

The Role of *Schistosoma Mansoni* Eggs in Protection against *Plasmodium Berghei* Infected Mice

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ABSTRACT

The co-occurrence of malaria and schistosomiasis is common in tropical regions of the world. Malaria remains a global burden with no vaccine discovered yet. These necessitated the need to look at the immune responses that could be triggered in a coinfection setting. Malaria induces a strong Th1 response while schistosomiasis skews the response to a Th2. This study examined the role of *Schistosoma mansoni* eggs on malaria disease progression in BALB/c mice infected with *Plasmodium berghei*. The objectives were to determine the immune correlates to protection. Two groups of mice were used: the experimental and control groups. Experimental were injected with a triple dose of *S. mansoni* eggs at ten day interval before being challenged with *P. berghei* while controls were infected with *P. berghei* only. Five mice from both groups at each time point were euthanized and spleen and serum collected. Mice were euthanized at day 3, 6, 9 and 12 post-challenge with *P. berghei*. Parasitaemia was monitored daily using Giemsa stained blood smears. Results showed that experimental mice exhibited lower levels of *P. berghei* parasitaemia (15.52%) as compared to the controls (23.06%). IgG levels were high in the experimental mice compared to controls following stimulation by soluble egg antigen (SEA). Differences in IgG levels between the two study groups were not significant ($p > 0.05$). Levels of IFN- γ and IL-4 were high in the experimental mice than the control group though the difference was not significant ($p = 0.213$). *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 grounds for further studies in non-human primates to better understand the immunomodulatory role of schistosome eggs on malaria progression.

Keywords: *Schistosoma mansoni*, *Plasmodium berghei*, iSoluble egg Antigen

1. Introduction

Concomitant parasitic infections are common in tropical and subtropical regions worldwide [1]. Malaria is the most important parasitic diseases causing approximately 1 million deaths annually. In contrast, schistosomiasis is estimated to affect about 200 million people globally, results in fewer deaths, but is associated with considerable morbidity [2]. 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.

On one hand, schistosomiasis infections protect against cerebral malaria [3] while on the other hand, they are associated with increased malaria severity [4]. Helminth infected patients have decreased cyto-adherence and decreased splenic clearance; the IgE immune complexes formed have an important role in influencing clinical presentation of severe malaria and establishing malaria tolerance [4]. These are as result of regulation in inflammatory factors as also shown in studies on Senegalese children [5]. Other studies, have reported on the role of both cellular and humoral responses. 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 [5, 6] which are explained by cross reactive epitopes between schistosome and *Plasmodium* antigens [7, 8]. 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 [9]. Malaria infections are characterized by an overproduction of IFN- γ and an underproduction of IL-10 [10]. Infections by schistosomiasis on the other hand results in an increase in IFN- γ and IL-2 (Th1) prior to egg laying [11] and this is followed by a dramatic increase in type 2 cytokines (IL-4, IL-5 and IL-10) which coincides with peak granuloma formation that leads to a decline in type 1 cytokine levels [12]. 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 [13]. 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 [13]. 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 [14]. 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. We focused on the co-infection of *Plasmodium berghei* (*P. berghei*) and *S. mansoni* in mice and we determined the immune responses involved. We also sought to understand whether only the egg antigen or other intrinsic factors contribute to protection against malaria. We hypothesized that *S. mansoni* eggs do not protect against *P. berghei* infections.

2. Methods

Six-week-old BALB/c mice bred at the Institute of Primate Research (Nairobi, Kenya), were maintained in cages of 10 mice each 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. *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 numbers counted microscopically before infecting the mice. For malaria, *P. berghei* maintained as frozen aliquots in liquid nitrogen were used. Three mice were infected with *P. berghei*. For all infections, a concentration of 10^5 parasitized red blood cells was administered to every mouse. All the procedures were carried out in accordance with institutional guidelines for animal care. The protocol was approved by the ethics review committee at the Institute of Primate Research (IRC/19/11).

2.1 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, we conducted this experiment. The aim 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).

We tested the single egg dosage along with a triple egg dosage administered at ten day intervals. Two sets of experiments were conducted, 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, 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, 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. 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. Mice were each infected with 10^5 RBCs parasitized with *P. berghei*. Both 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.

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

In this set up, we also used two groups of mice: control and the experimental groups. Each group consisted of 25 mice. Approximately 4000 *S. mansoni* eggs obtained from mice that had previously been infected with cercariae were injected into each of the *S. mansoni* eggs and *P. berghei* group of mice (IP). This was repeated twice with 10 day interval between every egg dosage (Based on results from the optimization experiments). Ten days after the third dose of eggs was administered to the experimental group of mice, both the control and experimental groups of mice were challenged with 10^5 RBCs parasitized with *P. berghei* ANKA strain.

Parasitaemia was 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 for spleen and blood. Five naive mice that were parasite free were also euthanized and serum and splenocytes collected. These provided the baseline samples (day 0). Blood was processed for serum and spleen for splenocytes. Serum samples were used for antibody specific ELISA. 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 *S. mansoni* eggs and *P. berghei* groups of mice (experimental).

Ten mice (control n=5 and experimental n=5) were left for the whole time during the experiment and their parasitaemia and clinical symptoms monitored daily from day 3 post *P. berghei* infection. This provided the

survivorship information and determine if indeed the *S. mansoni* eggs protect against *P. berghei* or not. None of these mice were euthanized. Mice infected with *P. berghei* succumbed to the parasite.

2.3 Infection of BALB/c Mice with *S. mansoni* cercariae

Mice were placed in 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. Mice were then returned to their cages and left for seven weeks to allow for schistosomiasis to develop to acute stage.

2.4 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. This was then left to stand in a shaking waterbath (37°C for 3hours). 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 700rpm 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 500rpm 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 for the *S. mansoni* egg vaccinations and *P. berghei* challenge experiments.

2.5 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 2000 rpm. Serum samples were then collected and stored at -20°C for analysis.

2.6 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 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. Fifty (50µl) of detector antibody, anti-IgG ALP diluted (1:1000), was then added per well and incubated for an hour. Washing was then done five times with PBS-Tween and 50µl per well of p-nitrophenyl phosphate (pNPP) added. Thirty minutes was then allowed for color to develop and the measurements for optical densities taken at 405nm.

2.7 Isolation of splenocytes from spleen

Briefly, spleens from both control and experimental arms 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 stainer into a petri dish, keeping the cell stainer suspended in complete RPMI 1640 (10% FBS, 1% L-glutamine and Gentamycin). Cells were then collected and centrifuged at 1500 rpm 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 then washed twice with RPMI 1640 supplemented with 5% FBS solution with spinning being done at 1500rpm for 10 minutes (Hettich Zentrifugen, Rotanta 460 R, Germany). Splenocytes were then resuspended in complete media for counting. These were freeze stored until use.

2.8 Culture of Splenocytes

Splenocytes from the controls, experimental and non infected arms 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 1500rpm for 10 minutes (Hettich Zentrifugen, Rotanta 460 R, Germany) and then suspended in 5ml complete medium and counted. A suspension of 4×10⁵ splenocytes was cultured at 37°C and 5% CO₂ in 48 well flat bottomed culture plates (Falcon, Lincoln park). Cells were stimulated by SEA (5ug/ml) and *P. berghei* antigen (3ug/ml). Media alone was used as the negative control, this does not lead to any T-cell stimulation and no proliferation is expected while concanavalinA (conA) was used to stimulate the cells and was the positive control (5ug/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, 100ug/ml Penicillin and 10% FBS (Sigma, MO USA). Supernatant was collected 72hrs after the start of culture and stored at -20°C.

2.9 Cytokine 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. 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 five times and blocked with 0.1% BSA PBS-Tween solution and left to stand for an hour. Fifty (50) μ L samples, 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. A properly diluted streptavidin phosphatase (1.1000) was added, incubated at room temperature for about an hour before the appropriate substrate (TMB), 50 μ l per well was added to all the wells. This was then allowed sufficient time to develop and readings taken at 630nm for both cytokines.

Data Analysis

P values were 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.

3.0 Results

We conducted route optimization experiments which laid the foundation for the main experiment. We adopted the IP route and a triple *S. mansoni* egg dosage (results not shown).

a) Clinical signs of mice vaccinated with eggs versus those infected with *P. berghei* only

In general mice that were vaccinated with a triple *S. mansoni* egg dosage appeared healthy and remained active for most part of the experiment. The control group of mice showed deteriorating health from day 6 post infection with parasitaemia rapidly increasing in these mice (Figure 4). They had poor appetite and restricted movement from day 6 post infection. They were shivering and had raised hair. One control mouse succumbed to *P. berghei* infection on day 6 while two succumbed on day 10 post infection. Mice vaccinated with the *S. mansoni* eggs remained active and started showing deteriorating clinical signs from day 10 post infection. Although this group of mice finally succumbed to infection just like the controls, their parasitaemia was lower and they survived longer (Figure 5). *S. mansoni* eggs therefore did not protect these mice but contributed to delaying their death. They slowed down the rate at which the mice infected with *P. berghei* succumbed to infection. Mice in the control group succumbed faster to *P. berghei* infection as compared to those inoculated with both *S. mansoni* eggs and *P. berghei* (experimental).

b) Parasitaemia profile of mice vaccinated with eggs versus the controls

Comparing the parasitaemia levels of the two groups of mice involved in this study, we found that the mice vaccinated with eggs showed lower parasitaemia compared to the controls (Figure 4). Parasitaemia increased rapidly in mice infected with *P. berghei* only as compared to mice inoculated with both the *S. mansoni* eggs and *P. berghei*. Mice receiving eggs had the highest parasitaemia as 16.24% while the controls had 23.12%. Due to the high parasite levels, only one control mouse was alive on day 11 post infection which finally succumbed on day 13. On the other hand three mice vaccinated with eggs were alive on day 11 post infection (Figure 5). The eggs led to a delay in the death by controlling the malaria parasitaemia but they did not protect as the mice ultimately died on day 15 post infection. Although the parasitaemia levels appeared lower in mice vaccinated with eggs, the differences were not statistically significant in the different days post infection (day 5, $p=0.220$, day 7, $p=0.213$, day 9, $p=0.199$).

c) Cytokine profiles

Splenocytes from both the control mice and the experimental mice were retrieved from liquid nitrogen, counted and cultured in a 48 well culture plate. Recovery following retrieval of the splenocytes was approximately 40%. Media and conA were used as negative and positive controls respectively. Experimental mice had their splenocytes stimulated with both *P. berghei* and SEA antigens while controls had *P. berghei* antigen only. Supernatants were collected after 72 hours and used for the determination of IFN- γ and IL-4 levels in both groups.

IL-4 profile

The minimum detectable concentration for IL-4 was 39pg/ml. There were no detectable levels of IL-4 in the naive mice (day 0). There was no response reported to SEA by the naive mice. The levels of IL-4 in these mice were below detection in response to SEA (Figure 1A). IL-4 levels were higher in the experimental mice

compared to the control mice. SEA induced higher levels of IL-4 in the experimental mice as compared to *P. berghei* antigen. Experimental mice showed the highest levels of IL-4 at day 3 post infection and these levels decreased gradually at day 6. 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 *S. mansoni* eggs and *P. berghei* group of mice (Day 3, Day 6, Day 12, $p=0.213$ and Day 9, $p=0.238$).

The levels of IL-4 were higher in the experimental group of mice as compared to the control mice (Figure 1A vs Figure 1B). However although this was the case, there was no significant difference comparing these levels in the two groups of mice at the various time points. 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 *S. mansoni* eggs and *P. berghei* mice (Day 3, Day 6, Day 12, $p=0.213$ and Day 9, $p=0.238$). IL-4 induced by SEA was high as can be seen in Figure 1A, 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* in the control mice at the four time points (Day 3, Day 6, Day 12, $p=0.213$ and Day 9, $p=0.287$).

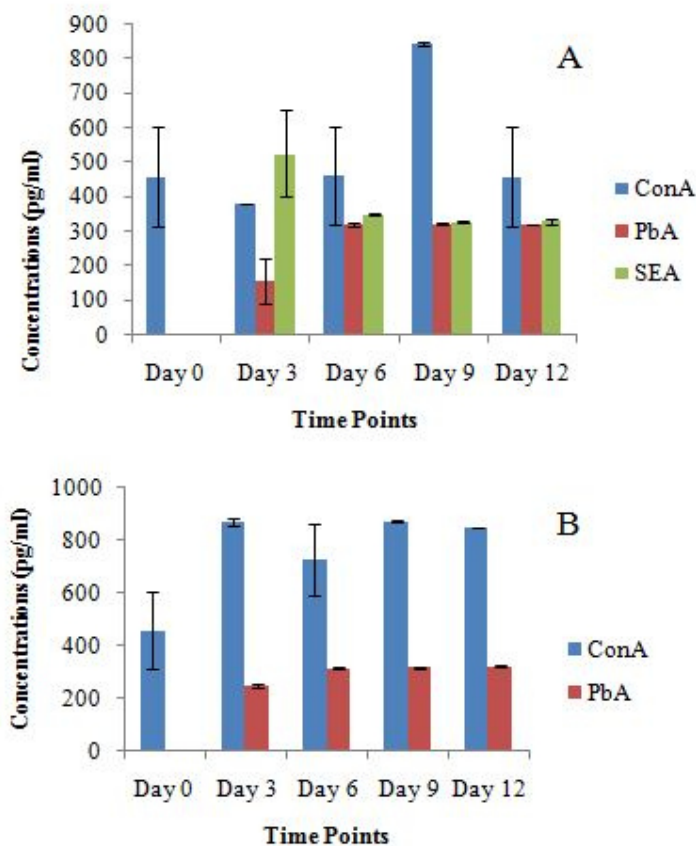


Figure 1: A) IL-4 profile in *S. mansoni* eggs and *P. berghei* inoculated mice. B) IL-4 profile in *P. berghei* only infected mice. In both groups ($n=4$), data represents the means and error bars represent SD.

IFN- γ Profile

We observed a gradual rise in the IFN- γ levels from day 3 to day 12 post infection (Figure 2A). 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 ($p>0.05$).

IFN- γ levels were higher in experimental mice (Figure 2B). Similar to the controls, 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 2A vs Figure 2B). We however note that these differences were not significant between the experimental and control groups (Day 3, $p=0.213$, Day 6, $p=0.287$, Day 9, $p=0.213$ and Day 12, $p=0.092$). There was also 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, $p=0.238$).

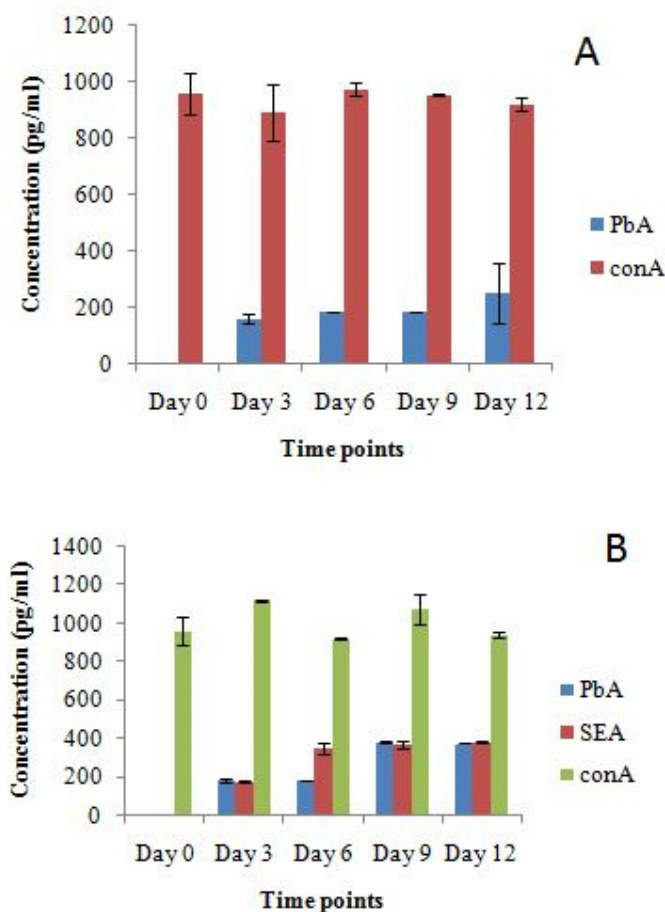


Figure 2: A) IFN- γ profile in *P. berghei* only infected mice. B) IFN- γ profile in *S. mansoni* eggs and *P. berghei* inoculated mice. In both groups (n=4). Data represents the means and error bars represent SD.

d) IgG levels

We determined IgG levels in serum. To determine the IgG levels induced as a result of infection by *P. berghei* or the *S. mansoni* eggs, these antigens were used for coating the microtitre plates. Levels in control mice increased gradually from day 0 to day 3 post infection and by day 6 they remained stable (Figure 3A). Differences were however not significant at the various time points ($p>0.05$).

In the experimental group of mice the IgG levels were higher compared to the control group of mice (comparing the IgG levels in terms of ODs obtained)(Figure 3B). IgG levels in the control mice were induced as a result of the *P. berghei* antigen. However, 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 better response in the *S. mansoni* eggs and *P. berghei* 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*. Levels of IgG induced in response to the egg antigen were higher compared to levels by the *P. berghei* antigen (Figure 3B). This was a clear indication that high IgG levels were in response to the *S. mansoni* eggs. In addition, although the IgG levels were higher for experimental mice compared to the controls, there was no significant difference in these levels for the various time points (intergroup comparison, $p>0.05$)

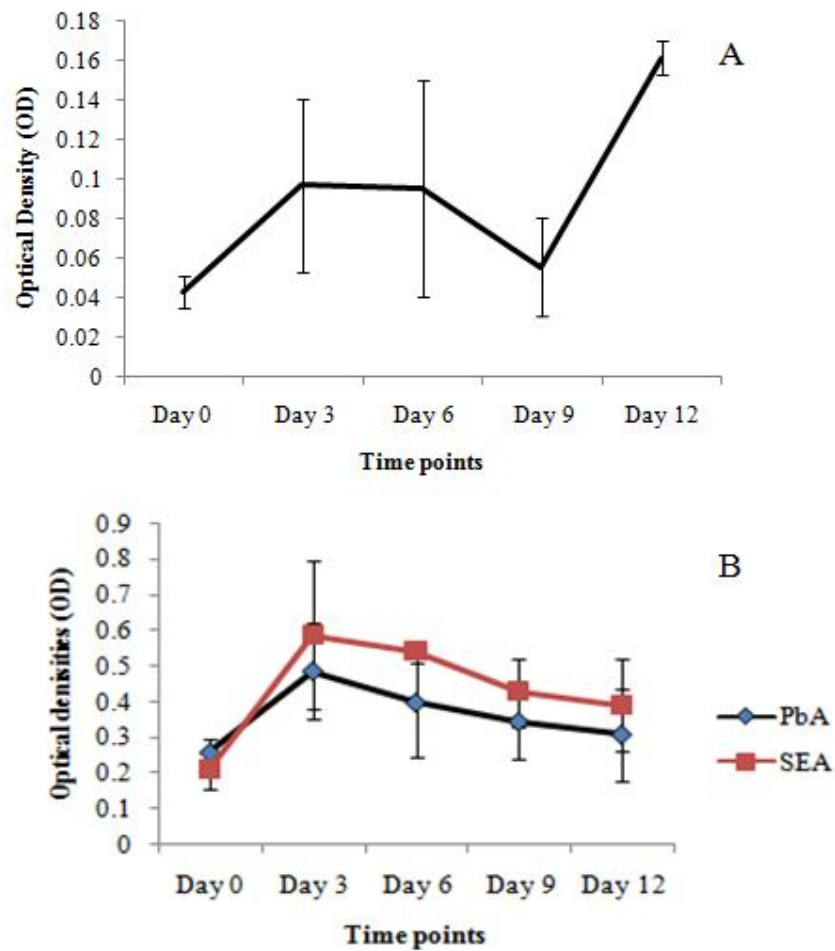


Figure 3: A) IgG profile in mice infected with *P. berghei* only. B) IgG profile in mice inoculated with *S. mansoni* eggs and *P. berghei*. Each time point represents n=4 mice. Data represents means and error bars represent SD. The days shown in the graph (day 0, 3, 6, 9 and 12) are days post *P. berghei* challenge

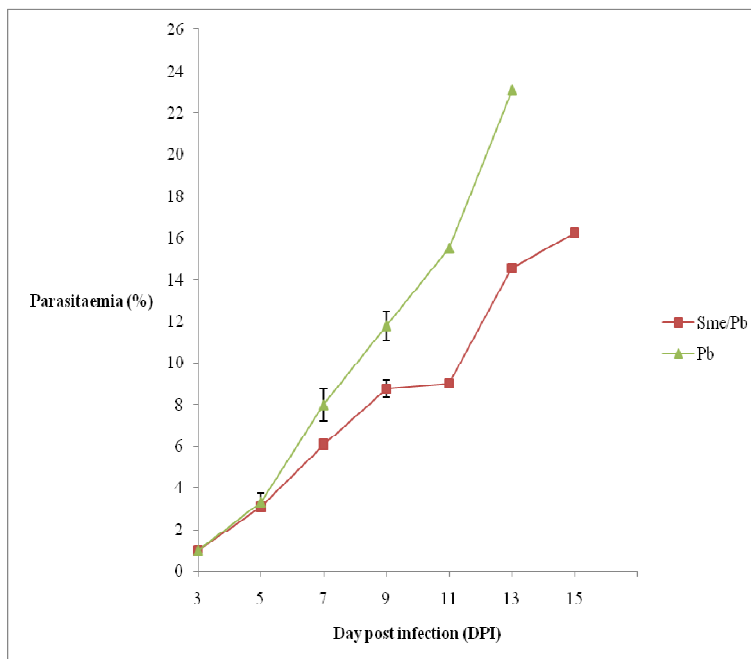


Figure 4: Parasitaemia curve of *S. mansoni* egg vaccination and *P. berghei* challenge experiments. The control group was infected with *P. berghei* only while the *S. mansoni* eggs and *P. berghei* group were inoculated with *S. mansoni* eggs and *P. berghei*. The data represents means. Error bars represent Standard deviation (SD).

e) Survivorship profile of mice

Ten (10) mice were left untouched during the *S. mansoni* egg vaccinations and *P. berghei* challenge experiment, 5 from each group. These mice were monitored till they succumbed to infection and death was only as a result of the infections the mice had. This provided crucial information on the effect the eggs had and if indeed they resulted in any form of delay or protection. The experimental group of mice survived longer than the control mice. At day 9 post infection, 80% of the experimental mice were still alive while 60% of the control mice were alive (Figure 5). None of the control mice was alive on day 14 while the experimental mice had 40% of the mice still alive on this day which finally succumbed on day 15 post infection.

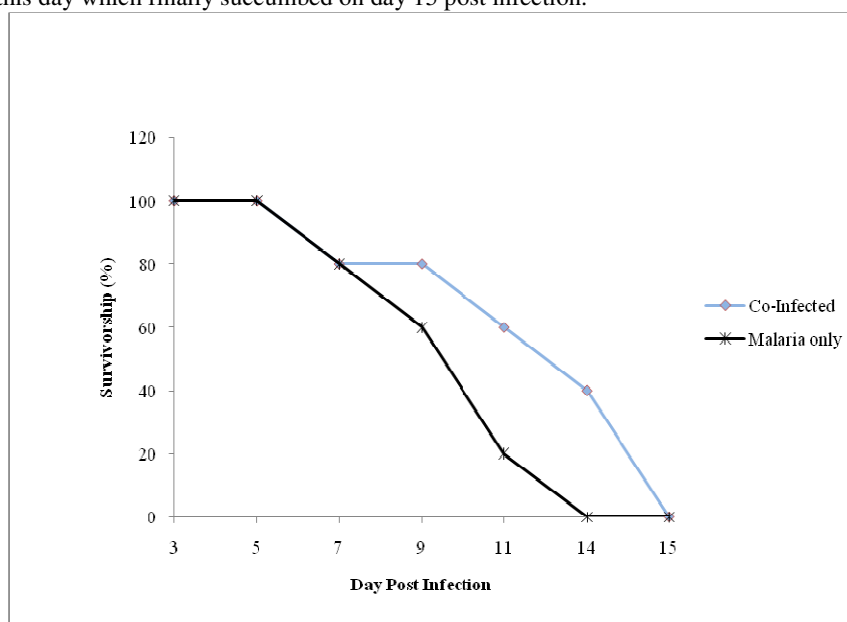


Figure 5: Survivorship curve of *S. mansoni* eggs and *P. berghei* mice and those infected with *P. berghei* only.

Discussion

Various 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 [4, 7, 15, 16] which implies that an antihelminthic intervention could result in reduced frequency of malaria attacks and/or malaria parasite densities in areas where both malaria and helminth infections are endemic. One explanation of this finding could be that the impact of helminth infections on malaria infection is intensity dependent such that heavy helminth infections are required to induce a shift in Th1/Th2 balance which in turn would be reflected in differences in the observed malaria infection between children in the intervention and control groups. This view has also been supported by findings of the study of Sokhna et al (2004) in Senegal who observed that the incidence rate of malaria attacks was significantly higher in children infected with *S. mansoni* particularly those carrying the highest egg loads as compared to uninfected children.

Here we utilized the *S. mansoni* eggs but their effect was weak on the progression of *P. berghei* parasitaemia in mice. This could be attributed to the fact that the eggs that were used were already aged in the mice tissues by week seven when perfusion was done and the further process of isolation may have again interfered with their quality. This could have contributed to the findings of this study where much as the eggs are shown to have an immunological impact in terms of controlling the rapid increase in malaria parasitaemia, the difference between parasitaemia, IgG, IFN- γ , and IL-4 levels between the controls and the experimental groups was not significant ($p > 0.05$).

Comparing the IP versus the IV route of application (optimization experiments), we found the IP to be more suitable for administration as compared to the IV which was more invasive to the mice and led to the death of two mice during the process (Nyangahu, unpublished data). 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 buildup steadily in both the *S. mansoni* eggs and *P. berghei* mice and the controls from day 3 to day 12 post-*P. berghei* challenge.

Comparing the parasitaemia and survivorship of the control mice and the mice vaccinated with eggs, we reported lower parasitaemia levels and enhanced survivorship in the mice that were vaccinated with the *S. mansoni* eggs. *S. mansoni* egg *P. berghei* inoculated mice survived longer by two days and had their highest parasitaemia (16.24%) slightly lower than that of the controls (23.12%). However these differences were not significant. This led us to conclude that the eggs did not protect against the *P. berghei* infection but contributed to enhanced survivorship of the *S. mansoni* eggs and *P. berghei* mice and also delayed the progression of parasitaemia in the same group of mice. Moreover, all the control mice that were used in this experiment died in the course of the experiment (100%) while 60% of the experimental mice died during the experiment (Figure 5). However, *S. mansoni* eggs do not contribute to protection from the *P. berghei* infection since the mice ultimately succumbed to infection and died. The protection due to the effect of the eggs was therefore shortlived. The remaining two mice vaccinated with eggs ultimately succumbed to infection later on day 15 post infection.

The effectiveness of *S. mansoni* eggs in contributing to protection to malaria may have been hampered by age and 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 [7]. 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* [18, 19]. 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 in their co-infection study reported reduced *P. chabaudi* parasitaemia in mice that were co-infected with both *S. mansoni* and *P. chabaudi*. Administration of a triple egg dosage in mice led to some delay in death of the mice being reported (Figure 6). This can be explained by the fact that schistosomes elicit a type 2 response [14]. The injection of the eggs to the mice leads to biasing of the immune response to a Th2. Therefore, for some delay effect to be seen, there needs to be a stable type 2 response which is brought in by the *S. mansoni* eggs (primary stimulus in a schistosome infection).

IL-4 findings demonstrate that eggs evoked a stronger immune response compared to the *P. berghei* antigen particularly at day 3 (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 [14]. The injection of the *S. mansoni* eggs resulted in immunomodulation 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 will take about 8 days to mount an immune response which will continue up to the 12th day [17]. 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 [20]. IgG in the experimental group of mice exhibited a similar pattern to IL-4 (Figure 3B). Higher levels of IgG were reported at day 3 post infection in the *S. mansoni* eggs and *P. berghei* mice with a gradual decline to day 12. These high levels were induced by SEA; this again 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 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. It is therefore clear from this study that the *S. mansoni* eggs slowed down the progression of the *P. berghei* infection.

Overall, the results of this study have shown that *P. berghei* parasitaemia was lower in the experimental mice as compared to mice infected with *P. berghei* alone 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 stronger compared to *P. berghei* antigen. There were higher levels of IgG, IFN- γ and IL-4 in the experimental mice compared to mice infected with *P. berghei* only in the days sampled. Finally, we provide evidence that the *S. mansoni* eggs though not protective against *P. berghei* infections, they do delay the build-up of *P. berghei* parasitaemia in infected mice and enable the *S. mansoni* eggs and *P. berghei* mice to survive longer than mice infected with *P. berghei* alone. Future studies should aim to replicate this in non human primates and provide further insights that can be translated to human studies.

Conclusions

We found that *S. mansoni* egg antigens contributed to delaying the death of mice infected with *P. berghei* and also resulted in a slow progression of *P. berghei* parasitaemia in these mice. The high levels of IL-4 among the experimental mice contributed to delay in death exhibited in these mice by polarizing the immune system to a Th2. This IL-4 levels might have led to reducing the pathological effects of severe malaria that are experienced at the latter stages.

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

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