THE ROLE OF *SCHISTOSOMA MANSONI* EGGS IN IMMUNE PROTECTION AGAINST *PLASMODIUM BERGHEI* INFECTED MICE

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NOVEMBER, 2013
DECLARATION

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

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DEDICATION

I dedicate this thesis to my mum, my brother Ondigo and two sisters. May the Lord bless you.
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# ABBREVIATIONS AND ACRONYMS

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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>HEPES</td>
<td>Hydroxyethyl-piperazineethanesulfonic acid</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral Blood Mononuclear cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>SEA</td>
<td>Soluble Egg Antigen</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

The co-occurrence of malaria and schistosomiasis is common in tropical regions of the world. Malaria induces a strong Th1 response while schistosomiasis skews the response to a Th2. Several studies demonstrate a non-consistent effect of schistosomiasis infection on progression of malaria. On one hand, schistosomiasis infections protect against cerebral malaria while on the other hand, they are associated with increased malaria severity. This study examined the role of *Schistosoma mansoni* eggs on *Plasmodium berghei* malaria progression in BALB/c mice. The objectives were to determine the changes in Th1, Th2 cytokines and IgG levels which are markers associated with malaria and schistosomiasis protection and also determine if *S. mansoni* eggs lead to protection from *P. berghei* malaria. Two groups of mice were used: the experimental group and the control group. The experimental group was injected with a triple dose of *S. mansoni* eggs at ten day interval before being challenged with *P. berghei*. The control group was infected with *P. berghei* only. Five mice from both groups were euthanized at each time point (day 3, 6, 9 and 12 post challenge with *P. berghei*) and the spleen and serum collected. Five mice from each group were monitored throughout the experiment. Parasitaemia was monitored daily using Giemsa stained blood smears. The results showed that the experimental mice exhibited lower levels of *P. berghei* parasitaemia (15.52%) as compared to the controls (23.06%). However the difference was not significant (*p*>0.05). IgG levels were found to be higher in the experimental mice compared to controls due to stimulation by soluble egg antigen (SEA). The differences in IgG levels between the two study groups was not significant (*p*>0.05). The levels of IFN-γ and IL-4 were higher in the experimental mice than the control group though the difference was not significant (*p*=0.213). The levels of IgG and IL-4 in experimental mice could be responsible for the delay in death reported in these mice and enhanced survivorship. In conclusion, *S. mansoni* eggs did not induce significant differences in cytokine and IgG levels; nevertheless they contributed to delaying death in the experimental mice by two days by enhancing levels of IgG and IL-4. These findings provide a pointer for further research in this field using higher animal model such as the non-human primates for a better understanding of the immunomodulatory role of schistosoma eggs on progression of malaria.
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background
Concomitant parasitic infections are common in tropical and subtropical regions worldwide (Brooker et al., 2007; Laranjeiras et al., 2008). Malaria is the most important parasitic diseases causing approximately 1 million deaths annually (WHO, 2010). In contrast, schistosomiasis is estimated to affect about 200 million people globally, results in fewer deaths, but is associated with considerable morbidity (Chitsulo et al., 2004). The overlap of Plasmodium falciparum (P. falciparum) with helminth infections including Schistosoma haematobium (S. haematobium) and Schistosoma mansoni (S. mansoni) are known to profoundly affect each other immunologically and in the degree of pathology they cause in the host (Chitsulo et al., 2004).

On one hand, schistosomiasis infections protect against cerebral malaria (Bucher et al., 2011; Waknine-Grinberg et al., 2010) while on the other hand, they are associated with increased malaria severity (Legesse et al., 2004; Sokhna et al., 2004). Helminth infected patients have decreased cytoadherence and decreased splenic clearance; the IgE immune complexes formed have an important role in influencing clinical presentation of severe malaria and establishing malaria
tolerance (Nacher et al., 2002). These are as result of regulation in inflammatory factors as also shown in studies on Senegalese children (Diallo et al., 2004; Bonnard et al., 2004). Other studies, have evaluated the role of both cellular and humoral responses in cases of co-infections. Schistosomiasis infections has been shown to evoke both the cellular and humoral arms of the immune system that can unbalance the regulation of inflammatory factors in uncomplicated cases of malaria (Diallo et al., 2004; Druilhe et al., 2005; Lyke et al., 2005) which are explained by cross reactive epitopes between schistosome and Plasmodium antigens (Helmby, et al., 1998; Naus et al., 1999). Cytokines are important mediators of the immune system. Malaria leads to production of Th1 cytokines majorly IFN-γ. IFN-γ has multiple immunoregulatory functions that mediate host defense against various pathogen infections. It regulates T cell polarization towards Th1, cellular proliferation and apoptosis (Boehm et al., 1997). Malaria infections are characterized by an overproduction of IFN-γ and an underproduction of IL-10 (Mitchell et al., 2005). Infections by schistosomiasis on the other hand results in an increase in IFN-γ and IL-2 (Th1) prior to egg laying (Wynn et al., 1993) and this is followed by a dramatic increase in Th2 cytokines (IL-4, IL-5 and IL-10) which coincides with peak granuloma formation that leads to a decline in type 1 cytokine levels (Lukacks and Boros, 1993). The production of antibodies by B cells occurs via T cell switching from Th1 to Th2. The control of malaria parasitaemia is mediated via antibodies specifically cytophilic IgG1 and IgG3 isotypes (Hagan et al., 1991).
Th2 polarization leads to an expansion of eosinophils, mast cells and basophils leading to production of Type 2 cytokines and IgG4 and IgE which are associated with control of schistosome infections (Hagan et al., 1991). The major cause of schistosomiasis pathology is caused by eggs which may become lodged within the host organs such as the livers, spleen and kidneys. Eggs have therefore evolved to be highly immunogenic and capable of inducing potent T-helper responses (Pearce, 2005). The deposition of eggs is a major stimuli for the production of Th2 cytokines in S. mansoni infections. Since malaria and schistosomiasis appear in the same geographical region, the question as to whether schistosomiasis infections affect the malaria parasite and its course to disease arises. This study focused on the co-infection of Plasmodium berghei (P. berghei) and S. mansoni in mice and determined the immune responses involved. It also sought to determine whether only the egg antigen or other intrinsic factors contribute to protection against malaria.
1.2 Statement of the Problem

There are several studies in animal models that demonstrate a non-consistent effect of schistosoma infection on malaria development which is mostly expressed as enhancement of parasitaemia (Sangweme et al., 2009; Wakanine-Grinberg et al., 2010). Schistosomes are antigenetically very complex organisms and stage specific antigens are found among schistosomulum, cercariae, eggs or adult worms. In early schistosomiasis infection, the immune system is skewed towards Th1 response prior to egg-laying and then progresses to Th2 response due to a peak in granuloma formation (Farah et al., 2000). An effective immune response against malaria requires a strong Th1 response and the presence of helminthes, which lead to the development of a strong Th2 response could potentially decreases the development of protective immunity against malaria. Therefore, there is need to investigate how *S. mansoni* egg antigens contribute to immune responses protecting against malaria. Sexually dimorphic adult worm pairs that reside in mesenteric veins lay eggs at an average rate of 300-3000 eggs/day/worm. Eggs that remain in the host continue the life cycle evoking host immuno-pathological responses that result in major patho-physiological consequences. Therefore, this study examined the role of egg antigens in malaria co-infection in mice that have been extensively utilized to study basic biology of malaria parasites, test vaccines and investigate immune responses they elicit.
1.3 Justification

Schistosoma eggs secrete soluble egg antigens (SEA) that mediate T cell responses and result in increased IgM and IgG2a levels. With malaria on the other hand, CD4 T cells are important in inducing blood stage immunity while CD8 subset are cytolytic against liver stages of the parasite (Torre et al., 2002). Protective responses to malaria also result in production of IgG1 and IgG3 cytophilic subclasses (Druilhe, 2005). How regulation of inflammatory factors in presence of S. mansoni infections affect malaria disease progression still remains poorly understood. S. mansoni, egg antigens evoke a primary stimulus, eggs were used in this study to dissect and characterize immune responses induced during malaria infections. The findings of this study will improve our understanding of immunological responses in co-infections, information that is necessary during drug or vaccine administration in endemic areas.
1.4 Null Hypothesis

Schistosoma mansoni infection is not protective against Plasmodium berghei infection in mice.

1.5 Objectives

1.5.1 General Objective

To assess the immune correlates of protection in mice infected with both S. mansoni eggs and P. berghei.

1.5.2 Specific Objectives

1. To determine the appropriate route of S. mansoni egg administration and optimal egg dosage.

2. To determine the changes in Th1 (IFN-γ) and Th2 (IL-4) cytokine levels in mice inoculated with both S. mansoni eggs and P. berghei.

3. To determine the changes in total IgG levels in mice inoculated with both S. mansoni eggs and P. berghei.

4. To determine the survival of mice inoculated with both S. mansoni eggs and P. berghei.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria Infection

Malaria is a common infection in hot tropical regions of the world but rarely occurs in temperate climates where it transmitted mainly through travelers who have visited endemic areas. It is a life threatening disease caused by *Plasmodium* parasites that are transmitted to people through the bites of infected female *Anopheles* mosquitoes. Though it is preventable and curable, malaria kills nearly a million children in the sub-Saharan Africa per year (WHO, 2010). Vector control is the primary public health intervention for reducing malaria infections at the community level. Drugs can be used to prevent malaria especially for those travelling to endemic areas with efforts still ongoing to find a vaccine. Where malaria is highly endemic, adults tend to develop partial immunity to the symptoms of the disease. However, young children bear the burden in terms of morbidity and mortality. The overall impact of malaria on human capital development in children remains largely unexplored and unquantified.
In Kenya, an estimated 11% of school days are lost by primary school children every year, while secondary school students miss up to 4.3% school days because of malaria (Sachs and Malaney, 2002). The same study has reported up to 50% of medically related school absenteeism to malaria (Sachs and Malaney, 2002). In addition, malaria affects the cognitive development and learning ability since children with malaria have a poorer nutritional status than non-malarial children, an outcome that can impair brain development (Malaney et al., 2004). Long term demographic impacts of malaria affect saving rates. The direct cost of prevention (buying of mosquito nets, insecticides and mosquito coils) and treatment of the disease eat into the disposable income of poor families, as do the cost of lost productivity (Sachs and Malaney, 2002).

Malaria distribution is based on vector distribution and climatic factors such as temperature, humidity and rainfall. Malaria is mainly transmitted in tropical and subtropical areas where Anopheles mosquitoes can survive and multiply (Figure 2.1) with the highest transmission found in sub-Saharan Africa. In cooler regions, transmission is less intense and more seasonal. In such climatic areas, Plasmodium vivax (P. vivax) might be more prevalent because it is more tolerant of lower ambient temperatures (WHO, 2009; Waters et al., 1991). P. falciparum is found in tropical and subtropical areas (Figure 2.1). On the other hand P. vivax is found mostly in Asia, Latin America and in some parts of Africa. P. ovale is found mostly in Africa (especially West Africa) and the Islands of the Western Pacific (World Health Organization, 2009; Waters et al., 1991). P. malariae is
widespread throughout sub-Saharan Africa, much of Southeast Asia, Indonesia and in areas of the Amazon Basin of South America (Westling et al., 1997).

![Map of the world showing the distribution of malaria risk](Acknowledgement: Adapted from World Health Organization)

**Figure 2.1:** Global distribution of risk of malaria transmission (World Health Organization, 2009).

### 2.1.1 Malaria Parasites

Malaria is transmitted by female *Anopheline* mosquitoes: *Anopheles gambiae* and *Anopheles funestus* which are the main vectors of the disease in Africa. Human malaria is caused by *Plasmodia* species. Five species are involved *P. vivax*, *P. falciparum*, *P. malariae*, *P. ovale* (Duffy et al., 1998) and more recently *P. knowlesi* (Cox-Singh et al., 2008; Singh et al., 2004; White, 2008). Amongst the
five malarial species infecting man the most pathogenic is *P. falciparum*, a parasite that differs from most other *Plasmodium species* in the regulation of its sexual phase of development. The second most important human pathogen is *P. vivax*, which (together with *P. ovale*) has a dormant phase in the liver (the hypnozoite) that creates significant challenges in attempts to eradicate the disease(s) because it persists as a metabolically down regulated cell and is therefore difficult to target directly with drugs or possibly vaccines (Duffy *et al.*, 1998). *Plasmodia* species infecting mice are of interest because they provide practical models for the experimental study of mammalian malaria.

These parasites have proved to be analogous to the malaria of man and other primates in most essential aspects of structure, physiology and life cycle (Carter and Diggs, 1977). This study will focus on *P. berghei* which belongs to a group of four *Plasmodium* parasites that infect murine rodents from Central Africa namely *Plasmodium vinckei* (*P. vinckei*), *Plasmodium chabaudi* (*P. chabaudi*), *Plasmodium yoelii* (*P. yoelii*) and *P. berghei*.

### 2.1.2 Life cycle of human malaria parasites

The life cycle of the malaria parasite is complex (Figure 2.2). Sporozoites are transmitted to the vertebrate host by the bite of an infected Anopheline female mosquito (CDC, 2010). Once in the blood circulation, they enter human liver cells where they develop into the pre-erythrocytic (exo-erythrocytic) schizonts. The period of pre-erythrocytic schizont development varies from one species of
parasite to another. Some species like *P. vivax* and *P. ovale* have a dominant hypnozoite stage where the parasite remains dormant in the liver for weeks or even years before they develop into pre-erythrocytic schizonts. The pre-erythrocytic schizonts usually contain millions of merozoites which are released into the blood circulation where they invade the red blood cells (CDC, 2010). Merozoites develop within the erythrocytes through ring, trophozoite and schizont stages (erythrocytic schizogony). Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocyte membrane with ligands on the surface of the merozoite. A small portion of the parasites differentiates from newly invaded merozoites into sexual forms, which are macrogametocyte (female) and microgametocyte (male). During a blood meal, mosquitoes ingest the gametocytes. In the mosquito, mature macrogametocytes are taken into the midgut (Silvie *et al.*, 2008). When the microgamete fertilizes, a zygote is formed which further develops into an ookinete and eventually forms an oocyst.

After infection in the mosquito and depending on the *Plasmodium* species and ambient temperature, thousands of sporozoites are released which then invade the salivary gland epithelium. When an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again (Silvie *et al.*, 2008). The life cycle of *Plasmodium* species and pattern of infections in both human and rodent species are similar (Jambou, 2011). Therefore, this study analyzed *P. berghei* infection in BALB/c mice.
2.2 Schistosomiasis Infection

Schistosomiasis is a parasitic disease caused by blood flukes (trematodes) of the genus *Schistosoma*. After malaria and intestinal helminthiasis, schistosomiasis is the third most devastating tropical disease in the world, being a major source of morbidity and mortality for developing countries in Africa, South America, the Caribbean, the Middle East, and Asia (Blanchard, 2004). The disease is due to the
immunologic reactions elicited by the schistosoma eggs trapped in the tissues and organs including the kidneys, livers, spleen among others. Antigens released from the egg stimulate a granulomatous reaction involving T cells, macrophages, and eosinophils that results in clinical disease (Pearce, 2002). In humans, urinary schistosomiasis leads to damage of bladder, urethras and kidneys. In intestinal schistosomiasis, there is enlargement of spleen, intestinal damage and hypertension of the abdominal vessels (WHO, 2006). These in turn lead to severe morbidity, in children helminths contribute to malnutrition a reduction in growth, impairment of cognitive functions. This study utilized *S. mansoni* which is responsible for intestinal schistosomiasis and its effect on progression of *P. berghei* parasitaemia determined.

2.2.1 Epidemiology of Schistosomiasis

Schistosomiasis is one of the most widespread of all parasitic infections of man. The World Health Organization (WHO) estimated that schistosomiasis and soil transmitted helminths represent more than 40% of the global disease burden caused by all tropical diseases, excluding malaria (WHO, 2006). Schistosomiasis is the most common parasite transmitted through contact with fresh water. It is endemic in more than 70 low income countries where it occurs in rural areas and the fringes of cities (WHO, 2006). Over 650 million people globally are at risk of infection, with more than 200 million people infected. Of these, 120 million are estimated to have symptoms, with 20 million people experiencing serious
complications. The economic effects and health implications of schistosomiasis are extensive. Higher disease rates occur in children (Gryseels et al., 2006) with infection frequently found in those less than 14 years in many risk areas (WHO, 2007).

Figure 2. 3: Global distribution of Schistosomiasis, map adopted from US Centre for Disease Control and Prevention

Schistosoma haematobium, S. japonicum and S. mansoni are the species that cause majority of human disease. They are predominant in various regions in the world. Other schistosome species such as S. intercalatum, S. mekongi, S. malayensis and S. mattheei also cause human disease though they occur in limited
S. haematobium (urinary) is highly prevalent in East Africa particularly around Lake Malawi, the islands of Mauritius and Madagascar and also in the Middle East. S. japonicum (hepatic/intestinal) is found predominantly in East and South East Asia and the western pacific. S. mansoni (hepatic/intestinal) is distributed throughout sub Saharan Africa and the Middle East, but is also found in some Caribbean islands, Brazil, Venezuela and the coast of Suriname (Figure 2.3).

2.2.2 Life cycle of Schistosomes

Schistosomes have a complex life cycle in which cercariae, free living in fresh water can penetrate healthy skin losing its tail and transform to the schistosomulum. This then finds its way to the venous circulation and is carried to the lungs (5-7 days of penetration). They then travel through the circulatory system to the hepatic circulation (after 15 days) where they mature into adult worms and mate. Depending on the schistosome species, they leave the liver as adult worms and migrate to their sites of infection either in the intestines or the bladder (Gryseels et al., 2006). The female then begins to lay eggs. The eggs attach and penetrate the wall of the lumen and are expelled in urine or faeces. The eggs when released hatch on contact with water bodies to release miracidia which seek out a suitable snail host. They enter a sporocyst stage that eventually results in free living cercariae which infect a human host (Figure 2.4).
2.3 Malaria-Schistosomiasis co-infections

Malaria and schistosomiasis are the major parasitic diseases in developing countries and their epidemiological coexistence is frequently observed, particularly in Africa (Brooker et al., 2007). Co-infection by these two parasites may have an important influence on the regulation of inflammatory factors associated with the development of these infections and their respective morbidity (Diallo et al., 2004). Malaria infections and other parasitic infections are
widespread in developing countries. There is evidence from some studies that intestinal worm infections may increase the risk of febrile malaria (Tchinda et al., 2012). However, the evidence is mixed, in studies conducted among malaria endemic populations in Africa, helminth have been reported to increase susceptibility to clinical malaria (Spiegel et al., 2003), reduce the risk (Lyke et al., 2005) or make no difference (Bejon et al., 2008).

2.3.1 Susceptibility to Malaria in presence of Schistosomiasis

Various species of rodent Plasmodia have been used to mimic human malaria infections (Carter and Diggs, 1977). Although they all possess features that differ from human disease, they have been useful to study susceptibility to infection and the regulation of immune responses. There are a number of studies that have analyzed the role of helminths on the course of malaria infection, with contrasting results. An early study on concurrent S. mansoni and malaria infections in Swiss TO mice concluded that there was no difference in malaria parasitaemia or the development of anaemia in mice infected with both S. mansoni (3 or 5 weeks of infection) and (P. yoelii) (Lewinsohn, 1993).

However, a more recent study in Swiss albino mice showed that co-infection of S. mansoni and a lethal strain of P. berghei (ANKA), where injection of infected erythrocytes took place 7 weeks after infection with S. mansoni, resulted in increased parasitaemia, mortality, and delayed reduction or clearance of parasitaemia (Legesse et al., 2004). Likewise, mice with concomitant S. mansoni
and *P. chabaudi* infections had higher parasitaemias than mice infected with *P. chabaudi* only, and some of the co-infected mice died compared to none of the mice infected with *P. chabaudi* only. C57BL/6 mice infected for 8 weeks with *S. mansoni* were then infected intraperitoneally with blood-stage *P. chabaudi* (Helmby et al., 1998). Although no differences were seen for *P. chabaudi*-induced IFN-γ responses, TNF-α production was significantly reduced in doubly infected mice. The study by Helmby and others (1998) examined not only the reactivity to malarial antigen but also responses to schistosome antigens were measured. This led to the conclusion that the proliferative and Th2 responses to schistosome antigens were suppressed up to 1 month after malaria infection showing that malaria may be able to control responses to helminthes. Different experiments have shown that the outcome in a case of co-infection may also depend on the malaria parasite strain.

Lwin and others in 1992 found that CBA mice infected for 8 or 12 weeks with *S. mansoni*, when co-infected with *P. chabaudi*, showed lower parasitaemia compared with mice not infected with *S. mansoni*. However, they found no effect of *S. mansoni* infection on the parasitaemia caused by *P. yoelii*, unless they used a more virulent *P. yoelii* strain.

*S. mansoni* have been shown to eliminate susceptibility to *P. chabaudi* in A/J mice. This is due to Th1 and Th2 responses it facilitates in the host. The A/J mice did not succumb to death on infection with *S. mansoni* but instead there was production of Interferon gamma to malaria antigens. Treatment with Interferon
gamma monoclonal antibodies in A/J mice infected with *S. mansoni* abolished the resistance to malaria indicating that the Interferon gamma was responsible for the resistance to *P. chabaudi* in *S. mansoni* infected mice (Yoshida *et al.*, 2000). The adverse outcome of non lethal *P. yoelii* malaria during co-infection with *S. mansoni* has been studied in BALB/c mouse model.

This study was done in female BALB/c mice to determine the effects of time and stage of concomitant disease infection on malaria disease outcome (Sangweme *et al.*, 2009). The study showed a complex relationship between schistosome co-infection and malaria disease outcome in which the timing of malaria infection in relation to schistosome acquisition is critical to disease outcome and pathology. Another study on *S. mansoni* in a murine model of cerebral malaria observed protection against brain pathology but severe disease was not prevented (Bucher *et al.*, 2011). A similar study has been conducted on the effect of *S. mansoni* infections on murine cerebral malaria (Wknine-Grinberg *et al.*, 2010).

The study showed that concomitant *S. mansoni* and *P. berghei* ANKA infections lead to reduction in cerebral malaria in ICR HSD mice. This protective effect was shown to be dependent on infection schedule and infecting cercariae number and was correlated with a Th2 response. In an experiment involving Schistosomal egg antigen injection, Wknine-Grinberg and others (2010) observed a delay in the death of *Plasmodium* infected mice indicating the involvement of the immune response.
Similar studies have been conducted in humans to explore the influence of helminth infections on the course of malaria. Most studies are longitudinal of helminth infections with 6–12 months follow-up to evaluate one or several aspects of malaria infection, such as susceptibility to infection, level of parasitaemia, development of immunity and protection from symptoms of severe malaria (Hartgers and Yazdanbakhsh, 2006). In addition a study conducted in Northern Senegal showed that the incidence of malaria attacks was higher in children positive for infection with *S. mansoni*, especially in subjects with the highest helminth loads (Sokhna *et al.*, 2004). The association of helminth infection with an increased risk for malaria incidence has been confirmed in another study which saw a positive association of intestinal helminths and infection with *P. falciparum* in adults in Thailand (Nacher *et al.*, 2004).

However a different study has contradicted the findings above and showed some protective response following infection with *S. haematobium* on *P. falciparum* infection (Briand *et al.*, 2005). A study performed in Senegal showing that *S. haematobium* had a protective effect on infection by decreasing *P. falciparum* densities as compared to helminth-free children (Briand *et al.*, 2005).
2.3.2 Mice as models for malaria and schistosomiasis studies

Rodent hosts with extensively characterised genetic backgrounds and transgenic lines are valuable and available tools for immunological studies. The BALB/c mouse model is advantageous for immunological studies, being an inbred strain with a defined major histocompatibility complex haplotype (Wykes and Good, 2009) and is susceptible to *P. berghei* infection. Rodent parasites are valuable model parasites for investigating the developmental biology of malaria parasites, parasite-host interactions, vaccine development and drug testing. The molecular basis of drug-sensitivity and resistance show similar characteristics in rodent and human parasites. Malaria *Plasmodia* are highly similar in their patterns of infection and life cycle, making the *P. berghei* strain, which is not transmittable to humans, a safe choice for experimentation and observation (Carter and Diggs, 1977).

In addition, mice have also been extensively used as a model for both immunity and immunopathology in schistosomiasis. It is a permissive host, in the sense that adult worms mature, form pairs and lay eggs, leading to the development of a severe morbidity associated with the deposition of eggs in the liver (Pearce, 2005). As in humans, these deposited eggs elicit granulomatous reactions and fibrosis, leading to portal hypertension with hepatosplenomegaly and the formation of porto-systemic shunts (Butterworth and Thorne, 1993). Meanwhile, the Th2 response with IL-4 production that is seen in chronically infected animals
is at least partly responsible for the development and maintenance of the egg granulomatous reaction, which can be down-regulated by IFN-γ or indirectly by IL-12 (Wynn et al., 1994). Although tumour necrosis factor (TNF) is also associated with the development of the granulomatous reaction, the overall conclusions are that, in mice, Thl responses are associated with protection and Th2 responses with immunopathology (Joseph, 1993). Considering therefore the importance that the rodent models have in helping in pre-clinical research, and the fact that they are also able allow immunological interactions similar to what happens in human, they were utilized in this study. BALB/c mice were used to examine the effect of *S. mansoni* eggs on the susceptibility to *P. berghei*.

### 2.3.3 Malaria and Schistosomiasis cytokine profile

Both malaria and schistosomiasis have stage-specific cytokine production patterns where immunologic balance is critical. It has been demonstrated that malaria is an inflammatory disease in which cytokine levels correlate with disease severity (Lyke et al., 2005). Parasitaemia is initially controlled by Th1 cytokines, and there is a gradual shift toward Th2 cytokine predominance (von der et al., 1996). Interleukin-4 is critical to memory induction in both the humoral arm (Luoni et al., 2001) and the development of memory CD8+ T-cell responses (Cavalho et al., 2002). The role of regulatory T cells and the associated cytokines IL-10 and transforming growth factor β in the host's immune response to malaria has captured interest, as they have recently been associated with increased parasite-
induced virulence in humans (Walther et al., 2005). Furthermore, mice with underlying helminth infection, in contrast to helminth-free mice, appear to generate regulatory T cells in response to murine malaria vaccines (Druilhe et al., 2005). Following egg deposition, the immune response in schistosome-infected individuals becomes Th2-skewed, which might interfere with the development of an adequate Th1 response necessary to control parasitaemia and the resultant malaria attacks. Regarding anti-inflammatory responses, chronic infections with helminths are known to be associated with the induction of a regulatory network that dampens immune responses to other antigens. The induction of a regulatory network of anti-inflammatory cytokines such as IL-10 and TNF-α might prevent pathology in the later phases of malaria infection (Hartgers and Yazdanbakhsh, 2006). Indeed, a small study in Ghana, showed increased IL-10 to TNF-α ratios in response to malaria antigens in whole blood of helminth-infected children living in a rural area endemic for both S. haematobium and P. falciparum (Hartgers, personal communication). These regulatory responses might be involved in controlling malarial pathology.

2.3.4 Antibodies involved in malaria and schistosomiasis infections

Antibody isotype responses can be useful as indicators of immune bias during infection. In studies of parasite co-infection however, interpretation of immune bias is complicated by the occurrence of cross-reactive antibodies (Fairlie-Claire Clarke et al., 2010). Antibodies of the IgG2a isotype are mainly produced by B cells in
response to IFN-γ in mice (Else and Finkelmann, 1998) whereas the Th2 cytokine IL-4 switches B cells to produce IgG1 (Purkerson and Isakson, 1992). Although the generation of IgG1 as a marker for Th2 cells is less definitive than IgG2a as a marker of a Th1-type response, the ratio of IgG1 to IgG2a provides a powerful indicator of immune bias (Li et al., 2004). Despite being present at a low concentration in human serum, IgE antibody plays unparalleled roles in allergy and parasitic infection. It is a marker in Th2 response polarization during the infection of *Schistosoma*. Relatively abundant circulating IgE antibodies which are induced by cytokines, such as IL-4 and IL-13, bind the Fc-epsilon specific receptors on mast cells, basophils and eosinophils and trigger the degranulation of these cells, thereby increasing vascular permeability and killing the parasites (Zhang and Mutapi, 2006).

### 2.4 Role of schistosome eggs in malaria pathology

In schistosome infection, the human immune system is exposed to schistosome larvae (cercariae and schistosomulum), adult worms and egg antigens. Animal studies as well as in vitro studies have demonstrated immunological changes and regulatory mechanisms associated with these different stages in the life cycle. The schistosome eggs represent the primary stimulus. They are major Th2 triggers as demonstrated in murine studies (Pearce *et al.*, 2004). Egg proteins (e.g. omega 1, alpha-1 and peroxiredoxin) are involved in the Th2 biasing activity (Everts *et al.*, 2009; Schramm *et al.*, 2007). In addition, schistosome eggs have
immunomodulatory potential inducing the alternative activation of macrophages and regulatory T-cell expansion (Schramm et al., 2007).

These eggs have been utilized extensively in various studies using mouse models where they have been shown to be direct activators of basophils which produce IL-4 (Anyan et al., 2010; Schramm et al., 2007). In another study in mice, the protein component, alpha-1 on the surface of the schistosome eggs has been shown to induce protection against cerebral malaria (Waknine-Grinberg et al., 2010). However, several studies in animal models demonstrate a non-consistent effect of schistosoma infection on malaria development (Sangweme et al., 2009; Waknine-Grinberg et al., 2010). The role of the schistosoma egg in the control of parasitaemia is not well elucidated. In addition, the effect of egg vaccination at various time intervals on malaria progression is also poorly understood. This study utilized the *S. mansoni* eggs in BALB/c mice and examined the effect on the progression of *P. berghei* infection.
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site
This study was conducted at the Institute of Primate Research (IPR), a research institute under the National Museums of Kenya (NMK) located in 400 acres of Ololua forest near Karen, approximately 20kms from Nairobi.

3.2 Sample Size
A total of 182 BALB/c mice of either sex were used in this study. The sample size was determined from literature review of similar work where the minimum number of mice per treatment group has been shown to be between 5 and 7 (Bucher et al., 2011).

The resource equation:

E=N (number of animals per treatment × number of treatments)-T (number of treatments) was also used (source N3Rs, page Experimental and design/statistics).

Ninety two (92) mice were infected with 350 cercariae each and left in cages for 7 weeks and livers harvested to obtain eggs. The remaining 90 mice were used in experimental work which was divided into three sections:
a. The route optimization and egg dosage experiments (n=20mice).

b. Malaria antigen preparation (n=20mice).

c. *S. mansoni* eggs vaccination and *P. berghei* challenge (n=50mice).

### 3.3 Host and parasites

Six-week-old BALB/c mice bred at the IPR (Nairobi, Kenya), were maintained in cages in groups of 10 mice and fed on commercial pellets and water. The mice were kept under a natural light-dark cycle of 12/12 hours with ambient temperatures of 25°C and relative humidity of 50-60%. For helminth infection, a Kenyan isolate of *S. mansoni* originally derived from human patients and maintained using *Biomphalaria pfeifferi* snails and baboons (*Papio Anubis*) was used to infect mice. The *Biomphalaria* snails maintained at the snail room at IPR were checked for viability and exposed to light in a beaker. These were then given thirty minutes to shed off cercariae and the numbers were then counted microscopically before infecting the mice.

*P. berghei* parasites maintained as frozen aliquots in liquid nitrogen were used. Three donor mice were infected with *P. berghei*. Briefly, cryovials were retrieved, thawed and mixed with an equal volume of sterile PBS. This was then injected to the donor mice which were monitored daily from day 3 post-infection until parasitaemia was 50%. The parasitaemia was determined by microscopy as described previously (Hoffman *et al.*, 1984). The mice were then euthanized and bled by cardiac puncture to give rise to parasitized red blood cells which were
used for infection of a new group of mice. This provided sufficient quantities
needed to infect the two treatment groups of mice present used for the study. For
all infections, a concentration of $10^5$ parasitized red blood cells was administered
to every mouse.

3.4 Optimization of route of administration and egg dosage experiments
To optimize the appropriate route of administration of *S. mansoni* eggs prior to
challenge with *P. berghei* parasitized red blood cells, an experiment was done.
The aim of the experiment was to establish whether *S. mansoni* eggs elicit any
protection against *P. berghei*. Previous studies have utilized a single egg dosage
intraperitoneally (Anyan et al., 2010; Waknine-Grinberg et al., 2010).

This study tested the single egg dosage along with a triple egg dosage
administered at ten day intervals. Two sets of experiments were conducted with
each experiment having ten mice. Each set of experiment had its own control
mice. In each set of experiments, two groups of mice were used, the control group
infected with *P. berghei* only (n=5) and the experimental group inoculated with
both *S. mansoni* eggs and *P. berghei* (n=5). In the first experiment (Figure 3.1),
experimental mice were injected intravenously (IV) with approximately 6600 *S.
mansoni* eggs followed by *P. berghei* infected red blood cells after ten days. The
control mice were infected with *P. berghei* only. In the second (Figure 3.2),
experimental mice were injected intraperitoneally (IP) with a triple dose of
approximately 4000 *S. mansoni* eggs at ten day intervals. They were later
challenged with *P. berghei* ten days after the final dose of *S. mansoni* eggs. The control mice were infected with *P. berghei* only. This was done simultaneously with the experimental mice. In both sets of experiments, *S. mansoni* eggs were suspended in 1% normal saline. The mice were each infected with $10^5$ RBCs parasitized with *P. berghei*. Both the control and experimental mice in the two experiments were returned to their cages and parasitaemia monitored for seven days starting on the 4th day post-infection.

**Figure 3. 1:** Experiment 1, single dose of *S. mansoni* eggs administered intravenously (IV)
3.5 Infection of BALB/c Mice with S. mansoni cercariae

Mice were placed into re-strainers mounted on a wooden rack with their tails dipped into tubes containing a solution of 350 S. mansoni cercariae. Masking tape was used to hold the re-strainers in position to avoid any movements. They were then left to stand for 30 minutes to ensure that cercariae penetrated through the tail as has been described by Coulson and others (1989). Mice were then returned to their cages and left for seven weeks to allow for schistosomiasis to develop to acute stage of the disease.
3.6 Procedure for collection of *S. mansoni* eggs

3.6.1 Perfusion procedure for Mice

To obtain eggs, infected mice were anaesthetized using ketamine xylazine mixture (0.03ml per 30g body weight) followed by a perfusion procedure which was done 7 weeks post cercariae infection. Mice were dissected to open abdominal and thoracic cavities. After carefully severing the ribs near the spinal column on the left side of the thoracic cavity of a mouse, a small slit in the hepatic portal vein was made. A 20 gauge needle was inserted into the descending aorta.

The perfusate (citrated saline: 8.5g sodium chloride and 15g sodium citrate per litre) was collected in a beaker by pumping perfusion fluid through the needle. Using phosphate buffered saline, worms were collected and any adhering to mesenteric veins were removed using forceps.

3.6.2 Isolation of *S. mansoni* eggs

To obtain a clean preparation of eggs, mice were perfused to remove adult worms. The livers were then pooled together in a clean glass beaker and homogenized using a blender for 5 minutes in saline solution (2% normal saline). The preparation was then given a fine homogenization after addition of 0.1g of trypsin and left to stand in a shaking waterbath (37°C for 3 hours). Afterwards, this was poured over a wire sieve, 250µm and 150µm, into a beaker. The supernatant was poured into 50ml tubes followed by centrifugation at 55g for 5 minutes (Hettich Zentrifugen, Rotanta 460 R, Germany). The pellet at the base of the tubes were
re-suspended then pooled together and washed in 2% normal saline. This was then centrifuged for a final time at 28g for three minutes and suspended in 5ml of saline. The quantity of eggs within this pellet was determined by microscopy before administration to mice. Approximately 4000 *S. mansoni* eggs were injected into each mouse intraperitoneally for the *S. mansoni* egg vaccinations and *P. berghei* challenge experiments.

3.7 *S. mansoni* egg vaccinations and *P. berghei* challenge experiments

Two groups of mice were used in this experiment: the control group infected with *P. berghei* only and the experimental group inoculated with both *S. mansoni* eggs and *P. berghei*. Each group consisted of 25 mice. Approximately 4000 *S. mansoni* eggs obtained from mice that had previously been infected with cercariae (section 3.5, 3.6 and 3.6.1) were injected into each of the experimental mice (IP). This was repeated twice at 10 day interval between every egg dosage (Based on results from second experiment of, section 3.4). Ten days after the third dose of eggs was administered to the experimental group of mice, both the control and experimental groups were challenged with $10^5$ RBCs parasitized with *P. berghei* ANKA strain. Parasitaemia was then monitored from day three post *P. berghei* infection. Five mice from each group were euthanized at day 3, 6 9 and 12 post *P. berghei* infection and the spleen and blood were obtained (Figure 3.3). Five naive mice that were parasite free were also euthanized and serum and splenocytes collected.
These provided the baseline samples (day 0). The blood was processed and serum collected while the spleen was processed for splenocytes. The serum samples were used for antibody specific ELISA. The splenocytes were frozen and stored in liquid nitrogen. They were later retrieved and cultured to provide supernatants for cytokine ELISAs. The IgG and cytokine ELISAs provided a monitor of the immune responses both in the controls and the experimental arms of mice.

Ten mice (control n=5 and experimental n=5) were monitored for 15 days starting on day 3 post P. berghei infection. Their parasitaemia and clinical symptoms were monitored after every two days. These mice were to provide the survival information and determine if indeed the S. mansoni eggs protected against P. berghei or not. None of these mice were euthanized until after 15 days. Any deaths reported were due to P. berghei infection. All the procedures were conducted in accordance with IPR’s regulations of animal use, care and welfare.
Figure 3.3: *S. mansoni* eggs vaccination and *P. berghei* challenge experiment, triple dose of *S. mansoni* eggs administered intraperitoneally (IP)

### 3.7.1 Determination of Parasitaemia

The tail of the mice was sterilized by wiping with spirit and a pair of scissors used to snip off the tip of the tail (1-2mm). On subsequent days though, a cotton gauze swab was used to disturb the wound to obtain blood from the tail. Blood collected was used to prepare thin blood smear on clean frosted ended 1” x 3” microscope slides. This was then air dried before fixing the thin smear with methanol for 5 seconds. Staining was done with freshly prepared 10% Giemsa solution for ten
minutes. Excess stain was rinsed off under tap water and slides left to dry. Presence of parasites was determined using immersion oil under a binocular microscope (Zeiss Standard 20, Germany) using a 100x objective lens. Infected and uninfected erythrocytes in different fields of view were identified and counted. A total of about 2000 erythrocytes were counted per slide and the percentage parasitaemia determined using the below formulae.

\[
\text{Parasitaemia (\%)} = \frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes counted}} \times 100
\]

3.7.2 Serum Preparation

Blood was collected from euthanized mice using the heart puncture technique. This was then processed individually for every mouse. Blood was left to stand overnight at room temperature and then centrifuged for 10 minutes at 445g. The serum samples were then collected and stored at -20°C for analysis.

3.7.2.1 Preparation of \textit{P. berghei} antigen

The \textit{P. berghei} antigen was prepared according to the method which has been described previously (Kima \textit{et al.}, 1992). Mice (n=20) infected with \textit{P. berghei} were left to reach a parasitaemia not exceeding 30% (predominant parasite stages were trophozoites and schizonts). At this point, blood was collected into heparinized tubes. Blood was then separated into various blood components using ficoll paque. RBCs were then washed three times with 1×PBS at 445g for 5 minutes. After each wash, supernatant was discarded to remove white blood cells.
and other factors. Two times (2×) volume of 0.1% saponin in PBS was added to the RBCs to facilitate lysis. This was incubated for ten minutes with occasional mixing at room temperature. PBS (5×) was then added to the mixture and centrifugation done at 3000rpm for 30 minutes at 4°C. The supernatant and the RBCs ghost was removed and washing done 2 to 3 times until the supernatant was clear. The preparation was then sonicated at 18Amp for four cycles for 30 seconds with one minute intervals in ice to lyse the parasite walls. A thin smear was made from this preparation and microscopic confirmation that the parasite walls were broken done. The lysate was then transferred to an eppendorf tube and centrifuged at 14000rpm for an hour at 4°C. The supernatant which is the antigen was collected and its protein concentration determined using the protein assay method. The antigen was then tested by an ELISA where positive and negative serum samples from mice were used. It was stored at -20°C until use.

3.7.2.2 Protein Assay

To determine the protein concentration of the crude antigen preparation, a microtitre plate was prepared in duplicate for double dilutions as previously described (Lowry et al., 1951). The standard protein, Bovine serum albumin, (BSA) was put in the first row columns and the test antigen in the following two columns. In the first row of wells, the standard antigen and test antigen (40μl) were added in duplicates. In the subsequent wells, 20μl of 1× PBS was added. Serial dilutions were then done and the final 20μl discarded. The dye was then
added (prediluted 1:4) at 200μl per well starting with the lowest concentration and read at 630nm after 5 minutes. With the standard having a concentration of 1.42mg/ml the concentration of the test antigen was determined to be 177μg/ml.

3.7.2.3 Total IgG ELISA Protocol

The levels of IgG were determined from the collected serum samples. Briefly each well of a microtitre plate (Nunc, Maxisorp) was coated with 50μl of either SEA (soluble egg antigen) at 2μg/ml or P. berghei antigen at 5μg/ml. This was left at 4°C overnight. After washing twice with PBS solution, 100μl per well of blocking buffer (3% BSA) were added and incubated for two hours at room temperature per the manufacturer’s instructions. Samples of serum diluted with PBS were then prepared at a dilution of 1: 20 and 50μl added to each of the wells. These were then left to stand for two hours at room temperature. This provided optimal conditions for binding to coating antibody. Fifty (50μl) of detector antibody, anti-IgG ALP diluted (1:1000), was then added per well and incubated for an hour. Washing was done five times with PBS-Tween and 50μl per well of p-nitrophenyl phosphate (pNPP) added. Thirty minutes was allowed for color to develop and the measurements for optical densities taken at 405nm.
3.7.3 Isolation of splenocytes from spleen

Briefly, spleen samples from both the control and experimental mice were collected at the four sampling points using a 70 μm cell stainer (BD Bioscience CA USA) and plunger of syringe, splenic cells were passed through the cell strainer into a petri dish, keeping the cell strainer suspended in complete RPMI 1640 (10% FBS, 1% L-glutamine and Gentamycin). Cells were then collected and centrifuged at 250g for 10 minutes at 4°C and pellets collected. Red blood cells in pellets were lysed with RBC lysis buffer (4.15g Ammonium chloride (NH₄Cl), 50ml 0.1M Tris HCl, made upto 500ml with H₂O distilled, pH 7.5 and filtered with 0.22μm pore size filter unit (Millipore Co, USA). The cell containing pellets were washed twice with RPMI 1640 supplemented with 5% FBS solution with spinning being done at 250g for 10 minutes (Hettich Zentrifugen, Rotanta 460 R, Germany). Splenocytes were then resuspended in complete media for counting and later freeze stored until use.

3.7.3.1 Culture of Splenocytes

Splenocytes from the controls, experimental and non infected groups of mice were retrieved from liquid nitrogen. The cells were thawed in the water bath at 37°C, washed with a mixture of RPMI and FBS thawing media twice, spinning was done at 250g for 10 minutes (Hettich Zentrifugen, Rotanta 460 R, Germany) and then suspended in 5ml complete medium and counted. Cells were stained using trypan blue and counted using a haemocytometer chamber. A suspension of
4×10^5 splenocytes was cultured at 37°C and 5% CO₂ in 48 well flat bottomed culture plates (Falcon, Lincoln park). Cells were stimulated by SEA (5μg/ml) and P. berghei antigen (3μg/ml). Media was used as the negative control. This does not lead to any T-cell stimulation and no proliferation was expected while concanavalinA (conA) was used to stimulate the cells and was the positive control (5μg/ml). This stimulates the proliferation of unprimed T-cells by indirectly cross linking the T-cell receptors (TCR). This concentration of the antigen gave the optimum stimulation for cytokine production based on preliminary experiments. Culture medium was RPMI 1640 supplemented with 20mM HEPES, 2mM L-glutamine, 100μg/ml Penicillin and 10% FBS (Sigma, MO USA). Supernatant was collected 72hrs after the start of culture and stored at -20°C.

3.7.3.2 Cytokine analysis by ELISA

The level of IL-4 and IFN-γ from the stimulated splenocytes was determined using the ELISA technique as per manufacturer’s instructions (MABTECH). The detection methods that were used are similar with minor differences. The coating antibody for IL-4 was filtered (0.22μm) while that IFN-γ was not. Briefly, the microtitre plates (Nunc, Maxisorp) were coated with respective monoclonal antibodies for the two cytokines and left to stand overnight at 4°C. The plates were then washed the next day with PBS five times and blocked with 0.1% BSA PBS-Tween solution and left to stand for one hour. Fifty (50) μl samples of supernatants, controls and standards were then prepared and added to the
respective IL-4 and IFN-γ antibody coated microtitre plate wells (Nunc, Maxisorp). This was left to stand at room temperature for two hours. The respective secondary antibody (Biotinylated) for the various cytokines was then added and then incubated for an hour followed by five washes with PBS-Tween. Fifty (50) μl of diluted streptavidin phosphatase (1.1000) was added per well, incubated at room temperature for one hour before the appropriate substrate (TMB), 50 μl per well was added to all the wells. This was then allowed sufficient time to develop and optical density readings taken at 630nm for both cytokines.

3.7.3.3 Determination of survivorship in mice

Survivorship of mice was used to determine how long the mice survive under infection following administration of only *P. berghei* in one group and *S. mansoni* eggs and *P. berghei* in another. Survivorship was used to understand if the mice with *P. berghei* infection alone survived longer than those with *S. mansoni* eggs and *P. berghei* or vice versa. Five mice from each group were left undisturbed (were not euthanized) and gave the survivorship profiles. To determine these profiles, the number of surviving mice was recorded along with the mice that died. As the mice succumbed to infection and died, the number of surviving mice was expressed as a percentage and used to draw the survivorship curve in the 15 days that were analysed. This gave information on the role of the eggs on the course of *P. berghei* infection in mice.
3.8 Data Analysis

Data management was done on an Excel spreadsheet. All entries were checked for any keyboard errors. Graphs and charts were presented using MS Excel spreadsheet. Statistical significance was calculated using the chi square test for non-parametric data both for intra-group and inter-groups comparisons. P values less than 0.05 were considered statistically significant. The statistical package for social sciences (SPSS) version 16 was used for analysis.

3.9 Ethical approval

The ethical approval to conduct this study was obtained from the Ethics review (IRC) committee at the Institute of Primate Research (IRC/19/11).
CHAPTER FOUR

4.0 RESULTS

The route optimization and egg dosage experiments laid the foundation for the *S. mansoni* egg vaccinations *P. berghei* challenge experiment where 25 mice in both the control and the experimental groups were used.

4.1 Optimizing the route of administration and appropriate *S. mansoni* egg dosage

The first experiment on assessment of egg injections via the IV route followed by *P. berghei* infection revealed that mice receiving eggs developed higher parasitaemia as compared to those infected with *P. berghei* only (Figure 4.1). A rise in parasitaemia was observed in both the controls and the experimental groups from day 6 post infection (Figure 4.1). A mean parasitaemia of 22.7% in control mice was observed at day 9 post-infection while the experimental mice had higher parasitaemia of 61.7%. In terms of the physical appearance, the control mice were healthy throughout the entire experiment while the experimental mice started showing signs of weakness and deteriorating health from day 8 post infection and this progressed until the end of the experiment.
One mouse was euthanized on both day 7 and 8 post infection from the experimental group due to their poor health. Euthanized mice showed signs of shivering, poor appetite, raised hair, limited movement and weakness. On day 10 post infection, two experimental mice succumbed to death. In contrast, the control mice survived well and were strong with only one mouse being euthanized on the final day of monitoring parasitaemia. A single egg injection followed by *P. berghei* infection was found not to protect mice against the infection. Parasitaemia developed rapidly in mice with the egg injections compared to those with *P. berghei* only.

This study therefore opted to assess a vaccine scenario where several egg injections are given to mice. This led to investigation of a slower method of release of eggs into the blood circulation via the IP route. Here mice were vaccinated with a triple dose of *S. mansoni* eggs before being challenged with *P. berghei*. Experimental mice had a lower mean parasitaemia (15.52%) compared to control mice (23.06%). The experimental mice were healthy until day 10 post infection as compared to the controls which started showing deteriorating health from day 6 post infection (Figure 4.2). There was shivering and poor appetite in one of the control mice. Day 6 post infection, one of the control mice succumbed to malaria and had a parasitaemia of (14.51%). The remaining two control mice progressively increased in parasitaemia compared to the experimental group of mice. They finally succumbed to death on day 10 post infection with a final parasitaemia of 19.12% and 19.79% (Figure 4.2). Day 9 post infection, one of the
experimental mice died at a parasitaemia of 9.54%. Two experimental mice had high parasitaemia levels of 14.97% and 15.52% and appeared weak and in poor health.

These experimental mice were shivering and with limited movement and raised hair. In line with animal regulations to reduce pain, these two mice were euthanized leaving two experimental mice and no control mice at all. The two remaining experimental mice had very low parasitaemia (1%) at day 12 and 13 but their health was poor and they were shivering and with raised hair (Figure 4.2). They were euthanized on day 13. The state of these mice considering the low parasitaemia could be due to the parasite sequestering deep in microvasculature and are thus not detectable in blood.

When IV and the IP route were compared, the IV route was found to be invasive to mice and led to the death of two mice in the first experiment during the injection process. The IP route, which was a slower method of release of eggs was a suitable route as no mice succumbed to death during the injections. This was therefore adopted in the study. In addition, the triple dose of *S. mansoni* eggs led to delay in the build-up of parasitaemia in the experimental mice. Control mice in the second experiment succumbed faster to infection compared to experimental mice. The triple egg dosage was adopted for the *S. mansoni* vaccination and *P. berghei* challenge experiment.
Figure 4.1: Parasitaemia profile of mice infected intravenously (IV). Pb shows the control mice while Sme/Pb represents experimental mice. Error bars represent standard deviation.
Figure 4.2: Parasitaemia profile of mice infected intraperitoneally (IP). Pb shows the control mice while Sme/Pb represents experimental mice. Error bars represent Standard deviation.

4.2 Cytokine profiles in control and experimental mice

4.2.1 IFN-γ Profile

The figures below (Figure 4.3 and 4.4) show the levels of IFN-γ in both the control and experimental mice. There were high levels of IFN-γ induced by conA from day 0 to day 12 post infection in the control group of mice (958.3, 891.6, 975.8, 952.3 and 921.6 pg/ml respectively). Cytokine levels induced by P. berghei antigen were below the detection levels for day 0 set of mice (naive mice). The minimum detectable concentration was 39 pg/ml. There was a gradual rise in the IFN-γ levels from day 3 to day 12 post infection (Figure 4.3).
The lowest levels of this cytokine were seen at day 3 with the highest at day 12. However comparing the levels of IFN-γ among the various time points induced by *P. berghei* antigen, there was no significant difference (day 3, 6, p=0.213, day 9, 12, p=0.238).

![Graph showing IFN-γ levels](image)

**Figure 4.3**: IFN-γ levels in control mice (pg/ml). PbA represents the *P. berghei* antigen and conA, concanavalin antigen, the positive control which were used to stimulate the splenocytes. Each time point indicates (n=4) mice.

The IFN-γ levels were higher in experimental mice compared to controls (Figure 4.4). Similar to the control group, the naive mice did not show any response to SEA. The levels of IFN-γ induced by *P. berghei* antigen were higher in the experimental mice compared to levels induced by the same antigen for the control
mice (Figure 4.3 and Figure 4.4). *P. berghei* antigen induced higher levels in experimental mice (day 3 experimental=180.5pg/ml, control=155pg/ml, day 9 experimental=380pg/ml, control 186pg/ml and day 12 experimental=376pg/ml, control=312pg/ml). There was a gradual rise in levels of this cytokine in the experimental mice from day 3 to day 12 post infection. SEA induced higher levels of IFN-γ at day 6 post infection with similar levels of cytokine production as *P. berghei* seen with the other time points. Highest levels of IFN-γ in the experimental group of mice were seen at day 9 and day 12 post infection (376pg/ml and 380pg/ml). Comparing these IFN-γ levels between the two groups, day 3 levels for both groups (controls and the experimental groups) were similar. The levels were higher for the experimental mice at day 9 post infection due to the effect of SEA (376pg/ml) as compared to the control mice at the same time point. IFN-γ levels were also higher at day 9 and day 12 in the experimental group as compared to the controls at the same time points (Figure 4.3 and Figure 4.4). However, much as the experimental group of mice appeared to have higher IFN-γ levels, there was no significant difference between the *P. berghei* induced IFN-γ in the controls and *P. berghei* induced IFN-γ in the experimental group (Day 3, p=0.213, Day 6, p=0.287, Day 9, p=0.213 and Day 12, p=0.092). There was no significant difference between the *P. berghei* induced IFN-γ levels in the control group of mice and the SEA induced IFN-γ levels in the experimental group of mice across the four time points (Day 3, p=0.213, Day 6, p=0.238, Day 9, p=0.213 and Day 12, p=0.238).
4.2.2 IL-4 profile

Figures 4.5 and 4.6 show the IL-4 profile in the control and experimental groups of mice. The minimum detectable concentration for IL-4 was 39pg/ml. There was a gradual rise in the IL-4 levels from day 3 to day 6 post infection before being constant at day 9 and day 12 post infection in control mice (Figure 4.5). High levels of this cytokine were reported at day 9 and day 12. The levels of IL-4 in naive mice (day 0) were below detection in response to SEA (Figure 4.6). The IL-4 levels were higher in the experimental mice compared to the control mice.
induced higher levels of IL-4 in the experimental mice as compared to *P. berghei* antigen (Figure 4.6). There was a gradual rise in the levels of IL-4 stimulated by *P. berghei* from day 3 to day 6 and stabilized on day 9 and day 12 post infection (Figure 4.6). Experimental mice showed the highest levels of IL-4 at day 3 post infection and these levels decreased gradually at day 6 before stability at day 9 and day 12. These high levels were induced by SEA. Response therefore could be due to the effect of the schistosome eggs. Although the levels of IL-4 stimulated by SEA were higher than those by *P. berghei*, there was no significant difference in these levels in the experimental group of mice (Day 3, Day 6, Day 12, p=0.213 and Day 9, p=0.238).

![Figure 4.5: IL-4 profile in control mice (pg/ml). Concentrations were determined post *P. berghei* infection. PbA represents the *P. berghei* antigen and conA, concavalin which was the positive control and were used to stimulate the splenocytes. Each time point indicates (n=4) mice.](image-url)
Figure 4. 6: IL-4 profile in experimental mice (pg/ml). Concentrations were determined post *P. berghei* infection. PbA represents the *P. berghei* antigen and SEA, the soluble egg antigen which were used to stimulate the splenocytes. Concanavalin A was the positive control. Each time point indicates (n=4) mice.

The levels of IL-4 were higher in the experimental group of mice as compared to the control mice (Figure 4.5 and Figure 4.6). There was no significant difference in *P. berghei* induced IL-4 levels in the control mice compared to the *P. berghei* induced IL-4 levels in the experimental mice (Day 3, Day 6, Day 12, p=0.213 and Day 9, p=0.238). The levels of IL-4 induced by SEA were higher in experimental
mice (Figure 4.6). However there was no significant difference between the levels induced by SEA in the experimental mice and levels of IL-4 induced by *P. berghei* antigen in the control mice at the four time points (Day 3, Day 6, Day 12, p=0.213 and Day 9, p=0.287)

### 4.3 IgG profile in control and experimental mice

The IgG levels in the control mice increased gradually from day 0 to day 3 post infection. These levels remained stable between day 3 and day 6 before dropping (Figure 4.7). IgG then rose sharply on day 9 post infection. However there was no significant difference in levels reported at the various time points (p=0.213).

![IgG profile in control mice](image)

**Figure 4.7:** IgG profile in control mice. IgG levels increased gradually from day 0, stabilized between day 3 and 6, dropped before increasing again on day 9 post infection. Each time point indicates (n=4) mice. These levels were in response to *P. berghei* antigen.
In the experimental group of mice the IgG levels were higher compared to the control group of mice. In this group, IgG was induced in response to the two antigens that the mice had been infected with (Figure 4.8). IgG levels in the control mice were induced as a result of the *P. berghei* antigen only (Figure 4.7) while the levels in experimental mice were in response to both the *P. berghei* antigen and SEA (Figure 4.8). The levels induced by *P. berghei* antigen in the experimental mice were higher than the levels of IgG induced by the same antigen in the control mice. *P. berghei* therefore led to a stronger response in the experimental group as compared to the response exhibited by the same antigen among the controls. SEA on the other hand induced highest levels of IgG in mice that were inoculated with both *S. mansoni* eggs and *P. berghei* (Figure 4.8). Levels of IgG induced in response to the egg antigen were higher compared to levels by the *P. berghei* antigen (Figure 4.8). This was a clear indication that high IgG levels were in response to the *S. mansoni* eggs.

In experimental mice, high levels of IgG were reported on day 3 post infection. These levels were high both for the *P. berghei* induced and the SEA induced IgG levels. They then decreased gradually from day 3 to day 12 post infection. Though the levels of IgG induced by SEA were higher than those by *P. berghei* in the same group, an intragroup comparison between these IgG levels at the different time points showed no significance difference (p>0.05). In addition, although the IgG levels were higher for experimental mice compared to the
controls, there was no significance difference in these levels for the various time points (integroup comparison, p>0.05)

**Figure 4.8**: IgG profile in experimental mice. High levels were reported on day 3 and these declined gradually to day 12 post infection. Naive mice had the lowest IgG levels. SEA initiated a stronger response compared to *P. berghei* antigen. Each time point indicates (n=4) mice.

### 4.4 Survivorship profile of mice

Ten (10) mice were left to survive during the entire period of the *S. mansoni* egg vaccinations and *P. berghei* challenge experiment, 5 from each group. Figure 4.9 shows the survivorship data for the control and experimental groups of mice. The experimental group of mice survived longer than the control mice. At day 9 post
infection, 4 mice (80%) among experimental group were healthy and living as opposed to 3 mice (60%) among the controls (Figure 4.9).

Figure 4. 9: Survivorship curve of experimental and control mice. Experimental (co-infected mice) survived longer compared to control (malaria only) mice. Control mice succumbed faster to *P. berghei* infection compared to experimental mice. 5 mice per group were used for survivorship information.
On day 11 post infection, 60% of the experimental mice were healthy and living while only 20% of the control mice were surviving. None of the control mice was surviving on day 14 while the experimental group had 2 mice (40%) of the mice healthy on this day which finally succumbed to death on day 15 post infection. These survivorship data is in agreement with the earlier results from second set of experiment during route optimization which showed that the experimental mice kept the parasitaemia low and survived longer as compared to the control mice which developed higher parasitaemia levels and succumbed to infection faster.

4.5 Testing Hypothesis

Overall, the injection of the *S. mansoni* eggs resulted in differences in levels of IgG, cytokines (IFN-γ, IL-4) and also the parasitaemia levels. Mice injected with the eggs showed a stronger immune response and kept the parasitaemia low and enhanced survivorship. The effect of the eggs was however not significant in all markers measured. The Null hypothesis was accepted.
CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

A number of studies have monitored immunological responses involved in co-infections of malaria and schistosomiasis. Previous studies had observed that helminth infections increased the risk of malaria infection (Helmby et al., 1998; Sokhna et al., 2004) which implies that an antihelmintic intervention could result in reduced frequency of malaria attacks and/or malaria parasite densities in areas where both malaria and helminth infections are endemic (Druilhe et al., 2005).

The schistosome eggs represent the primary stimulus and have been largely attributed to be responsible for pathology associated with schistosomiasis and they trigger the immune response. In this study, S. mansoni eggs were used but their effect was weak on the progression of P. berghei parasitaemia in mice. This could be attributed to isolation procedure of the eggs which may have interfered with their quality. This could have contributed to the findings of this study where the eggs are shown to have an immunological impact in terms of controlling the rapid increase in malaria parasitaemia; however there is no significant differences in the disease markers that were measured (chi test, p>0.05).
In the route optimization experiments, this study found the IP route was more suitable for administration of S. mansoni eggs. IV is more invasive to the mice and led to the death of two mice during the process. These findings agree with various other studies in mice that have utilized the IP route for injection of the schistosome eggs (Waknine-Grinberg et al., 2010; Anyan et al., 2010). The study by Anyan and others (2010) also showed that S. mansoni eggs are direct inducers of IL-4 production by basophils which agrees with the findings of this study. The experimental group of mice had higher IgG and IL-4 levels at day 3 post-P. berghei challenge and these levels decreased gradually to day 12 post challenge. IFN-γ levels build-up steadily in both the experimental mice and the controls from day 3 to day 12 post-P. berghei challenge.

5.1.1 Parasitaemia from route optimization experiments

The results suggest that S. mansoni eggs played a role in delaying the progression of the P. berghei infection by keeping the parasitaemia low. This was observed in the route optimization study where the experimental mice had their highest parasitaemia at 15.52% at day 11 post infection while the control mice had their highest parasitaemia at 23.06% at day 9 post infection (results, section 4.1). This shows that the mice inoculated with both the eggs and P. berghei kept the parasitaemia lower and delayed the P. berghei infection as compared to those that only had the P. berghei infection.
It took longer for the experimental group of mice to reach high levels of parasitemia (day 11) as compared to the controls (day 9). In addition there was a difference in the parasitaemia attained with the control mice only taking 9 days post infection to reach a parasitaemia of 23.06% while the *S. mansoni* eggs and *P. berghei* mice took 11 days to reach a parasitaemia that was lower than that of the controls (15.52%). This difference was however not significant. This shows that the *S. mansoni* eggs did contribute to enhanced survivorship of the experimental mice and also delayed the progression of parasitaemia in the same group of mice. Moreover, the 5 control mice that were used in this experiment died in the course of the experiment (100%) while 3 mice (60%) from the experimental group died during the experiment (results, section 4.4). However, *S. mansoni* eggs do not protect from the *P. berghei* infection since the mice ultimately succumbed to infection and died. The effectiveness of *S. mansoni* eggs in contributing to protection to malaria may have been hampered by the extraction procedure from livers.

The findings of this study disagree with other similar studies that have been done on co-infections and using different species/strains of rodent malaria. In a co-infection study of *S. mansoni* and *P. chabaudi*, remarkable higher parasitaemia were reported in mice co-infected with *S. mansoni* and *P. chabaudi* as compared to those infected with *P. chabaudi* alone (Helmby *et al.*, 1998). Similarly, a study by (Yoshida *et al.*, 2000) demonstrated increased susceptibility to *P. chabaudi*, increased mortality and elevated *P. chabaudi* parasitaemia in *S. mansoni* and *P.*
chabaudi co-infected mice than mice infected with P. chabaudi alone. Another similar study examined the parasitaemia in mice co-infected with both S. mansoni and P. berghei. Higher susceptibility to P. berghei and parasitaemia levels were reported in mice that were co-infected as compared to mice that were infected with P. berghei alone (Legesse et al., 2004). Some of these differences can be attributed to the fact that different types of mice were used of different age and also different strains of the parasites were also used as compared to the present study.

Contrary to the above studies though, the findings of this study agree with other studies that showed reduced parasitaemia in mice that were co-infected with S. mansoni and P. berghei (Lewinsohn, 1975; Lwin et al., 1992). Lewinsohn showed in his study that mice co-infected with P. yoelii and S. mansoni had severe anaemia. Malaria dominated the picture before the parasitaemia finally cleared and there was moderate splenomegaly towards the end of the experiment. Lwin and others (1992) in their co-infection study reported reduced P. chabaudi parasitaemia in mice that were co-infected with both S. mansoni and P. chabaudi. The findings of the first experiment showed that a single dose of the S. mansoni eggs contributed to increased susceptibility to P. berghei. Mice that were infected with the single egg dosage and later challenged with P. berghei succumbed to death faster and had high parasitaemia as compared to those that were infected with P. berghei alone (results, section 4.1).
However when the triple egg dosage is administered to the mice, delay in death of the mice is reported (results, section 4.1). This can be explained by the fact that schistosomes elicit a type 2 response (Pearce et al., 2004). The injection of the eggs to the mice led to biasing of the immune response to a Th2. Therefore, for the delay effect to be reported there needs to be a stable type 2 response which is brought in by the *S. mansoni* eggs (primary stimulus in a schistosome infection). Boosting of the egg dosage leads to a stable antigenic concentration beyond the required threshold that leads to delay in the progression of *P. berghei* parasitaemia. The high antigen concentration achieved by boosting of the egg dosage thrice, ensures a Th2 bias and it could be the effect of this type 2 response that leads to the delay in death and reduced *P. berghei* parasitaemia that is seen in experimental mice.

The survivorship profile that was seen in the *S. mansoni* egg vaccination and *P. berghei* challenge experiments (results, section 4.4) is an additional piece of evidence of the role the *S. mansoni* eggs played in enhancing the survivorship of the experimental mice. The mice that were inoculated with both the *S. mansoni* eggs and *P. berghei* survived longer compared to those infected with *P. berghei* alone.
5.1.2 IgG and Cytokines (IFN-γ and IL-4)

In *S. mansoni*-infected mice, an initial Th1-dominated immune response switches to a Th2 profile upon the deposition of eggs as in humans; eggs lodging in the liver provoke the predominant pathology of liver fibrosis (Pearce *et al.*, 2002). This study examined the immunological changes that were introduced by injection of *P. berghei* onto mice with a pre-existing *S. mansoni* infection. This was in comparison to another set of mice with only *P. berghei* infections. Control mice exhibited gradual rise in IFN-γ from day 3 to day 12 post infection though the levels were low. On the other hand mice that were inoculated with both *S. mansoni* eggs and *P. berghei* showed a similar IFN-γ pattern building up from day 3 to day 12 post infection. However there were higher levels of IFN-γ in the experimental mice starting at day 6 before becoming stable at day 9 and day 12 post infection. Despite appearing to have higher levels of IFN-γ in the experimental group, there was no significant difference between the two groups (p=0.213).

Considering that malaria leads to a Th1 response and schistosomiasis leads to a Th2 with the laying of eggs (Pearce *et al.*, 2004), at day 6 post infection in the experimental mice, it was 16 days after the injection of eggs. It is not yet clear therefore why the levels of IFN-γ (Th1) induced by SEA remained high at these time points. However, the high IFN-γ levels in the experimental mice towards day 12 might have played a role in regulation of malaria pathogenesis and reducing
the burden of the malaria parasite in blood; which resulted in lower parasitaemia levels in these set of mice and thus enhanced their survival. These findings however contradict those of a similar study that showed higher levels of IFN-γ in mice prior to egg laying and a complete shift to Th2 (IL-4, IL-10) cytokines following deposition of eggs (Waknine-Grinberg et al., 2010). Differences with the present study can are attributed to the methods that were used. Waknine-Grinberg and others (2010) determined levels of cytokines in serum while the present study examined these levels using supernatants.

IL-4 was reported to rise gradually from day 3 to day 12 post infection in the control mice. A similar pattern of IL-4 was reported in the experimental mice induced by the *P. berghei* antigen with stability of these levels from day 6 to day 12 post infection. However, there were high levels of IL-4 in the experimental mice induced by SEA at day 3 post infection. These levels decreased gradually to day 12 post infection. It is clear from these findings that the *S. mansoni* eggs evoked a stronger immune response compared to the *P. berghei* antigen particularly at day 3 post infection (13 days post *S. mansoni* egg injection) leading to high levels of IL-4. IL-4 being a signature Th2 cytokine plays a central role in Th2 biasing (Pearce et al., 2004).
The injection of the _S. mansoni_ eggs resulted in immuno-modulation and an upregulation of IL-4 levels which could have contributed to delaying the deaths of mice and delaying the progression of _P. berghei_ parasitaemia in these set of mice. Day 3 in this study was 13 days after the injection of the _S. mansoni_ eggs; it has been shown that once the eggs are injected, they take about 8 days to mount an immune response which continues up to the 12th day (Anyan _et al._, 2010).

These findings therefore agree with those by (Anyan _et al._, 2010) which showed high IL-4 levels in mice injected with eggs, 8 days post injection. Th2 polarization might be down modulating malaria induced pro-inflammatory response and limit severe pathology at the later stages of the disease (Hartgers and Yazdanbakhsh, 2006).

IgG in the experimental group of mice exhibited a similar pattern to IL-4. Higher levels of IgG were reported at day 3 post infection in the experimental mice with a gradual decline to day 12. These high levels were induced by SEA; this shows effect the eggs had in triggering a stronger antibody response. The levels of IgG induced by _P. berghei_ were lower compared to those induced by SEA (difference however not significant) clearly showing a higher response due to the effect of the eggs. High IgG levels at day 3 coincided with high IL-4 levels at the same time point.
The delay in death for two days in the experimental mice can be attributed to the higher levels of antibody compared to the control mice. The gradual decrease in IgG and IL-4 levels from day 3 to day 12 post infection can be explained by the fact that the *S. mansoni* eggs are clearing up from the system with time. By the time *P. berghei* challenge was done, it was 13 days post *S. mansoni* egg injections. The immunological effect of the eggs starting declining from day 3 to day 12 post *P. berghei* infection. These findings agree with those of Anyan and others (2010) who have defined the period between which *S. mansoni* eggs initiate a strong immune response. The immune response elicited by the eggs has been shown to be optimal between the 8th and 12th day post injection (Anyan et al., 2010) and therefore this immune effect is fading away with time. It is therefore clear from this study that the *S. mansoni* eggs slowed down the progression of the *P. berghei* infection.

There were two major limitations experienced in this study. First, the colony of mice that was used was not uniformly picking up infections. During route optimization experiments, mice were infected with cercariae for egg generation which was repeated in the *S. mansoni* egg vaccination and *P. berghei* challenge experimental work. However, mice did not pick up infections uniformly as was expected and this led to the use of more mice than anticipated. Secondly, the ELISA results obtained both from IgG and cytokine ELISAs had very low ODs. However, this could be explained by the fact that recovery levels of the splenocytes was low (40%) and this could have led to poor proliferation which
might have interfered with the cytokine levels in the supernatants. This study is unable to justify the low levels of IgG (low ODs) that were reported but it has been shown that mice exhibit low IgG levels (Yole, personal communication).

Overall, results of this study have shown that *P. berghei* parasitaemia was lower in the experimental mice as compared to control mice and that the experimental mice survived longer compared to the mice infected with *P. berghei* only. These results also provide evidence that the immune response triggered by SEA antigen was much stronger compared to *P. berghei* antigen for example; there were higher levels of IgG, IFN-γ and IL-4 in the experimental mice compared to control mice.
5.2 Conclusions

1. The intraperitoneal route of *S. mansoni* egg administration is a better route of injection of eggs to mice. The triple dosage of *S. mansoni* eggs at ten day intervals is an optimal concentration that resulted in delay in death in experimental mice.

2. IL-4 and IFN-γ levels among the experimental mice contributed to delay in death that was reported in these mice. IL-4 in particular might have led to reducing the pathological effects of severe malaria that are experienced at the latter stages.

3. IgG levels induced by SEA in experimental mice contributed to delay in the death and reduced the rates of progression of *P. berghei* parasitaemia.

4. *S. mansoni* eggs had a role to play in enhancing the survival rates of the experimental mice. Mice inoculated with both *S. mansoni* eggs and *P. berghei* survived longer as compared to those with *P. berghei* only.
5.3 Recommendations

1. Further complementary studies need to be undertaken in this field with a focus on IgG sub-classes involved in malaria protection.

2. More studies need to be done with a better colony of mice that uniformly pick up infections and allow a better grasp of what happens in co-infections of schistosomes and plasmodia infections.

3. Further studies should be done targeting more anti and pro-inflammatory cytokines to enable a better understanding of the immunomodulatory roles of various cytokines in this co-infection.

4. More studies recommended that will isolate and examine the immunological effect of various surface proteins of schistosome eggs on progression of *P. berghei* in mice.
REFERENCES


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APPENDICES

Appendix I:

KENYATTA UNIVERSITY
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FROM:  Dean, Graduate School
TO:    Nyangahu David Donald
        C/o Department of Public Health

DATE:  17th December, 2011
REF:   P150/21567/2010

APPROVAL OF RESEARCH PROPOSAL

This is to inform you that the Graduate School Board at its meeting of 5th
December, 2011 approved your research proposal for M.Sc degree.

JOHN M. ODONGI
FOR:  DEAN, GRADUATE SCHOOL

c.c. Chairman, Department of Public Health

Supervisors:

1. Dr. Margaret W. Muturi
   C/o Department of Medical Laboratory
   Kenyatta University.

2. Dr. Lucy Ochola
   Tropical and Infectious Diseases Department
   Institute of Primate Research
   C/o Department of Public Health
   Kenyatta University.

JMO/2011
Appendix 11

INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: IRC/19/11

Dear Dr Lucy Ochola

It is my pleasure to inform you that your proposal entitled "Schistosoma egg extracts contribute to protection from Plasmodium berghei infections" in collaboration with Dr Margaret Muturi and Mr Donald Nyangahu of Kenyatta University has been reviewed by the Institutional Review Committee (IRC) at a meeting of 29th November 2011. 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 Owara

Signed
Secretary IRC: Dr. John Kagia

Date: 21st June 2012