,* 2013; Megnekou, Hviid, & Staalsoe, 2009). Other studies have equally confirmed the involvement of TNF-α in *Plasmodium* clearance. TNF-α probably induce parasite clearance by activating of other cells such as macrophages which leads to the release of NO (Langhorne *et al.,* 2002). Release of nitric oxide(NO) leads to killing of parasites (Taylor-Robinson *et al.,* 1993).

In this study, high serum IgG levels together with high IFN-γ levels were in 150pg/ml IFN-γ vaccinated mice. Despite the high levels of IgG detected in this mice, pre-patency period and survival time were shorter. This could suggest that, serum IgG may not be effective in promoting prolong parasite suppression and enhanced survivorship. This is in agreement with earlier studies (Achtman, Stephens, Cadman, Harrison, & Langhorne, 2007; De Souza *et al.,* 1997).

5.5. Conclusion

The study has demonstrated that, vaccination of mice with Transgenic *P . berghei* lysate with 75pg/ml IFN-γ may be safe and well tolerated in mice. The study has also shown that, repeated vaccination with transgenic *P. berghei* soluble lysate containing mIFN-γ has significant effect on IFN-γ and TNF-α production by spleen cells leading to enhanced malaria protection.

5.6. Recommendation

The study recommends the following;

i. comparative study should be carried out to determine conclusively whether parasite expressed IFN-γ is secreted out by the erythrocyte, or released upon schizont rupture.

ii. gross and histopathology studies should be performed to determine the effect of vaccination on tissues vital organ.

iii. Combination of cytokine expressing parasites with soluble lysate in a priming-boosting manner should be considered in future vaccination experiments.

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APPENDICES

Appendix 1: Reagents and Reagent Setups

Incomplete RPMI Media- 1000mL

Add 1 sachet of RPMI 1640 powder, supplemented with L-glutamine (GIBCO) to 900mL distilled water slowly under continuous stirring. Rinse the sachet completely into the medium. Add 5.95 g of HEPES to make 25mM. Top up with distilled water to 1000mL. Filter sterilize through a 0.20-µm filter in 1000mL sterile bottle. Store at 4^{o}C for up to 30−60d. When about to use, add 4.0mL of 5% sodium bicarbonate solution (24mM) and 0.5mL of Neomycin-sulphate stock per 100mL of stored media.

Complete RPMI Media- For 100mL

Add 25mL of FBS heat inactivated to 75mL of Incomplete RPMI media.

5% $NaHCO_3$ Stock solution:

Add 5 g of $NaHCO_3$ to 100mL of distilled water. Filter sterilize through a 0.20-µm filter and store at 4^{o}C in tightly capped bottles.

Incomplete RPMI Media for Spleencyte culture

For 100mL

Add 1mL of 5mM 2-mercaptoethanol stock solution to 0.2mL of 50mg/ml gentamicin. Top up with 98.8mL incomplete medium without neomycin sulfate.)

Complete RPMI Medium for spleenocyte culture

Add 10mL of FBS to 90mL of incomplete RPMI medium

10% Giemsa Working Solution:

For 100mL, take 10mL of Giemsa stock solution into 90mL of distilled water.

Phosphate Buffer Saline (10x)- For 500mL

NaCl ----------> 40gm

KCl----------> 1gm

Na_2HPO_4 ----------> 7.2g

KH_2PO_4 ----------> 1.2g

dH_2O ----------> 400mL

Adjust pH to 7.2 and volume to 500mL. Store at room temperature.

Phosphate Buffer Saline (PBS) 1X - For 1000mL

Take 100mL of PBS (10X) and top up with 900mL distilled water

RBC Lysing buffer

4.15g Ammonium chloride (NH_4Cl), 50ml 0.1M Tris HCl, made up to 500ml with distilled H_2O, pH 7.5 and filtered with 0.20μm pore size filter unit. Store at 4^oC.

Pyrimethamine Solution (0.007mg/ml)- For 400mL

Dissolve 28mg of pyrimethamine powder (5-4-Chlorophenyl- 6-ethyl 2,4-pyrimidinediamine) in 4mL DMSO to make a final concentration of 7 mg/ml. Vortex

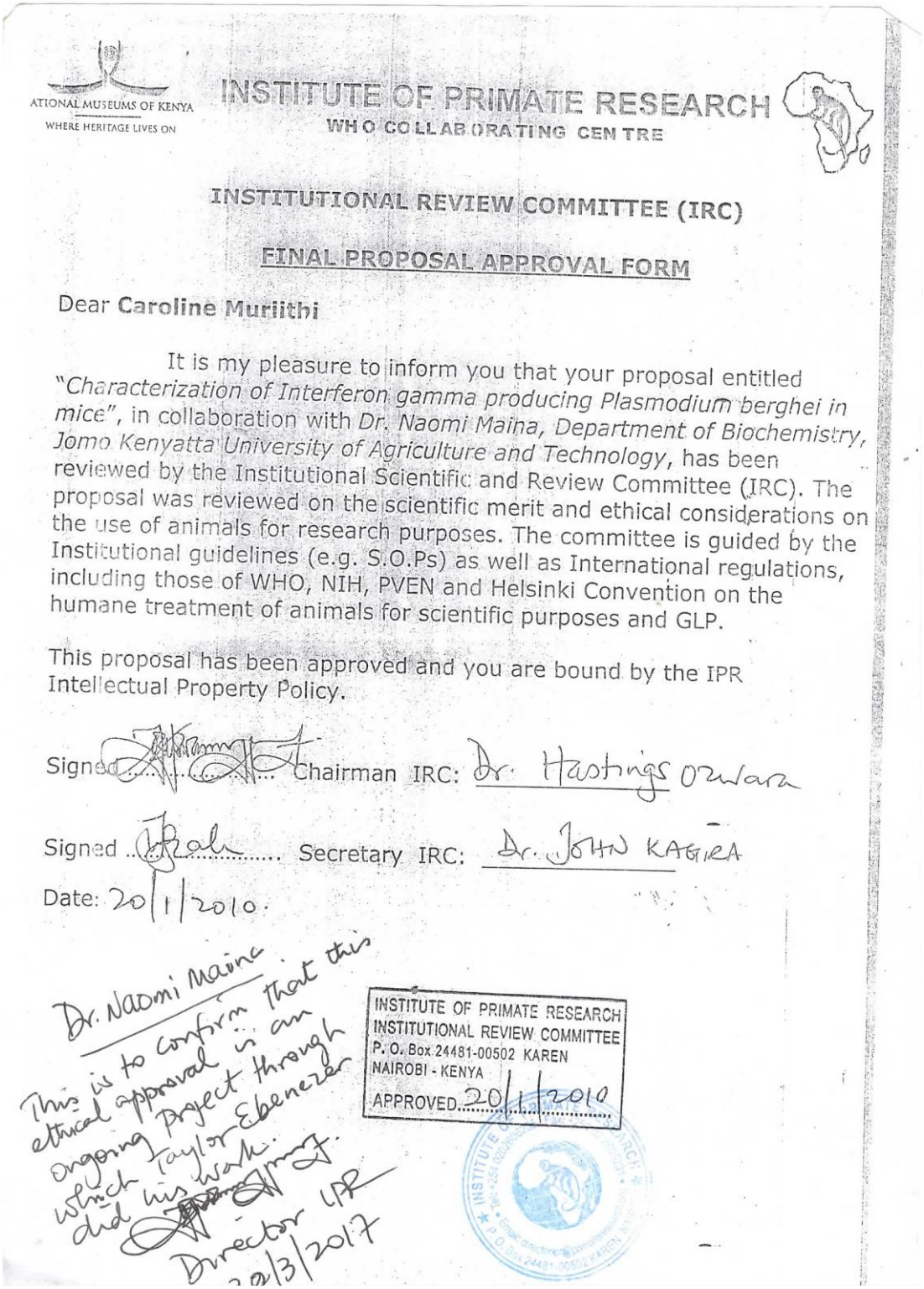
briefly. Add 350mL of normal tap water and adjust pH to 3.5–5.0 (with 1 M HCl). Volume to 400mL. Store at 4°C and use the solution for the drinking water of mice for a maximum of 7d.

COARTEM SOLUTION:

At a dose of 450mg/kg of tablet per day for three consecutive days. Each tablet contains 20/120mg Artemether Lumefantrine making a total of 140mg per tablet.

- Determine the average weight of all experimental mice. Assuming the average weight of the 60 experimental mice = Xg
- Then, mg of COARTEM required = (Xg x 450mg)/1000g
- Total mg required for 60 mice per day = ((Xg x 450mg))/1000g x 60
- Determine the number of tablet required and crushed into powder.
- Dissolve in 15ml of water (0.2ml per mouse for 60mice).
- Administer 0.1ml orally per mouse twice a day (morning and evening)

Appendix 2: Ethical Approval



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FINAL PROPOSAL APPROVAL FORM

Dear **Caroline Muriithi**

It is my pleasure to inform you that your proposal entitled "*Characterization of Interferon gamma producing Plasmodium berghei in mice*", in collaboration with *Dr. Naomi Maina, Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology*, has been reviewed by the Institutional Scientific and Review Committee (IRC). The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines (e.g. S.O.Ps) as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved and you are bound by the IPR Intellectual Property Policy.

Signed ..... Chairman IRC: Dr. Hastings Ozwara

Signed ..... Secretary IRC: Dr. JOHN KAGIRA

Date: 20/1/2010.

Dr. Naomi Maina
This is to confirm that this ethical approval is an ongoing project through which Taylor Ebenezer did his work.
Director IPR
20/3/2017

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED 20/1/2010

Appendix 3: Acknowledgement of Receipt of Manuscript for Publication

JKUAT EMAIL

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Immunization of Mice with Soluble lysate of Interferon gamma expressing Plasmodium berghei Induces high IFN-γ Production.
Ebenezer Mintah Taylor, MSc.; Faith Onditi; Naomi Maina; Hastings Ozwara

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