CYTOKINE (IL-5, IL-10, IL-13 AND IFN-γ) PROFILES OF SCHOOL CHILDREN INFECTED BY *SCHISTOSOMA MANSONI* UPON MULTIPLE TREATMENTS WITH PRAZIQUANTEL

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN IMMUNOLOGY IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

April 2011
DECLARATION

I, hereby declare that this thesis is my original work and has not been presented for a degree or any other award in any other University

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DEDICATION
This thesis is dedicated to my late parents, Mama Leah Sembeya and Baba Enock Ndombi Lianda.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BILIVAX</td>
<td>Bilharzia vaccine</td>
</tr>
<tr>
<td>BSA</td>
<td>Buffered Saline Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>DCs</td>
<td>Dentritic cells</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<tr>
<td>EPG</td>
<td>Eggs per gram</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute of Allergy and Infectious Diseases</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cells</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen Associated Molecular Patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered saline</td>
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<tr>
<td>pH</td>
<td>Power of hydrogen</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>PZQ</td>
<td>Praziquantel</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute Media</td>
</tr>
<tr>
<td>SEA</td>
<td>Soluble Schistosome Egg Antigenic (preparation)</td>
</tr>
<tr>
<td>Sm</td>
<td>Schistosoma mansoni</td>
</tr>
<tr>
<td>SWAP</td>
<td>Soluble Worm Antigenic Preparation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;1</td>
<td>T-helper 1 cells</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;2</td>
<td>T-helper 2 cells</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine peroxidise</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>T-regs</td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td>WHA</td>
<td>World Health Assembly</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Schistosomiasis is a parasitic disease caused by trematode worms. The most prevalent species of the worm in Kenya is *Schistosoma mansoni*. The disease is endemic in parts of Nyanza, Eastern and Coast provinces. Its prevalence in primary school children in Asembo area near Lake Victoria in Rarieda district of Nyanza province is about 17%. Children are more susceptible to infection than adults and thus bear a greater burden of schistosomiasis in terms of morbidity and intensity of infection. Cytokines are an important parameter which can be used to evaluate an individual’s immune responses and as an indicator of susceptibility, resistance and morbidity to the disease. This study sought to determine progressive cytokine profiles of a cohort of *Schistosomiasis mansoni* infected school children upon single or multiple treatments with Praziquantel. The children were recruited into two separate groups of about 90 children each. One group designated arm A were diagnosed, specimens collected and treated where necessary, 3 times a year, once in each term of the school year. The other group, designated arm B was diagnosed, specimens collected and treated at time zero and after 24 months. Infection intensity was based on eggs per gram of faeces. Cytokine responses from whole blood cultured with soluble egg antigens (SEA) and soluble worm antigen preparation (SWAP) were determined by ELISA. The cytokines assayed were IFN-γ, IL-5, IL-10 and IL-13. The relationship between cytokine production and infection intensity was determined by correlation test. The effect of multiple treatments on cytokine levels was also determined and comparisons made between cytokine levels of children treated once and those treated multiple times. Infection intensity in the children ranged from a minimum of 12 to a maximum of 2088 epg of faeces and a mean of 217.8 epg of faeces. At the end of the study following single or multiple treatment, infection intensity had dropped, ranging from 0 to a maximum of 828 and mean of 43.22 epg of faeces. Out of a total of 113 children who remained in the study, 36 were not re-infected, 10 in the multiple treatment group and 26 in the single treatment group at the end of the study. No significant correlations were obtained between cytokine levels and infection intensity except for IL-13 (P<0.05) from SEA stimulated samples from children who had low infection intensity. Repeated cycles of treatment did not have a significant effect on cytokine levels (P>0.05). There were also no significant differences in cytokine levels between children treated once (arm B) and those treated multiple times (arm A). Based on the findings of this study, it is recommended that other immunological parameters such antibody and B cell responses be used to assess possible immunity development in children following repeated treatment after reinfection in children. For control of schistosomiasis, a combination of strategies should be adopted besides treatment, including public education, improved sanitation and provision of clean drinking water. Finally, a follow-up period for retreatment of reinfected children should be more than three months but less than 2 years to realise meaningful gains in the control of schistosomiasis. More research should however be done to determine the most appropriate follow-up treatment time. Studies should also be done to elucidate other immunological responses elicited in children who undergo repeated treatment after reinfection.
CHAPTER ONE: INTRODUCTION

1.1 Background

Schistosomiasis is a parasitic disease caused by infection with trematode worms. The major human Schistosome parasites are *Schistosoma haematobium*, *S. mansoni* and *S. Japonicum* which are endemic in several parts of Africa, the Middle East, South America, China, Southeast Asia, and the Philippines. It is estimated that 200 million people are infected with the disease worldwide and those at risk of infection are 600 million (Chitsulo et al., 2000). Infection with the parasite occurs when an individual comes in contact with the skin-penetrating larval form called cercariae released from parasitized fresh water snails. Children in endemic areas get infected from the time they start to come in contact with fresh water. Generally, infection intensity increases as children age, peaks at around early adolescence and then drops in adulthood (Woolhouse and Hagan, 1999). After infection, worms can live in the bloodstream for many years while further infection and reinfection leads to chronic disease (Vennervald et al., 2004). In Kenya, the disease is endemic in parts of Nyanza, Eastern and Coast provinces. *Schistosoma mansoni* infection is highly endemic in areas around Lake Victoria. A survey in children attending schools near the Lake in Rarieda district in the year 2001 found a mean prevalence rate of 16.3% for *S. mansoni* and 0.2% for *S. haematobium* (Handzel et al., 2003).

Schistosomiasis causes two main clinical conditions: acute schistosomiasis and chronic schistosomiasis. Acute schistosomiasis is a debilitating febrile illness, which is also called Katayama syndrome (Ross et al., 2007). Its symptoms are less severe especially in *S. mansoni* and occur 6-8 weeks after infection and even before eggs appear. These include fever, fatigue, myalgia, malaise, non-productive cough, eosinophilia and patchy infiltrates in the lungs (Pearce and MacDonald, 2002; Ribeiro et al., 2002; Gryseels et al., 2006). The
chronic form of the disease results from the host’s immune response to Schistosome eggs and the antigens they secrete. In most infected individuals in endemic areas, this stage is relatively asymptomatic. In a small proportion of individuals, the granulomatous reaction evoked is more severe and results in fibrous deposition in host tissues, their subsequent enlargement and chronic fibro-obstructive disease (Ross et al., 2002). Hepatosplenomegaly is a common characteristic of the disease in these individuals at this stage and death may ultimately occur if not treated (Werf et al., 2003).

Diagnosis of schistosomiasis is mainly by the microscopic examination of urine and faeces for the presence of eggs. It can also be done using antibody-based assays which are quite sensitive. These cannot however differentiate between past and active infection and the possibility of cross-reaction with other helminths (Tsang and Wilkins, 1997; Rabello, 1997). Praziquantel is the drug of choice for the treatment of schistosomiasis and plays a central role in community-based programmes for the control of the disease including implementation of the World Health Assembly (WHA) resolution 54.19. The resolution states that all children at risk of acquiring schistosomiasis should be treated with PZQ (WHO, 2001). There is however no prophylactic vaccine available against the disease.

1.2 Problem statement

Findings from many studies report the development of resistance to reinfection following curative treatment of Schistosoma mansoni infection in individuals despite continued exposure to infection source. A study similar to this one in design but involving adult car washers who are occupationally exposed to cercarae-containing water reported that some individuals developed increased resistance to reinfection following multiple cycles of treatments, reinfections and retreatment with Praziquantel (PZQ) (Karanja et al., 2002). It is
however not known if similar results occur in children who are naturally exposed to infection. This study sought to determine the effect of treatment of schistosomiasis mansoni in 8 to 10 year old school children on immunity development as inferred on $T_H1$ and $T_H2$ cytokine responses. The study also sought to determine the effect of multiple treatments on the levels of these cytokine and whether multiple treatments following re-infection in children elicits cytokine responses associated with immunity.

1.3 Justification of the study

Children bear a greater burden of Schistosoma mansoni infections than adults. They are more susceptible, they harbour higher infection intensities and suffer severe morbidities. Unlike adults, it is not known whether children develop resistance to reinfection following repeated cycles of treatment after reinfection. Resistance to reinfection can be inferred on $T_H1$ and $T_H2$ cytokine responses after treatment. Findings from the study will be of great relevance to the Ministries of Health (MOHs) in countries where the disease is endemic especially in making decisions on the best way to implement WHA 54.19., especially on the follow-up retreatment period after reinfection.

1.4 Research questions

i. What are the effects of effective treatment of $S. mansoni$ infection in 8 to 10 year old school children on $T_H1$ and $T_H2$ cytokine levels?

ii. What are the effects of multiple treatments on $T_H1$ and $T_H2$ cytokine levels following subsequent reinfections with $S. mansoni$ in 8 to 10 year old school children?

iii. What are the differences in $T_H1$ and $T_H2$ cytokine levels between children treated multiple times and those treated once during a two year study period?
1.5 Null hypotheses

i. There are no changes in cytokine profiles of 8 to 10 year old school children upon treatment of *S. mansoni* infection.

ii. There are no differences in cytokine profiles between children who have had multiple rounds of treatment, reinfection and retreatment of *S. mansoni* infection and those treated once at the beginning of the study during a two year study period.

1.6 Objectives

1.6.1 General objective
To determine changes in cytokine profiles of *Schistosoma mansoni* infected 8 to 10 year old school children upon treatment with Praziquantel.

1.6.2 Specific objectives

i. To compare the infection intensity and cytokine levels at baseline and 2 years later in a cohort of *Schistosoma mansoni* infected school children.

ii. To determine the effect of repeated cycles of treatment using Praziquantel upon reinfection on cytokine levels.

iii. To compare the cytokine levels between children who have had repeated cycles of treatment (arm A) upon reinfection with those treated once at the beginning of the study and at the end of 2 years (arm B).
CHAPTER TWO: LITERATURE REVIEW

2.1 Schistosomiasis infection of humans

2.1.1 Schistosome species

Schistosomiasis, also called bilharzia is a disease of the tropics caused by blood dwelling flukes of the genus *Schistosoma*. Five species of *Schistosoma* are known to infect human beings: *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi*, *Schistosoma haematobium*, and *Schistosoma intercalatum* (Ross *et al.*, 2007). Of the five species, only three are of serious concern when it comes to human schistosomiasis. These are *S. mansoni* which is transmitted by *Biomphalaria* snails and causes intestinal and hepatic schistosomiasis in Africa, the Arabian peninsula, and South America; *S. haematobium*, transmitted by *Bulinus* snails and causing urinary schistosomiasis in Africa and the Arabian peninsula; and *S. japonicum*, transmitted by the amphibian snail *Oncomelania* and causing intestinal and hepatosplenic schistosomiasis in China, the Philippines and Indonesia (Engels *et al.*, 2002). *Schistosomiasis japonicum* is a zoonotic parasite that infects several other animals such as cattle, dogs, pigs and rodents. *Schistosoma mansoni* has also been reported to infect rodents and primates (Gryseels *et al.*, 2006).

Adult schistosomes are white or grayish worms about 7-20 mm in length. They have a cylindrical body with two terminal suckers, a complex protective tegument, a blind digestive tract and reproductive organs. The worms have separate sexes with the male forming a groove or gynaecophoric channel in which the long slender female dwells. The worms live as a permanently embraced couple within the perivesical for *S. haematobium* or mesenteric venous plexus for other species. They feed on blood and globulins by anaerobic respiration and regurgitate debris in the hosts blood (Gryseels *et al.*, 2006).
2.1.2 Life cycle of schistosomes

Human infection by schistosomes occurs when an individual comes in direct contact with fresh water that harbours the free swimming larval form of the parasite called cercariae. Cercariae are shed by parasitized fresh water snails. The larvae possess a forked tail and are non-feeding, utilizing their endogenous nutrients for energy production. The implication is that they must come in contact with the skin of their primary host within 12-24 hours after emerging from the snail. On contact with the skin, percutaneous penetration occurs by both mechanical activity and by the production of proteolytic enzymes. As they penetrate, the cercariae shed their tails and enter the lymphatic system from where they move to the blood circulatory system which transports them to the lungs. After a short stay in the lungs, the developing schistosomula then re-enter blood circulation for transport to the portal or vestibule circulation to complete the life cycle. It is from here that the schistosomula mature couple up for reproduction and migrate to their perivescular or mesenteric destination where egg production occurs (Gryseels et al., 2006).

Maturation of the larvae into adult male and female worms from infection time takes about 4 to 6 weeks before egg production commences. This continues for the rest of their life, which averages three to five years but may also be as long as thirty years (Gryseels et al., 2006). The females produce hundreds for the African species to thousands of eggs per day for the oriental species. The tough-shelled eggs (Fig 2.1) contain ciliated miracidium larva that secretes proteolytic enzymes. These enzymes help them to cross the endothelium and basement membrane of the vein and epithelium of the intestine or bladder to be shed with faeces or urine and complete the life cycle. How egg migration occurs across the venous and intestinal walls is not clearly understood although there seems to be an immunological component to it since egg excretion in immunocompromised mice is minimal (Doenhoff et
al., 1997). Another study reported a correlation between diminished egg excretion and decreased CD4\(^+\) T cell count in *S. mansoni*-infected HIV positive patients (Karanja *et al.*, 1997).

![Schistosoma mansoni egg](image)

**Figure 2.1 Schistosoma mansoni egg (Adapted from Ross *et al.*, 2007)**

Once excreted, eggs remain viable for up to 7 days, time during which the miracidia larvae are released when they come in contact with water. The larvae then, using light and chemical stimuli, search for their respective fresh water snail which serves as the intermediate host. Inside the snails, the miracidia larvae multiply asexually into multicellular sporocysts and later into cercarial larvae to continue the life cycle (Fig 2.2; Gryseels *et al.*, 2006).
Figure 2.2: Life cycle of *S. mansoni*, *S. japonicum*, and *S. haematobium*. (Adapted from McManus and Loukas, 2008)

### 2.2 Schistosomiasis distribution

#### 2.2.1 Global schistosomiasis distribution

Schistosomiasis is endemic in 76 countries and territories globally, according to Engels *et al.* (2002). Active transmission is reported from 67 countries. It was estimated in 2003 that 779 million people were at risk infection and 207 million people were infected (Steinman *et al.*, 2006). The three major schistosome parasites of humans are *S. haematobium*, *S. mansoni* and *S. japonicum* and are prevalent in parts of Africa, the Middle East, South America, China,
south-east asia and the philippines. *Schistosoma mansoni* is found in 54 countries, including part of Arabian peninsula, northern Africa, sub-Saharan Africa, Brazil and some Caribbean islands (Fig 2.3). *Schistosoma haematobium* is endemic in 53 countries in the Middle East and most of the African continent while *S. japonicum* is endemic in China, Indonesia and the Philippines (Chitsulo *et al.*, 2000).

![Figure 2.3: Global distribution of schistosomiasis (Adapted from Gryseels *et al.*, 2006)](image)

2.2.2 Schistosomiasis in Kenya

In Kenya, schistosomiasis is endemic in many parts, particularly in the Coastal and Lake Victoria region. Other areas of endemicity are Mwea and Machakos in Eastern Kenya and Nyando basin in Western Kenya, places that undertake irrigation farming. *Schistosoma haematobium* and *S. mansoni* are the commonest species in the East African region.
Prevalence of *S. mansoni* in the Lake Victoria region is found to increase with proximity to the lake while for *S. haematobium*, prevalence increases with distance from the lake. The most affected group around Lake Victoria in Kenya are men and school age children. This is attributed to the high frequency of water contact in these two groups (Handzel *et al.*, 2003).

### 2.3 Schistosomiasis disease

#### 2.3.1 Acute disease

The acute phase of *S. mansoni* infection is largely asymptomatic in individuals living in endemic areas possibly due to in-utero sensitization. In newly infected patients, especially those travelling to endemic areas for the first time, it is manifested as Katayama syndrome which is basically a systemic hypersensitivity reaction against the migrating schistosomula and eggs and manifests within two to twelve weeks after a primary infection. The percutaneous penetration of cercariae in newly infected persons can also trigger a rash on the skin called cercarial dermatitis. Symptoms for the systemic reaction manifest suddenly a few weeks to months after a primary infection. They include fever, fatigue, myalgia, malaise, non-productive cough and eosinophilia. In some patients, abdominal symptoms may develop later, caused by the migration and positioning of the mature worms (Gryseels *et al.*, 2006; Ross *et al.*, 2007). Most patients recover spontaneously after 2-10 weeks of infection. Other individuals develop persistent and more serious disease.

#### 2.3.2 Chronic disease

In the chronic phase, schistosomiasis mansoni may manifest two different clinical forms depending on an individual. It may cause a variety of subtle morbidities under the intestinal form of the disease, which affects most individuals, or it may develop into the severe hepatosplenic form in few cases. In intestinal schistosomiasis, migration of eggs through the
intestinal wall may provoke mucosal granulomatous inflammation, microalceration and superficial bleeding. The most common symptoms of this form of disease are chronic or intermittent abdominal pain and discomfort, loss of appetite and diarrhoea with or without blood. The frequency and severity of the symptoms in infected people is related to intensity of infection (Gryseels et al., 2006). The hepatosplenic form of the disease is characterised by hepatosplenomegaly, periportal fibrosis, portal hypertension, ascites oesophageal varices, collateral circulation, haematesis, and death if not treated (Werf et al., 2003; King et al., 2005). In the early stages of this chronic form of the disease, there is hepatic inflammation which is an early reaction to trapped eggs in the periportal spaces of the liver. This is the main cause of hepatic splenomegaly found in up to 80% of infected children and adolescents. It is however less common and intense in adults. Massive deposition of collagen in the periportal spaces may occur years later mostly in young and middle-aged adults leading to hepatic fibrosis. Fibrosis process takes 5 to 15 years and may eventually cause bleeding from gastro-oesophageal varices which is the most common cause of mortality in schistosomiasis (Gryseels et al., 2006).

2.4 Diagnosis of schistosomiasis

Microscopic detection of parasite eggs remains the preferred and most reliable method for diagnosing schistosomiasis infection. The eggs are fairly easily detected and identified in faeces due to their size and lateral spine (Fig 2.1). For detection of mild and light infections, concentration methods such as sedimentation in a glycerine solution or centrifugation in formolised ether are needed. The Kato-Katz method (Katz et al., 1972) is the most commonly used method of diagnosis since it allows quantification of the infection by egg counts, usually eggs per gram of faeces (EPG). It is a rapid, simple, and inexpensive technique that requires 40 to 50 mg of faeces. It is widely used in field studies and national control programmes
(Feldmeier, et al., 1993). This technique is not able to detect infection in early stages, that is, before egg secretion commences. It may also miss to detect eggs in mild or light infections.

Diagnosis can also be done using antibody detection techniques that are preferable in a few specific circumstances such as diagnosis in patients not excreting eggs, in field studies for defining regions of low-level endemicity where individual patients have low egg burdens. They may also be useful in determining re-emergence of infection in a region following an apparently successful control program (Ross et al., 2002). While antibody based assays are quite sensitive, they cannot distinguish past infection from active infection. They can also cross-react with other helminthes and are not easily applicable under field conditions. The antibodies routinely detected include IgG, IgM or IgE against soluble worm antigen or crude egg antigen (Gryseels et al., 2006). There are also tests to detect somatic schistosome antigens, such as circulating anodic antigen and circulating cathodic antigen. These are detected and quantified with labelled monoclonal antibodies in serum or urine of infected individuals (Gryseels et al., 2006).

2.5 Immunology of schistosomiasis

The immune response in the first three to five weeks is dominantly TH1 like with elevated levels of IFN-γ. As egg production begins, TH1 component declines with an associated emergence of a strong TH2 response (Pearce and MacDonald, 2002). The cellular proliferative responses of peripheral blood mononuclear cells (PBMCs) from patients with acute disease are higher to parasite soluble egg antigen (SEA) and soluble worm antigen preparation (SWAP) than those from patients with chronic disease (Malaquias et al., 1997). Many studies report a mixed TH1/TH2 cytokine profile during acute phase of the disease with the cytokines mostly reported being IFN-γ, IL-10 and IL-5 (Montenegro et al., 1999). The
immune response in the chronic phase of the disease is marked by a predominantly $T_H^2$ cytokine profile although a few studies have reported a $T_H^1$ profile (Mwatha et al., 1998). This underscores the variable nature of the immune response in chronic patients, which is a mix of both $T_H^1$ and $T_H^2$ cytokines (Ribeiro et al., 2004).

The early immunological mechanisms that lead to the induction of $T_H^2$ response during schistosomiasis infection are not well understood. The responses in the course of infection have been described as modified $T_H^2$-cell response with high IL-10 and IgG4 and low IgE reactions (Maizels et al., 2003). It has been suggested that the innate immune system recognizes conserved parasite motifs, preferentially triggering a $T_H^2$ response. It is known that whereas schistosomula and adult worm antigens do not induce strong inflammation locally, eggs induce inflammation and granulomatous hypersensitivity reaction in the liver, spleen and lungs. Eggs are also strong inducers of the $T_H^2$ responses. The egg antigens that play a large role in the immune system biasing are glycoconjugates and lipoconjugates. Studies involving animal models have identified several cell populations that may be mediators of this immune system biasing. These include a special compartment of B cells called B-1 cells, natural killer T (NKT) cells and Basophils. These cells have been shown to expand in response to schistome egg carbohydrates and produce immunoregulatory factors such as prostaglandin (PG) E$_2$, IL-10 and IL-4 all of which are capable of skewing the immune response towards $T_H^2$ profile (Capron et al., 2005).

Dendritic cells may also be involved in the $T_H^2$ biasing of the immune response during schistosomiasis infection. Dendritic cells are important antigen presenting cells whose maturation determines T-cell priming and differentiation from naive to $T_H^1$, $T_H^2$ or T-regulatory (T-reg) type cells. There is a possibility that the pathogen associated molecular
patterns (PAMPs) on schistosome eggs signal DCs via their pattern recognition receptors (PRRs) towards a Th2 driven pathway (Capron et al., 2005).

Animal models have substantially been used to understand *S. mansoni* infection. Rats and mice represent two divergent courses of infection. The rat is a semi permissive host in which parasites are spontaneously eliminated before maturity and therefore prior to egg production, with subsequent development of a strong immunity to reinfection. Mice are permissive hosts in which the infection proceeds to full pathology. Immunity to reinfection in the rat is essentially a Th2 response, which implies an antibody dependent cellular cytotoxicity mechanism (Mosmann and Coffman, 1989; Modlin and Nutman, 1993; Zurawski and de Varies, 1994). Mice on the other hand become resistant to reinfection when immunised by injection with irradiated cercariae. This elicits a Th1 inflammatory response that blocks migration of schistosomula and kills larvae (Capron and Dessaint, 1985).

### 2.6 Parasite survival mechanisms in the human host

Schistosomes are able to persist in the potentially hostile environment in the human host for many years. They must have mechanisms to protect themselves from immune attack while probably also actively intervening to render the response ineffective. The parasite is covered by a protective tegument which is actually a syncytial layer with a surface of normal plasma membrane overlain by an external secreted membranocalyx. The membranocalyx is predominantly composed of lipid and acts as a shield against immune attack (Wilson and Coulson, 2009). The membranocalyx is also coated with host erythrocyte blood group proteins which help to mask parasite proteins (Mclaren *et al*., 1982). The parasites gut has oesophageal glands whose secretions are thought to serve a protective role by denaturing antibodies and complement factors. Later stages of the parasite are much less susceptible to
in vitro killing by products of the oxidative burst from host phagocytes. This is attributed to antioxidant proteins released by the parasite (Wilson and Coulson, 2009). Several other parasite proteins have been identified with the ability to modulate the immune response in favour of the parasite. For example Sm16, a cercaria-specific protein has anti-inflammatory properties. There is also evidence that the host’s complement defence mechanisms might be turned against it to protect the parasite by the decay accelerating factor (DAF) detected on the parasite tegument (Wilson and Coulson, 2009).

2.7 Role of cytokines in schistosomiasis

Cytokines are small protein molecules secreted by different cell populations of the immune system. They mediate immune responses and hematopoiesis. They are predominantly produced by T cells and macrophages. Depending on the cytokine environment and signals sent by antigen presenting cells, naive CD4+ T helper (T\textsubscript{H} cells) differentiate after activation into either T\textsubscript{H}1 or T\textsubscript{H}2 cells, each of which secretes a restricted set of cytokines. Differentiation of T\textsubscript{H}1 cells is promoted by IL-12 and these cells secrete mainly IFN-\gamma and IL-2. On the other hand, differentiation of T\textsubscript{H}2 cells is promoted by IL-4, and the cells mainly secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Generally, T\textsubscript{H}1 subset promotes cell-mediated immunity while T\textsubscript{H}2 subset provides help to B cells in antibody production. The immune response during early schistosomiasis is predominantly T\textsubscript{H}1 but switches to a predominant T\textsubscript{H}2 response as the infection progresses (Malaquias \textit{et al.}, 1997).

The T\textsubscript{H}1 and T\textsubscript{H}2 cell subsets counter-regulate each in the course of schistosomiasis infection. This is observed from the levels of the various cytokines produced by each of the two sub-sets. The counter-regulation is mainly between IFN-\gamma and the other T\textsubscript{H}2 cytokines. Several previous studies have described the main cytokine players involved in the interaction
between the host and the parasite. Interleukin-4 is important in the orchestration of the T\textsubscript{H}2 responses, regulation of IgE production and counter-regulation of T\textsubscript{H}1 responses (De Vries \textit{et al.}, 1999). Interleukin-13 plays an important role in the regulation of IgE and has been shown to be the major fibrogenic cytokine in \textit{S. mansoni} infected mice. Interleukin-5 regulates eosinophils whose levels correlate with resistance to reinfestation. Interleukin-10 is important in modulating the immune responses during schistosomiasis thereby reducing potentially harmful parasite specific responses. Interferon-gamma is the most important T\textsubscript{H}1 cytokine whose role is to regulate and amplify T\textsubscript{H}1 responses. It is associated with the morbidity witnessed during schistosomiasis infection. It is also associated with resistance to infection seen in some few individuals living schistosomiasis endemic areas, usually referred to as ‘endemic normals’ Another morbidity associated cytokine is TNF-\textalpha (Joseph \textit{et al.}, 2004).

### 2.8 Morbidity in \textit{Schistosoma mansoni} infection

The major cause of morbidity in \textit{Schistosoma mansoni} infection is the host immune response to parasite eggs that become trapped in hepatic sinusoids. Granulomas form around the trapped eggs. This is mediated by CD4\textsuperscript{+} T cells and involves cellular infiltration, mainly of eosinophils, macrophages, fibroblasts and lymphocytes (Dunne \textit{et al.}, 1999). The granulomas resolve with death of eggs, leaving fibrotic plaques in the liver. It is the resulting fibrosis that often leads to portal hypertension. While granulomas themselves are pathogenic, they also serve a host-protective role. Mice tolerized against \textit{S. mansoni} egg antigens do not form granulomas after infection but suffer severe liver damage. The granulomas sequester egg-secreted toxins from hepatocytes. In addition, egg-antigen-specific antibodies may have a neutralising effect on the toxins (Fallon and Dunne, 1999).
Both T\textsubscript{H}1 and T\textsubscript{H}2 cytokines are associated with egg granuloma formation. In study that evaluated the cytokine profile in schistosomiasis patients developing hepatic fibrosis in prehepatosplenic and early hepatosplenic stages, T\textsubscript{H}2 cytokines IL-5, IL-10 and IL-13 cytokines were associated with hepatic fibrosis. IL-5 and IL-13 showed the strongest association with severe hepatic fibrosis (Ribeiro et al., 2004). Other studies have reported an association between low IFN-\(\gamma\) and TNF-\(\alpha\) (T\textsubscript{H}1 cytokines) with severe hepatic fibrosis (Henri et al., 2002; Booth et al., 2004). In another study involving severe combined immunodeficient mice, their injection with TNF-\(\alpha\) allowed the development of granulomatous lesions around eggs (Amiri et al., 1992). There is agreement from most studies including those involving mice about the protective role of IFN-\(\gamma\) in severe fibrosis of the liver (Hoffmann et al., 2000; Chiaramonte et al., 2001).

2.9 Chemotherapy and control of schistosomiasis

2.9.1 Praziquantel

Since the 1970s, effective, safe and simple drugs for the treatment of schistosomiasis have been available. Praziquantel is the most widely used and effective drug for the treatment of schistosomiasis. It is an acylated quinoline-pyrazine that is active against all schistosome species. It is commonly available in the market as 600 mg tablets. The recommended standard regimen is 40 mg/kg body weight in a single dose. It acts on the adult worm by paralysing it and damaging the tegument. It has mild side-effects which include nausea, vomiting, malaise and abdominal pain. It is safe for treatment of children and pregnant women. The drug however has little or no effect on eggs and immature worms. It is therefore recommended that follow-up treatment be done 4-6 weeks later. A single dose of the drug is nonetheless still quite effective since 70-100\% of patients cease to excrete eggs after treatment (Renganathan and Cioli, 1998; WHO, 2002; Dayan, 2003).
Treatment with Praziquantel is also effective in morbidity reduction in patients. Studies using clinical, radiographic, and sonographic observations have shown the regression over weeks to months of intestinal and vesical lesions, and reactive hepatomegaly. Additionally, while the cumulative fibrosis that occurs during infection is mostly irreversible, periportal fibrosis regresses following treatment with Praziquantel (Vennervald and Dunne, 2004; Singh et al., 2004).

After almost 30 years of intensive use, resistance to Praziquantel may be emerging. So far, many countries have implemented mass treatment programmes in schistosomiasis endemic areas using this drug. There are several reported cases of drug resistant *S. haematobium* and *S. mansoni* infections. Some of the cases were reported from regions of heavy exposure to Praziquantel in Kenya and Egypt (Ross et al., 2002). The drug however remains highly effective in many communities that have undergone multiple courses of treatment over a period of 10 or more years. It may take many years for resistance to Praziquantel to become an important clinical and public health issue. This is because worm reproduction in the mammalian host is sexual and the generation time is relatively long (Ross et al., 2002).

Praziquantel is also the drug of choice in the implementation of the World Health Assembly resolution 54.19 that calls for the regular treatment of at least 75% of all school-age children at risk of contracting schistosomiasis (WHO, 2001). Besides control of morbidity, treatment also seems to increase resistance to reinfection. In a study done in Kisumu, Kenya involving adult car washers occupationally exposed to schistosomiasis infection, some individuals in the cohort were highly susceptible to reinfection despite equal exposure at the beginning of the study. These individuals became increasingly resistant to reinfection after multiple subsequent retreatments after reinfections (Karanja et al., 2002). Other Studies on reinfection
rates after treatment indicate that adults in general have lower intensities of reinfection than children, even in situations where the level of exposure to the parasite is higher among adults than children (Kabaterine et al., 1999). It is also evident from epidemiological studies in areas of endemicity that adults suffer fewer infections compared to children, accounting for the level of exposure in the two groups. This implies that resistance to infection develops with age, manifesting near puberty years (Dunne and Mountford, 2001).

2.9.2 Other drugs for treatment of schistosomiasis

Oxamiquine is another drug that is effective in treatment of S. mansoni infection. Unlike Praziquantel, this drug is not effective against all species of Schistosoma. It also has more pronounced side-effects such as drowsiness, sleep induction and epileptic seizures. This drug has however limited availability in the market. Artemether, which is a well known anti-malaria drug, is also effective against immature stages of S. japonicum, S. mansoni, and possibly S. haematobium (Gryseels et al., 2006). It would therefore be a very effective drug for prophylaxis against schistosomiasis infection especially in workers involved in rescue operation during flooding. A combination of Praziquantel and artemether or some other derivative of artemisinin would be effective against all stages of the parasite in a human host (Fenwick et al., 2003). Widespread use of artemether in malaria-endemic areas for treatment of schistosomiasis is not recommended because it might lead to development of resistance in Plasmodium to the drug.

2.9.3 Control of schistosomiasis

Since the 1930s, many attempts have been made in different countries to control schistosomiasis. Most control efforts were based on chemical molluscicides for snail control
and use of drugs. Elaborate control programs have been run in countries such as china, Brazil and Egypt. The aims and strategies for schistosomiasis control have changed considerably in the past few decades. Transmission control aimed at the intermediate host has shifted to morbidity control especially since the introduction of Praziquantel in the market. This is because snail control using molluscicides is expensive, requiring substantial human and material resources. The chemicals are also toxic to other aquatic organisms. The method is however still used in Egypt and China (Gryseels et al., 2006)

It has been shown in Japan that schistosomiasis can be eliminated by behavioral changes, sanitation, and safe water supply. Public education to increase knowledge about the disease and how to avoid infection works well when combined with provision of safe water supplies and latrines (Gryseels et al., 2006). Several developing countries have realized a lot of success in preventing new infections and curing existing infections by combining treatment with socio-economic development (Fenwick et al., 2003).

World Health Assembly resolution 54.19, (WHA 54.19), forms the main component of most national schistosomiasis control programs. The resolution calls for member countries to ensure access to essential drugs against schistosomiasis in all health services in endemic areas for treatment of clinical cases and high risk groups such as children. The aim is to ensure that all school-age children in endemic areas are treated regularly during their childhood to improve their health, nutritional status and protection from serious morbidity. The resolution sets a target of administering chemotherapy to at least 75% of all school-age children at risk infection by the year 2010 (WHO, 2002). Several strategies have been applied to implement this resolution including indiscriminate mass treatment, active case finding and treatment of risk groups. Many sub-Saharan Africa countries face great challenges in implementing WHA 54.19. The main challenge is limitation in financial resources and prevalence of other more
serious diseases. Initiatives such as Partners for Parasite control Consortium, Schistosomiasis Control Initiative and Public-Private Partnerships supported by the Bill and Melinda Gates foundation are currently working to extend chemotherapy-based control of schistosomiasis in sub-Saharan Africa (Engels et al., 2002; Gryseels et al., 2006).

There are obvious and direct benefits of treatment of infected individuals. Adult worms are killed thus reducing exposure to morbidity-inducing eggs. It also changes host-parasite relationship, leading to augmented immunoregulation of morbidity. It is also beneficial in eliciting protective immunological responses, especially in adults (Colley and Secor, 2004). The mechanism by which treatment causes an increase in resistance to reinfection is not fully understood. Before treatment, schistosome worms live in the blood for many years, ranging from an average of three to five years, and some up to thirty years, all this time releasing antigens (van Dam et al., 1996). Some antigens are however concealed from the immune system until the death of the worm either naturally or by a drug like PZQ. The worm’s outer tegument is disrupted by PZQ thus exposing the underlying concealed antigens to the immune system consequently boosting anti-worm responses (Shaw et al., 1987). This is the possible explanation of the immunity to reinfection in adults and older children who are exposed to naturally dying worms, having harboured infections longer than younger children.

Several immune correlates of resistance have been described. A past study in a Kenyan population reported increased post-treatment worm-IgE which correlated with reinfection immunity (Webster et al., 1997). This implies a T\textsubscript{H}2 response involving larval killing affected by Eosinophils. Peripheral blood Eosinophilia has been reported as a correlate of immunity (Hagan et al., 1985). Other studies have reported production of IL-5 and IL-4 by PBMCs \textit{in vitro} in response to parasite antigens as correlates of resistance (Roberts et al., 1993). The T\textsubscript{H}1 cytokine IFN-\gamma has been linked to protection in ‘endemic normals’, uninfected
individuals living in endemic areas (Viana et al., 1994). Their PBMCs produce higher levels of parasite specific IFN-γ and lower levels of IL-10 than do infected individuals (Viana et al., 1994).

2.9.4 Schistosomiasis vaccine

The current schistosomiasis control strategy is based on the treatment of infected individuals and at risk groups in endemic areas by chemotherapy. The major challenge to achieving meaningful reduction in prevalence remains constant reinfection of individuals together with poor sanitary conditions prevailing in endemic areas especially in sub-Saharan Africa. There is also the concern of development of drug resistance by the parasite and the cost of implementing mass chemotherapy. There is therefore need for an effective vaccine against the disease, which together with chemotherapy would contribute enormously toward disease control. Schistosomula have been the primary source of the target antigens that are vaccine candidates. A high level of protection against S. mansoni infection has been attained in mice when immunised with irradiated with cercariae. Studies have shown that both T_{H}1 and T_{H}2 cell responses may contribute to protection (Capron et al., 2005; Oliveira et al., 2008; McManus and Loukas, 2008).

A lot of progress has been made in efforts to identify relevant schistosome antigens that may be involved in inducing protective immune responses (Oliveira et al., 2008; McManus and Loukas 2008 and Capron et al., 2005). The objective has been to develop a recombinant protein, synthetic-peptide or DNA vaccine. Sm 14 is one of the recombinant antigens that demonstrated protective properties. In a study involving mice challenged with 100 cercariae, Sm 14 was able to induce 67.9% of protection with Freud’s adjuvant and 64% without adjuvant. In the same study, other recombinant antigens that showed promising results
include Sm 22.9 and Sm 29 both of which were tried in mice (Oliveira et al., 2008). DNA-based vaccines hold promising prospects since they are able to induce both cellular and humoral immune responses. Administration of a DNA vaccine coding for three antigens, that is a 200 kDa glycosylphosphatidylinositol (GPI)-anchored surface protein, Sm 14, and IrV5 induced a 65% of protection. Use of synthetic peptides containing immunodominant epitopes of vaccine antigens constitutes another alternative strategy in vaccine development. Preliminary studies have demonstrated that immunodominant epitopes paramyosin and Sm 14 are able to induce levels of protection similar to those achieved by recombinant Sm 14 immunization (Oliveira et al., 2008; Ahmad et al., 2009).

Despite the discovery of numerous potentially promising vaccine candidate antigens from *S. mansoni* and to a less extent *S. haematobium*, only one vaccine has entered clinical trials. This is the *S. haematobium* BILHVAX, or the 28-kDa GST. Findings from numerous longitudinal studies of reinfection rates following curative treatment of people living in schistosomiasis-endemic areas indicate that some of these people acquire a form of protective immunity after years of exposure. These findings have served to encourage efforts to develop anti-schistosome vaccine (Oliveira et al., 2008).

Immune correlative studies suggest that acquired antischistosome protective immunity after curative chemotherapy is mediated largely by a Th2 response, orchestrated by IgE and eosinophils. Along with the high levels of protective IgE are also high levels of IgG4. Immunoglobulin G4 is thought to block the protective effects of other immunoglobulins and in most studies it correlates with susceptibility to infection. Immunity in schistosomiasis therefore depends on IgE/IgG4 balance and which in turn depends on IL-4 and IL-13 produced by Th2 cells. Production of IgG4 is also regulated by IL-10 and transforming growth factor (TGF-β) (Capron et al., 2005; Oliveira et al., 2008; Ahmad et al., 2009).
Studies involving mice immunised with irradiated cercariae have shown that protection is mediated by activated macrophages and T\textsubscript{H}1 cytokines IFN-γ and IL-2. Protection in people living in endemic areas and who remain uninfected despite exposure to infection is attributed to T\textsubscript{H}1 cytokines. An effective vaccine against schistosomiasis should therefore elicit a mixed T\textsubscript{H}1/T\textsubscript{H}2 response (McManus and Loukas, 2008; Oliveira et al., 2008).
CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design
This is a cohort study that involved recruitment of two groups of 8 to 10 year old *Schistosoma mansoni* infected school children who were followed up longitudinally for 2 years. One group designated arm A (n=88) was diagnosed, specimens collected and treated where necessary, 3 times a year, once in each term of the school year. The other group designated arm B (n=91) underwent the process of diagnosis, specimen collection and treatment at only two time points: beginning of the study and after 24 months which was the end of the study. Specimens collected were processed and analysed in the research laboratory. Stool samples were analysed for the presence and number of eggs per gram (epg) of faeces. Part of the blood sample collected was used to check for the presence of malaria parasite while the rest was used for culturing with schistosome parasite antigens and supernatants harvested and frozen to be assayed later for cytokines IFN-γ, IL-5, IL-10 and IL-13. Egg and cytokine data was later processed by Microsoft Office Excel 2007 and analysed by Graph Pad Prism statistical software version 4.

3.2 Study area
This study was conducted in western Kenya, in the Asembo Bay area, Rarieda District of Nyanza province. This area borders Lake Victoria and is highly endemic for schistosomiasis (prevalence of 16.3% in school children), according to a previous study (Handzel *et al.*, 2003). Samples were processed, stored and analysed at the Schistosomiasis laboratory located at the Kenya Medical Research Institute’s Centre for Global Health Research, Kisumu.
3.3 Study subjects

The subjects were school children, both male and female, between the ages of 8 to 10 years and attending primary schools within 3 km of Lake Victoria and with high schistosomiasis mansoni prevalence (>50%) (Handzel et al., 2003).

3.3 Inclusion criteria

Subjects enrolled were both male and female 8 to 10 years old school children. They were attending primary schools in the Asembo Bay area that are within 3 kilometres of Lake Victoria shoreline. They were egg-positive for *Schistosoma mansoni* upon diagnosis by stool examination. They were also willing to participate in the study and informed consent given by the guardian.

3.4 Exclusion criteria

Persons were excluded from the study if they:

- had haemoglobin values below 8gHb/dl and were evidently ill according to the assessment of the clinician,
- were unwilling to participate in the study or their parents declined to give consent,
- were not residing in the area,
- were outside the 8 to 10 year bracket.
3.5 Sample size determination

Sample size was determined using Epi Info statistical software, with the following parameters:

- Standard error of 1.96
- Prevalence of 16.3%
- At power = 80%

This gave n as 64

The assumption was that approximately 5% will not consent and about 10% migration per year, the number was adjusted to a minimum total of 179 subjects at the beginning. One hundred and eighty subjects were enrolled in each arm for screening considering the fact an initial survey had revealed a mean prevalence of schistosomiasis in pupils in each of the selected schools was about 45%. In total therefore, 360 children were enrolled for the two study arms. After screening and consenting, 88 subjects were recruited in the multiple follow-up group while 91 subjects were recruited in the single follow-up group.

3.5 Ethical considerations

This study was reviewed and approved by the Ethical Review Committee of the Kenya Medical Research Institute.

3.6 Informed consent process

Since the study involved school children, relevant ministry of education offices from the district level were consulted for permission. Permission was also sought from head teachers of participating schools. Both the parents/guardians and their children signed the consent and
assent forms respectively before witnesses (Appendix II). The forms were translated into the local language, Dholuo for parents who do not understand English (Appendix II).

3.7 Specimen collection and handling
The specimens from this study were standard blood and stool specimens. Blood collection was done aseptically and specimens handled using universal precautions. Approximately 6 ml of blood was collected by venipuncture into vacutainer tubes with heparin. Each specimen was labelled with the assigned subject number and date. They were then put in Styrofoam containers and transported to the Schistosomiasis Laboratory at KEMRI’s Centre for Global Health Research, Kisumu. The stool specimens were examined for the presence of Schistosome eggs while blood samples examined for malaria and the rest of the blood was cultured with parasite antigens and assayed for cytokine responses.

3.8 Laboratory procedures

3.8.1 Parasitology
Stools were examined for helminth eggs by standard Kato/Katz smear (Katz et al., 1972; WHO, 1994; Handzel et al., 2003) using three slides per stool. This technique diagnosed *S. mansoni, Ascaris lumbricoides*, hookworm, and *Trichuris trichuria*.

3.8.2 Whole blood culture
Whole blood culture was done using 6 ml of heparinised blood diluted to a ratio of 1:5 in RPMI-1640 containing penicillin-streptomycin and L-glutamine. The blood was then cultured in 24-well microtitre plates, 1.5 ml per culture in the presence of the mitogen phytohemagglutinin to determine responsiveness of immune cells in culture, schistosome
antigens (soluble worm antigen preparation-SWAP and soluble egg antigen-SEA), and RPMI. After 5 days of culture, the supernatant fluids were separately harvested and stored frozen (-20 °C) (Appendix I) until assayed by cytokine capture ELISAs.

3.8.3 Cytokine ELISA

Assays for cytokines were done by capture ELISA using commercial cytokine-specific monoclonal antibody pairs. The cytokines assayed from the frozen culture supernatant fluids were primarily IL-5, IL-10, IL-13 and IFN-γ. This was done as previously described (Mwinzi et al., 2001; Appendix I), with modifications. Ninety six-well ELISA plates were coated with 100 µl of a given monoclonal capture antibody diluted in PBS and incubated overnight at room temperature. They were then washed thrice (ELx405 Microplate washer, Biotek) with 0.05% Tween 20 (vol/vol), and blocked with PBS plus 10% BSA at 300 µl per well for 1 hour. The plates were then washed thrice and cytokine standards (R and D systems), serially diluted as per manufacturer’s directions in respective reagent diluents and samples added at 100 µl per well followed by 2 hour incubation at room temperature.

The plates were then washed thrice with the same wash buffer and working concentration of mAB was added at 100 µl per well for another 2 hour incubation at room temperature. This was followed by another three times wash before addition of Streptavidin-horseradish peroxidise conjugate (R and D Systems) diluted in respective reagent diluents and added at 100 µl per well. This was followed by 20 minute incubation at room temperature before another round of 3 washes. Tetramethylbenzidine peroxidise substrate (TMB) was added at 100 µl per well and colour developed at room temperature. After development of colour (about 15 minutes), stop solution (1N sulphuric acid) was added at 50 µl per well. The optical density of each well correlated to cytokine quantity and was determined immediately using
an automated microplate reader at 450 nm. IFN-γ Reagent diluent was 1xPBS+10% BSA while reagent diluent for IL-5, IL-10 and IL13 was Trizma base+1% tween20 +1% BSA (Appendix I).

3.8.4 Data analysis

Data was processed first by Microsoft Office Excel 2007 and further analysis was done using Graph Pad Prism 4 statistical software. Kruskall-Wallis test and Mann-Whitney U-test were used to assess differences in the intensity of infection in eggs per gram of faeces and cytokine production between arm A and arm B children and between different time points. The non-parametric tests were used since the cytokine data was not normally distributed. The spearman rank correlation test was used to analyse the relationship between intensity of infection and cytokine production. Results were considered significant at P<0.05.

Attempts were made to determine commonalities in immune profiles in subjects showing a trend of fewer incidences of reinfections and or low infection intensities in eggs per gram. Children who were reported to have had no water contact were excluded from this analysis since lack of water contact would be a confounder. It was therefore safe to conclude that significant reductions in the prevalence of the disease in the children will be due to treatment and resulting immune profiles that may mirror those seen in resistant adults. Comparisons were made between study Arm A and Arm B to determine which treatment regime is best not only in morbidity reduction but also in eliciting immune responses that may confer a level of resistance to reinfection.
CHAPTER FOUR: RESULTS

4.1 Overview

A total of 179 8 to 10 year-old *Schistosoma mansoni* infected school children were enrolled for this study. They were then randomly assigned into two parallel groups designated arm A with 88 children and arm B with 91 children. Children in study arm A were diagnosed and treated at baseline, and followed-up for diagnosis and treatment if positive after every four months throughout the two-year study period. Children in arm B were diagnosed and treated at the beginning of the study and after 2 years. A total 23 children in arm A missed at least one follow-up or dropped out entirely from the study while only 45 children in arm B were available for the final follow-up. Stool and blood samples were collected from the children at the baseline and at each follow-up time point. Infection intensity in eggs per gram (EPG) of faeces was determined while cytokine response was determined from supernatants harvested following whole blood culture. Background cytokine production by unstimulated cells was determined from blood cultured in media alone while responsiveness of lymphocytes was determined by culturing whole blood in phytohemagglutinin (PHA). Specific cytokine responses were determined by assaying supernatant from blood cultured with Schistosome egg (Soluble egg antigen; SEA) and worm antigens (Soluble worm antigen preparation; SWAP). Assays were done for IFN-\(\gamma\), IL-5, IL-10 and IL-13.

4.2 Infection intensity of the children at the beginning of the study

Cytokine and infection intensity (in eggs per gram of faeces, epg) data was available for a combined total of 113 children from both the multiple follow-up group (study arm A) and the single follow-up group (Study arm B) at baseline and 108 at the last follow-up. Infection intensity ranged from 12-2088 epg. The mean infection intensity was 217.8 and 43.22 epg of faeces at baseline and last follow-up respectively (Table 4.1).
Table 4.1: Infection intensity (EPG) summary statistics at baseline and at the 2-year follow-up for children all children in the study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline EPG of faeces</th>
<th>2-year follow-up EPG of faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of children</td>
<td>113</td>
<td>108</td>
</tr>
<tr>
<td>Minimum</td>
<td>12.00</td>
<td>0.0</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>36.00</td>
<td>0.0</td>
</tr>
<tr>
<td>Median</td>
<td>84.00</td>
<td>0.0</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>228.0</td>
<td>24.00</td>
</tr>
<tr>
<td>Maximum</td>
<td>2088</td>
<td>828.0</td>
</tr>
<tr>
<td>Mean</td>
<td>217.8</td>
<td>43.22</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>348.6</td>
<td>126.1</td>
</tr>
</tbody>
</table>

For purposes of this analysis, all the children were divided into three groups based on the intensity of their infection: a low infection intensity group, with less than 100 eggs per gram of faeces (1-99 epg), a medium infection intensity group, who had between 100 and 399 eggs per gram of faeces (100-399 epg) and a high intensity infection group which comprises children with 400 and above eggs per gram (≥400 epg). From the baseline egg count, 64 children were in the low infection intensity group, 31 had medium infection intensity and 13 had high infection intensity. A total of 108 children gave their blood and stool samples at the 2-year follow-up, which was the 6th follow-up for the group that had multiple follow-ups (arm A) and the first follow-up for the single follow-up group (arm B). Parasitological data at the last follow-up indicated a total of 37 re-infections among all the children in the study. Out of these, 18 were from the multiple follow-up group and 19 were from the single follow-up group. In terms of infection intensity, 26 children had low infection intensity, 7 had medium infection intensity and 4 had high infection intensity (Table 4.2).
Table 4.2: Number of children with low infection intensity, medium infection intensity and high infection intensity (in eggs per gram of faeces) at baseline and at last follow-up

<table>
<thead>
<tr>
<th>Infection intensity group</th>
<th>Number of children at baseline</th>
<th>Number of children at last follow-up (after 2 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low intensity (1-99 epg)</td>
<td>64</td>
<td>26</td>
</tr>
<tr>
<td>Medium intensity (100-399 epg)</td>
<td>31</td>
<td>7</td>
</tr>
<tr>
<td>High intensity (≥400 epg)</td>
<td>18</td>
<td>4</td>
</tr>
</tbody>
</table>

The number of re-infections in the multiple follow-up group (arm A) were considered for the entire study period. Out of the 68 children in this group that were followed up throughout the study period, 10 (14.7%) remained un-infected. Twenty children were re-infected once, 27 were re-infected thrice and 11 children were re-infected more than three times. Fifty eight children in this group therefore had at least two treatments during this study period. Despite this repeated treatment following re-infection, 18 children in this group were re-infected at the end of the 24 month follow-up. This is almost similar to the number of children re-infected in the single follow-up group (arm B), in which 19 (42.2%) out of 45 were infected at 2 years. Repeated treatment for two the years therefore did not lead to reduced re-infection prevalence levels compared to the group treated only once. In fact more children (57.8%) remained un-infected in the single follow-up group with one treatment compared to 14.7% in the multiple follow-up group (Table 4.3)
Table 4.3: Percentages of children re-infected and those not re-infected in the multiple follow-up group (arm A) and in the single follow-up group (arm B)

<table>
<thead>
<tr>
<th>Follow-up group</th>
<th>Children not re-infected (%)</th>
<th>Children re-infected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple follow-up</td>
<td>14.7</td>
<td>85.3</td>
</tr>
<tr>
<td>Single follow-up</td>
<td>57.8</td>
<td>42.2</td>
</tr>
</tbody>
</table>

4.3 Cytokine levels at baseline and at the last follow-up

Cytokine levels were determined from supernatants harvested from whole blood which had been cultured with SEA and SWAP. IFN-γ was detected in picograms per millilitre. The IFN-γ levels from many children samples were undetectable. For the low infection intensity group, the median IFN-γ levels were zero for both SEA and SWAP stimulated samples at baseline and at the final follow-up at 2 years. Mean levels of the cytokine from SEA and SWAP stimulated samples were slightly higher, at 3.978pg/ml and 5.155pg/ml respectively compared to 2.177pg/ml and 0.617pg/ml respectively. IL-10 was the cytokine with the highest mean and median levels from both SEA and SWAP stimulated blood samples. Its baseline means were 154.3pg/ml and 208.0pg/ml and medians 94.13pg/ml and 133.2pg/ml from SEA and SWAP stimulated samples respectively. Baseline mean and median cytokine levels were generally higher than those for the 24 months follow-up. The few exceptions include IL-13 mean and median for SWAP stimulated samples where levels of the cytokine from the last bleed were higher than baseline levels (Table 4.4).
Table 4.4: Cytokine (IFN-γ, IL-5, IL-10 and IL-13) levels for the low infection intensity group from both SEA and SWAP stimulated whole blood samples

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Baseline</th>
<th>Last Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SEA</td>
<td>SWAP</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>3.978</td>
<td>0.0</td>
</tr>
<tr>
<td>IL-5 (ng/ml)</td>
<td>86.03</td>
<td>1.15</td>
</tr>
<tr>
<td>IL-10 (ng/ml)</td>
<td>154.3</td>
<td>94.13</td>
</tr>
<tr>
<td>IL-13 (ng/ml)</td>
<td>22.72</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The medium infection intensity group had the lowest mean and median IFN-γ responses in both SEA and SWAP stimulated cultures, with most samples producing barely detectable levels. Just like the low infection intensity group, IL-10 in this group had the highest mean and median levels from both SEA and SWAP stimulated samples at baseline and at 24 months. Interleukin-5 and IL-13 were produced in comparatively similar amounts for both SEA and SWAP stimulated whole blood samples. Baseline cytokine levels were generally higher than levels at the last bleed for all cytokines and for both SEA and SWAP stimulated blood samples (Table 4.5). The high infection intensity group recorded the highest levels for all the cytokines from both SEA and SWAP stimulated samples. The highest mean and median cytokine levels for this group were for SWAP stimulated IL-10 at baseline, which were 539.4ng/ml and 351.6ng/ml respectively. Interleukin-5 was the second most responsive cytokine, followed by IL-13 and lastly IFN-γ. It was also noted that baseline cytokine levels were higher than those produced at the 24 months for this infection group. Soluble worm antigen preparation stimulated samples yielded higher cytokine levels than those stimulated by SEA in this infection group just as was the case in the other infection groups (Tables 4.4, 4.5 and 4.6).
Table 4.5: Cytokine (IFN-γ, IL-5, IL-10 and IL-13) levels for the medium infection intensity group from both SEA and SWAP stimulated whole blood samples

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Baseline</th>
<th>Last Bleed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SEA</td>
<td>SWAP</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>0.4608</td>
<td>0.0</td>
</tr>
<tr>
<td>IL-5 (ng/ml)</td>
<td>8.676</td>
<td>0.0</td>
</tr>
<tr>
<td>IL-10 (ng/ml)</td>
<td>86.42</td>
<td>22.99</td>
</tr>
<tr>
<td>IL-13 (ng/ml)</td>
<td>13.15</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 4.6: Cytokine (IFN-γ, IL-5, IL-10 and IL-13) levels for the high infection intensity group from both SEA and SWAP stimulated whole blood samples

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Baseline</th>
<th>Last Bleed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SEA</td>
<td>SWAP</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>28.32</td>
<td>0.0</td>
</tr>
<tr>
<td>IL-5 (ng/ml)</td>
<td>41.67</td>
<td>0.8965</td>
</tr>
<tr>
<td>IL-10 (ng/ml)</td>
<td>151.8</td>
<td>115.8</td>
</tr>
<tr>
<td>IL-13 (ng/ml)</td>
<td>50.35</td>
<td>15.35</td>
</tr>
</tbody>
</table>

4.4 Correlation between infection intensity in eggs per gram of faeces and cytokine levels.
Correlation was done between cytokine levels from SEA and SWAP stimulated blood samples in picograms for IFN-γ and nanograms for IL-5, IL-10 and IL-13 and infection intensity in eggs per gram of faeces using Spearmans Rank Correlation coefficient test. This
analysis was done separately for each of the infection intensity groups, that is, the low, the medium and the high infection intensity groups for both baseline and at 24months/2 years.

4.4.1 Correlation between IFN-γ and infection intensity

Interferon gamma (IFN-γ) levels were generally very low for both SEA and SWAP stimulated whole blood samples. Samples from most children did not produce detectable levels of the cytokine. As a result, no correlation could be done for SEA stimulated samples in the last follow-up of the medium and the high intensity infection group (Table 4.7). Soluble worm antigen preparation (SWAP) stimulated IFN-γ levels for the high intensity infection group, last follow-up gave the highest Correlation coefficient (r= 0.7746), although this was not significant (P>0.05). Levels of the cytokine for the low intensity infection group correlated negatively with infection intensity except for SEA stimulated baseline cytokine levels. None of these correlations for IFN-γ were significant (P>0.05; Table 4.7).
Table 4.7: Correlation analysis between IFN-\(\gamma\) levels and *S. mansoni* infection intensity in children with low, medium and high infection intensity

<table>
<thead>
<tr>
<th>Antigen type</th>
<th>Correlation coefficient (r) and P value</th>
<th>Low infection intensity (1-99 epg)</th>
<th>Medium infection intensity (100-399 epg)</th>
<th>High infection intensity (≥400 epg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline Last bleed</td>
<td>Baseline Last bleed</td>
<td>Baseline Last bleed</td>
</tr>
<tr>
<td>SEA</td>
<td>r</td>
<td>0.1157 -0.1754 0.1086</td>
<td>0.1418</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.3745 0.3914 0.5680</td>
<td>-</td>
<td>0.5746 -</td>
</tr>
<tr>
<td>SWAP</td>
<td>r</td>
<td>-0.1218 0.02035 0.2639</td>
<td>-</td>
<td>0.3976 0.7746</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.3458 0.9214 0.5680</td>
<td>-</td>
<td>0.1023 0.333</td>
</tr>
</tbody>
</table>

r= Spearman rank correlation co-efficient

Significance at P<0.05 (two tailed)

4.4.2 Correlation between IL-5 and infection intensity

There was a very weak correlation between IL-5 levels from both SEA and SWAP stimulated whole blood samples in all the infection categories. The low intensity infection group recorded the lowest correlation coefficients of 0.00901 and 0.058 for baseline SEA and SWAP levels respectively (P>0.05). Correlation coefficients for the last follow-up in this category were negative just like those for the high intensity infection group, last follow-up Table 4.8). The medium intensity infection group (last follow-up) gave the highest positive correlation coefficient, (r=0.6487) but this was not significant (P0.1389) from SWAP stimulated whole blood samples. The r value of -0.9487 for the high intensity infection group
from SEA stimulated whole blood samples was the highest negative correlation observed though not significant (P>0.05; Table 4.8).

**Table 4.8: Correlation analysis between IL-5 levels and *S. mansoni* infection intensity in children with low, medium and high infection intensity**

<table>
<thead>
<tr>
<th>Antigen type</th>
<th>Correlation coefficient (r) and P value</th>
<th>Low infection intensity (1-99 epg)</th>
<th>Medium infection intensity (100-399 epg)</th>
<th>High infection intensity (≥400 epg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>Last bleed</td>
<td>Baseline</td>
</tr>
<tr>
<td>SEA</td>
<td>r</td>
<td>0.009012 (n=60)</td>
<td>-0.0662 (n=25)</td>
<td>0.1255 (n=30)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.9455</td>
<td>0.7531</td>
<td>0.5086</td>
</tr>
<tr>
<td>SWAP</td>
<td>r</td>
<td>0.05799 (n=60)</td>
<td>-0.1933 (n=25)</td>
<td>0.1981 (n=30)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.6599</td>
<td>0.3545</td>
<td>0.2939</td>
</tr>
</tbody>
</table>

r= Spearman rank correlation co-efficient

Significance at P<0.05 (two tailed)

**4.4.3 Correlation between IL-10 and infection intensity**

The low intensity infection group recorded the lowest correlation coefficient for IL-10 just like what was observed with IL-5. The r values were -0.04464 and -0.05931 at baseline for whole blood stimulated with SEA and SWAP respectively (Table 4.9). Another reflection of what was observed with IL-5 is that the highest cytokine-infection intensity correlation coefficient was in the medium infection intensity group. Soluble worm antigen preparation (SWAP) stimulated whole blood samples for this group gave a positive correlation although
not significant \(r=0.7042; P>0.05; \text{Table 4.9}\). Soluble worm antigen preparation (SWAP) stimulated whole blood samples for the high infection intensity at the last follow-up did not yield detectable cytokine levels and therefore no correlation test could be done for this group.

**Table 4.9: Correlation analysis between IL-10 levels and *S. mansoni* infection intensity in children with low, medium and high infection intensity**

<table>
<thead>
<tr>
<th>Antigen type</th>
<th>Correlation coefficient ((r)) and (P) value</th>
<th>Low infection intensity ((1-99\ epg))</th>
<th>Medium infection intensity ((100-399\ epg))</th>
<th>High infection intensity ((\geq 400\ epg))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Last bleed</td>
<td>Baseline Last bleed</td>
<td>Baseline Last bleed</td>
<td>Baseline Last bleed</td>
</tr>
<tr>
<td>SEA</td>
<td>(r)</td>
<td>-0.04464 (n=59) 0.03025 (n=37)</td>
<td>0.06339 (n=30) 0.3706 (n=7)</td>
<td>-0.2739 (n=18) 0.4000 (n=4)</td>
</tr>
<tr>
<td></td>
<td>(P)</td>
<td>0.7371 0.4092</td>
<td>0.7393 0.3956</td>
<td>0.2874 0.7500</td>
</tr>
<tr>
<td>SWAP</td>
<td>(r)</td>
<td>-0.05931 (n=59) -0.0383 (n=26)</td>
<td>0.1121 (n=30) 0.7042 (n=7)</td>
<td>0.1997 (n=18) -</td>
</tr>
<tr>
<td></td>
<td>(P)</td>
<td>0.6555 0.8525</td>
<td>0.5553 0.0881</td>
<td>0.4269 -</td>
</tr>
</tbody>
</table>

\(r=\) Spearman rank correlation co-efficient

Significance at \(P<0.05\) (two tailed)

**4.4.4 Correlation between IL-13 and infection intensity**

In contrast to IL-10, the low intensity infection group gave a fairly strong correlation between IL-13 levels and infection intensity. During the last follow-up, SEA stimulated whole blood samples for this group gave a correlation coefficient which was significant \(r=0.4368; P<0.05; \text{Table 4.10}\). The medium infection intensity group had a weak correlation for SWAP
stimulated whole blood samples (r= -0.036 and 0.089 for baseline and the last follow-up respectively). The high intensity infection group gave negative IL-13 versus infection intensity correlation except for SWAP stimulated whole blood samples at the last follow-up, but correlation was not significant (r=0.3162; P>0.05; Table 4.10).

Table 4.10: Correlation analysis between IL-13 levels and S. mansoni infection intensity in children with low, medium and high infection intensity

<table>
<thead>
<tr>
<th>Antigen type</th>
<th>Correlation coefficient (r) and P value</th>
<th>Low infection intensity (1-99 epg)</th>
<th>Medium infection intensity (100-399 epg)</th>
<th>High infection intensity (≥400 epg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline Last bleed</td>
<td>Baseline Last bleed</td>
<td>Baseline Last bleed</td>
</tr>
<tr>
<td>SEA</td>
<td>r</td>
<td>0.1862 0.4368</td>
<td>-0.03631 0.08909</td>
<td>-0.04361 -0.7746</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=60) (n=26)</td>
<td>(n=30)</td>
<td>(n=18) (n=4)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.1544 0.0257*</td>
<td>0.8489 0.8397</td>
<td>0.8636 0.333</td>
</tr>
<tr>
<td>SWAP</td>
<td>r</td>
<td>0.1581 -0.0841</td>
<td>0.2132 0.5345</td>
<td>-0.4490 0.3162</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=61) (n=26)</td>
<td>(n=30)</td>
<td>(n=18) (n=4)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.2238 0.6829</td>
<td>0.2580 0.2357</td>
<td>0.0616 0.7500</td>
</tr>
</tbody>
</table>

r= Spearman rank correlation co-efficient

Significance at P<0.05 (two tailed)

*Significant correlations in bold
4.5 Effect of multiple treatments after re-infection on cytokine levels

Among the 68 children followed up during the two year study period, 10 remained negative when their stools were tested for the presence of *S. mansoni* eggs. These ten therefore had only one treatment with Praziquantel (PZQ) during the entire study period. Twenty children were re-infected once at different time-points during the study period and therefore had 2 PZQ treatments. Twenty seven children were treated thrice following two re-infections and 11 children had more than four treatments (Table 4.11) Cytokine levels in each of the four categories, namely children with a single treatment, two treatments, three treatments and four or more treatments were analysed separately. This was to determine the effect if any of number of treatments on cytokine levels for each of the four cytokines.

<table>
<thead>
<tr>
<th>Percentage of children</th>
<th>Re-infection incidences</th>
<th>Number of treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>29.4</td>
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<tr>
<td>16.2</td>
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**Table 4.11: Percentages of children and their re-infection incidences**

4.5.1 Interferon-gamma

IFN-γ produced from SEA stimulated whole blood was generally very low, with most samples not yielding enough levels to be detected. There was no significant change in IFN-γ levels after treatment in the one treatment group (P>0.05). The post-treatment levels were however slightly lower than baseline levels (Fig. 4.1). The situation was however the reverse for children with two treatments and for those with three treatments. Interferon-gamma (IFN-γ) produced from SEA stimulated blood samples increased after the first treatment for
children with two treatments and after the second treatment for children with three treatments. However, the increase was not significant (P>0.05; Fig 4.2 and 4.3).

Figure 4.1: Change in IFN-γ in children who had only 1 treatment when their whole blood samples were stimulated with soluble egg antigen (SEA). Levels of IFN-γ are expressed in picograms per millilitre, error bars represent standard error of the mean. Comparison of medians between baseline and after the first treatment using Mann Whitney test did not give a significant result (P>0.05)
Figure 4.2: Change in IFN-γ produced by soluble egg antigen (SEA) stimulated whole blood samples in children who had two treatments. Interferon-gamma (IFN-γ) levels are expressed in pictograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when medians at baseline, after the first and second treatment were compared using Kruskall-Wallis test (P>0.05).
Figure 4.3: Change in IFN-γ produced by soluble egg antigen (SEA) stimulated whole blood samples in children who had three treatments. Interferon-gamma (IFN-γ) levels are expressed in pictograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when medians at baseline, after the first, second and third treatment were compared using Kruskall-Wallis test (P>0.05).

Slightly higher levels of IFN-γ were detected in SWAP stimulated whole blood samples compared to SEA stimulated whole blood samples. Like IFN-γ from SEA stimulated whole blood samples, post-treatment IFN-γ levels from SWAP stimulated whole blood samples were lower than baseline levels in children with only one treatment. A comparison of their medians at baseline and after first treatment did not give a significant result (P>0.05; figure 4.4).
Figure 4.4: Change in IFN-γ produced by soluble worm antigen (SWAP) stimulated whole blood samples in children who had two treatments. Interferon-gamma (IFN-γ) levels are expressed in pictograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when medians at baseline and after the first treatment were compared using Mann Whitney test (P>0.05).

Samples from groups with two treatments and above had a general trend where IFN-γ levels increased with each successive treatment from baseline and peaked at the second treatment before dropping again. This trend is well typified by the three treatment group whose medians at baseline and after successive treatments did not however differ significantly (P>0.05; figure 4.5).
**Figure 4.5: Change in IFN-γ produced by soluble worm antigen (SWAP) stimulated whole blood samples in children who had three treatments.** Interferon-gamma (IFN-γ) levels are expressed in picograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when medians at baseline, after the first, second and third treatment were compared using Kruskal-Wallis test (P>0.05)

A comparison was also made between baseline and 24 months follow-up IFN-γ levels for children in three treatment groups: one treatment, two treatment and three to five treatment groups. This was done to determine which treatment group, if any, had the greatest change in cytokine levels by the end of the study. For SEA stimulated whole blood samples, baseline cytokine levels were higher than levels at the last follow-up for the single treatment and the 3-5 treatment groups. Last follow-up mean cytokine level for the single treatment group was undetectable. The group that underwent 2 treatment had the highest IFN-γ production (Fig 4.6). Its baseline mean was 0.91±0.49 SEM pg (Fig4.6). None of the three groups gave a significant difference between baseline and 24 months IFN-γ medians (P>0.05; Fig 4.6).
Figure 4.6: Comparison of baseline and 24 months IFN-γ levels from SEA stimulated whole blood samples for children with 1, 2 and 3-5 treatments. Interferon-gamma (IFN-γ) levels are expressed in pictograms per millilitre; error bars represent standard errors of the mean. There was no significant difference when baseline and 24 months medians for the single treatment, two treatment and three to five treatment groups were each compared using Mann Whitney test (P>0.05)

Interferon-gamma (IFN-γ) levels from SWAP stimulated whole blood samples at baseline for the three treatment groups were higher than last follow-up levels. The two treatment group had the highest last follow-up mean of 3.30±2.27pg compared to 0.94±0.52pg for the 3-5 treatment group and less than 0.1pg for the single treatment group. Baseline and last follow-up IFN-γ medians for each of the three treatment groups were not significantly different (P>0.05; Fig 4.7).
Figure 4.7: Comparison of baseline and last follow-up IFN-γ levels from SWAP stimulated whole blood samples for children with 1, 2 and 3-5 treatments. Interferon-gamma (IFN-γ) levels are expressed in picograms per millilitre; error bars represent standard errors of the mean. There was no significant difference when baseline and last follow-up medians for the single treatment, two treatment and three to five treatment groups were each compared using Mann Whitney test (P>0.05)

4.5.2 Interleukin-5

There was a general increase in IL-5 SEA samples after the first treatment for children with 1 treatment only and for those with two treatments. This increase was however not significant (P>0.05). Cytokine levels then dropped after the second treatment for children who had two treatments and after the third treatment for those who had three treatments (Fig 4.8)
Figure 4.8: Change in IL-5 from SEA stimulated whole blood samples after successive treatments in children with 2 treatments. Interleukin-5 levels are expressed in nanograms per millilitre, error bars represent standard error of the mean. The IL-5 medians at baseline, after the first and second treatments were compared by Kruskal-Wallis test. The medians were not significantly different (P>0.05)

IL-5 responses from SWAP stimulated whole blood samples were relatively higher than responses to SEA. Levels of the cytokine were highest before treatment for all categories of children except for those who had more than four treatments. The levels then declined after the first treatment though not significantly except for children who had two treatments whose IL-5 (SWAP) levels at baseline and after first treatment were significantly different (P<0.05; figure 4.10). children who had more than four treatments had low baseline IL-5 levels which then marginally increased after the first treatment, peaking after the second treatment and declining thereafter (Fig 4.11)
Figure 4.9: Change in IL-5 from SEA stimulated whole blood samples after successive treatments in children who had 3 treatments. Interleukin-5 levels are expressed in nanograms per millilitre, error bars represent standard error of the mean. The IL-5 medians at baseline, after the first, second and third treatments were compared by Kruskal-Wallis test. The medians were not significantly different (P>0.05).

Figure 4.10: Change in IL-5 from SWAP stimulated whole blood samples after successive treatments in children who had 2 treatments. Interleukin-5 levels are expressed in nanograms per millilitre, error bars represent standard error of the mean. Baseline IL-5 median was compared with median levels after first and second treatment respectively using Mann Whitney test. There was a significant change in IL-5 median between baseline and levels after first treatment (*P<0.05).
Figure 4.11: Change in IL-5 from SWAP stimulated whole blood samples after successive treatments in children who had 5 treatments. Interleukin-5 levels are expressed in nanograms per millilitre, error bars represent standard error of the mean. There was no significant difference when medians at baseline and all treatment groups were compared using Kruskall-Wallis (P>0.05). Comparison was done between baseline medians and medians for each of the treatment groups using Mann Whitney test. No comparison gave a significant result (P>0.05)

Baseline and last follow-up IL-5 levels were also compared for the single treatment, two treatment and 3-5 treatment groups for both SEA and SWAP stimulated whole blood samples. For SEA stimulated samples, the single treatment group had a slight difference in mean between baseline and last follow-up cytokine levels, 10.95±7.52 ng and 15.24±12.85 ng respectively. The two treatment group had the greatest difference in mean cytokine levels between baseline and last follow-up: 12.86±8.57 ng and 72.38±58.10 ng for baseline and last
follow-up respectively (Fig 4.12). The difference for the 3-5 treatment group was the reverse of what was observed in the two previous groups with baseline cytokine levels being higher than last follow-up levels (36.67±17.61 ng and 12.38±6.19 ng respectively). Baseline and last follow-up medians for the single, two and three treatment groups were not significantly different, with (P>0.05; Fig 4.12)

![Bar chart showing mean IL-5 levels](image)

**Figure 4.12: Comparison of baseline and 24 months IL-5 levels from SEA stimulated whole blood samples for children with 1, 2 and 3-5 treatments.** Interleukin-5 (IL-5) levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when baseline and 24 months medians for the single treatment, two treatment and three to five treatment groups were each compared using Mann Whitney test (P>0.05)

Consideration of baseline and 24 months IL-5 levels from SWAP stimulated whole blood samples for the single, two and three to five treatment groups gave a different picture from the SEA stimulated levels. The highest cytokine levels were for baseline in the single treatment group with a mean of 420.57±166.86 ng. Levels of the cytokine at 24 months for
the same group had a mean of 130.29±73.14 ng. The other treatment groups had mean cytokine levels not exceeding 250ng and with narrower differences in means between baseline and 24 months levels (Fig 4.13). Differences in baseline and 24 months medians for the three treatment groups were not significant (P>0.05; Fig 4.13)

![Figure 4.13: Comparison of baseline and 24 months IL-5 levels from SWAP stimulated whole blood samples for children with 1, 2 and 3-5 treatments. Interleukin-5 (IL-5) levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when baseline and 24 months medians for the single treatment, two treatment and three to five treatment groups were each compared using Mann Whitney test (P>0.05)](image)

**4.5.3 Interleukin-10**

Levels of IL-10 produced in response to SEA increased slightly after the first treatment for each of the groups, that is, from those with one treatment to the last group who had more than four treatments. The cytokine levels then fluctuated up and down from the second treatment to the last. The three times treatment group had a significant change in IL-10 median levels: an increase after the first treatment when compared to baseline, (P<0.05). When IL-10
medians at baseline and the first, second and third treatment points were compared, they were found to be significantly different (P<0.05; Fig 4.14)

![Image of bar chart](image)

**Figure 4.14: Change in IL-10 from SEA stimulated whole blood samples after successive treatments in children who had 3 treatments.** Interleukin-10 levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was a significant difference among the medians from baseline across all treatment times when comparison was done using Kruskall-Wallis (**P=0.0086). Comparison was also done between baseline median and those for each of the treatment groups using Mann Whitney test. There was a significance difference between IL-10 median at baseline and IL-10 median after the first treatment (*P=0.0411)

IL-10 levels produced in response to SWAP stimulated whole blood samples tended to decrease when baseline and post-treatment cytokine levels were compared. This drop in cytokine levels was observed after each successive treatment in all groups except in the group that had three treatments where IL-10 levels increased after the third treatment following an initial drop. It is also this group that recorded a significant change in cytokine levels (P<0.05)
when baseline levels were compared to levels after the first treatment. Comparison across all treatments in this group also indicated a significant change in IL-10 levels (P<0.05; Fig 4.15).

![Graph showing change in IL-10 from SWAP stimulated whole blood samples after successive treatments in children who had 3 treatments. Interleukin-10 levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was a significant difference among the medians from baseline across all treatment times when comparison was done using Kruskall-Wallis test (\(*\*P=0.0015\)). Comparison was also made between baseline median and those for each of the treatment groups using Mann Whitney test. There was a significance difference between IL-10 median at baseline and IL-10 median after the first treatment (\(*\*P=0.007\))](image)

**Figure 4.15:** Change in IL-10 from SWAP stimulated whole blood samples after successive treatments in children who had 3 treatments. Interleukin-10 levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was a significant difference among the medians from baseline across all treatment times when comparison was done using Kruskall-Wallis test (\(*\*P=0.0015\)). Comparison was also made between baseline median and those for each of the treatment groups using Mann Whitney test. There was a significance difference between IL-10 median at baseline and IL-10 median after the first treatment (\(*\*P=0.007\))  

Baseline and 24 months IL-10 SEA levels were compared for three treatment groups. Baseline cytokine levels were higher than 24 months levels for the three treatment groups. The greatest difference between baseline and 24 months was in the single treatment group, with their medians being significantly different (P<0.05). Baseline and 24 months IL-10
medians for the two treatment and 3 to 5 treatment groups did not differ significantly (P>0.05; Fig 4.16).

Baseline cytokine levels were also higher than 24 months levels for SWAP stimulated whole blood samples in the three to five treatment groups, just like what was observed with SEA stimulated samples. The single treatment group for SWAP stimulated samples also recorded the highest cytokine levels, with the mean baseline levels being 442.67±138.66 ng and 24 months mean being 394.67±174.00 ng. The groups that had two and three to five treatments recorded slightly lower means. The highest from these two groups was 312.00±90.67 ng which was produced by baseline samples for the 3-5 treatment group). Baseline and 24 months medians for each of the tree treatment groups were not significantly different (P>0.05; Fig 4.17).

**Figure 4.16: Comparison of baseline and 24 months IL-10 levels from SEA stimulated whole blood samples for children with 1, 2 and 3-5 treatments.** Interleukin-10 (IL-10) levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was a significant difference between baseline and 24 months medians for the single treatment group when compared using Mann Whitney test (*P=0.0412). Baseline and 24 months medians for the other treatment groups were no significantly different (P>0.05)
Figure 4.17: Comparison of baseline and 24 months IL-10 levels from SWAP stimulated whole blood samples for children with 1, 2 and 3-5 treatments. Interleukin-10 (IL-10) levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when baseline and 24 months medians for the single treatment, two treatment and three to five treatment groups were each compared using Mann Whitney test (P>0.05)

4.5.4 Interleukin-13
Levels of IL-13 produced by whole blood samples in response to SEA and SWAP were generally low. Samples from many children did not produce detectable cytokine levels in response to the two antigens. Cytokine quantities produced in response to SEA did not change significantly among the different treatment groups (P>0.05; figure 4.18; Fig 4.19). Levels in the two-treatment group remained fairly constant (Fig 4.18) while the three-treatment group had levels initially declining after the first treatment and rose after the second treatment (Fig 4.19).
Figure 4.18: Change in IL-13 from SEA stimulated whole blood samples after successive treatments in children who had 3 treatments. Interleukin-13 levels are expressed in nanograms per millilitre, error bars represent standard error of the mean. The IL-13 medians at baseline, after the first, second and third treatments were compared by Kruskal-Wallis test. The medians were not significantly different (P>0.05).

Interleukin-13 (IL-13) levels from SWAP stimulated whole blood samples decreased after the first treatment for all treatment categories except for the group with more than four treatments. These changes were however only significant in the three treatment group (P<0.05) when baseline IL-13 median was compared with median after the first treatment. Differences in IL-13 medians at baseline and after successive treatments were also significant (P>0.05; Fig 4.19).
Figure 4.19: Change in IL-13 from SWAP stimulated whole blood samples after successive treatments in children who had 3 treatments. Interleukin-13 levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was a significant difference among the medians from baseline across all treatment times when comparison was done using Kruskall-Wallis test (*P=0.0317). Comparison was also made between baseline median and medians for each of the treatment groups using Mann Whitney test. There was a significance difference between IL-13 median at baseline and IL-13 median after the first treatment (*P<0.05).

Comparison of baseline and 24 months IL-13 levels from SEA stimulated whole blood samples for different treatment groups gave mixed results. The single treatment group had higher baseline cytokine levels compared to 24 months while, just like the 3-5 treatment group, the two treatment group had higher 24 months levels compared to baseline levels. The lowest 24 months IL-13 level was 6.50±3.34 ng from the single treatment group, whose baseline mean was 25.77±10.69 ng. The 2 treatment group had the highest 24 months mean of 32.27±17.39 ng while its baseline mean was 19.28±15.62 ng. The 3-5 treatment group had a baseline mean of 36.67±16.97 ng and 24 months mean of 10.69±3.56 ng (Fig 4.20).
Figure 4.20: Comparison of baseline and 24 months IL-13 levels from SEA stimulated whole blood samples for children with 1, 2 and 3-5 treatments. Interleukin-13 (IL-13) levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was no significant difference when baseline and 24 months medians for the single treatment, two treatment and three to five treatment groups were each compared using Mann Whitney test (P>0.05)

Comparison between baseline and 24 months IL-13 medians from SWAP stimulated whole blood samples from the three treatment group yielded significant results (P<0.05; figure 4.22). Medians for the single and 3-5 treatment groups were however not significantly different (P>0.05). Baseline mean for the single treatment group was however higher than 24 months mean; 184.00±106.29 ng and 83.43±40.00 ng respectively. The 3-5 treatment group had the least difference between baseline and 24 months means, 60.57±18.29 SEM ng and 51.43±11.43SEM ng respectively (Fig 4.21).
**Figure 4.21:** Comparison of baseline and 24 months IL-13 levels from SWAP stimulated whole blood samples for children with 1, 2 and 3-5 treatments. Interleukin-13 (IL-13) levels are expressed in nanograms per millilitre, error bars represent standard errors of the mean. There was a significant difference between baseline and 24 months medians for the two treatment group when compared using Mann Whitney test (**P=0.0092). Baseline and 24 months medians for the other treatment groups were no significantly different (P>0.05)

4.6 Comparison of cytokine levels between children treated multiple times (arm A) and those treated only once at the beginning of the study (arm B)

Children in study arm A were followed up six times during the study period at an interval of about four months each. During each follow-up, cytokine levels were measured in culture samples for all children present at that time regardless of whether they had been re-infected or not. Out of the 68 children who remained in the study, 10 did not test positive for schistosomiasis following the first treatment. These 10 children from arm A were only treated once at the beginning of the study just like the children in study arm B. They were therefore excluded from the comparison between arm A children who had multiple treatments (more than one treatment) following re-infection with arm B children who had only one treatment at
the beginning of the study. At 24-months, cytokine levels from SEA and SWAP-stimulated cultures were compared between those treated severally (Arm A) and those treated only once during the same time (Arm B), using Mann Whitney test.

4.6.1 Interferon gamma

IFN-γ responses to SEA and SWAP in children in both study arms were very low. Values for most children were zero. Levels of the cytokine from SEA stimulated whole blood samples in arm A were marginally higher with a mean of 3.71±2.4 pg compared to 1.07±0.53 pg for arm B. Arm A IFN-γ from SWAP stimulated whole blood samples levels were also slightly higher than those from arm B (figure 4.22). Differences in median IFN-γ levels from both SEA and SWAP stimulated whole blood samples for the multiple treatment group and single treatment group were not significant (P>0.05 SEA; P>0.05 SWAP; Fig 4.22).
Figure 4.21: Comparison of IFN-γ levels from SEA and SWAP stimulated whole blood samples between schistosome infected children treated multiple times (arm A) and those treated once (arm B) with Praziquantel. Interferon-gamma (IFN-γ) levels are expressed in picograms per millilitre, error bars represent standard errors of the means. Comparison was made between arm A and arm B IFN-γ medians using Mann Whitney test. There was no significant difference between the arm A and arm B IFN-γ from SEA (P=0.938) and SWAP (P=0.7299) stimulated whole blood samples. The number of children in arm A and arm B for this comparison were 56 and 46 respectively.

4.6.2 Interleukin-5

There was no significant difference in IL-5 medians from SEA stimulated whole blood samples between the multiple treatment group and the single treatment group (P>0.05). IL-5 levels from SWAP stimulated whole blood samples also differed marginally between the two groups, with multiple treatment group having a mean of 109±22.86 ng while the single treatment group having a mean of 116.14±33.0 (Fig 4.23).
**Figure 4.22**: Comparison of IL-5 levels from SEA and SWAP stimulated whole blood samples between schistosome infected children treated multiple times (arm A) and those treated once (arm B) with Praziquantel. Interleukin-5 (IL-5) levels are expressed in nanograms per millilitre, error bars represent standard errors of the means. Comparison was made between arm A and arm B IL-5 medians using Mann Whitney test. There was no significant difference between the arm A and arm B IL-5 medians from SEA (P=0.6704) and SWAP (P=0.4986) stimulated whole blood samples. The number of children in arm A and arm B for this comparison were 56 and 46 respectively.

**4.6.3 Interleukin-10**

Interleukin-10 (IL-10) median from SEA stimulated whole blood samples in the multiple treatment group was not significantly different from that of the single treatment group (P>0.05). Responses from SWAP stimulated whole samples were a little more elevated in both study arms though levels for study arm A were slightly higher with a mean of 217.71±34.28 ng compared to 181.71±30.54 ng. Difference in their medians was not significant (P>0.05; Fig 4.24).
**Figure 4.23:** Comparison of IL-10 levels from SEA and SWAP stimulated whole blood samples between schistosome infected children treated multiple times (arm A) and those treated once (arm B) with Praziquantel. Interleukin-10 (IL-10) levels are expressed in nanograms per millilitre, error bars represent standard errors of the means. Comparison was made between arm A and arm B IL-10 medians using Mann Whitney test. There was no significant difference between the arm A and arm B IL-10 medians from SEA (P=0.4778) and SWAP (P=0.4576) stimulated whole blood samples. The number of children in arm A and arm B for this comparison were 56 and 46 respectively.

### 4.6.4 Interleukin-13

IL-13 is the only cytokine whose medians were significantly different (P<0.005; Fig 4.25) between the multiple and single treatment groups for SWAP stimulated whole blood samples. Levels of the cytokine were higher in the multiple treatment group with a mean of 58.93±10.40 ng compared to 37.73±1066 ng for the single treatment group. While the SEA stimulated whole blood IL-13 mean levels were also higher in the multiple treatment group compared to the single treatment group, their medians were not significantly different (P>0.005; Fig 4.25)
Figure 4.24: Comparison of IL-13 levels from SEA and SWAP stimulated whole blood samples between schistosome infected children treated multiple times (arm A) and those treated once (arm B) with Praziquantel. Interleukin-13 (IL-13) levels are expressed in nanograms per millilitre, error bars represent standard errors of the means. Comparison was made between arm A and arm B IL-13 medians using Mann Whitney test. There was a significant difference between the arm A and arm B IL-13 medians from SWAP stimulated whole blood samples (*P=0.0165). The difference was not however significant for the SEA stimulated IL-13 medians (P=0.7782). The number of children in arm A and arm B for this comparison were 58 and 46 respectively.
CHAPTER FIVE: DISCUSSION

5.1 Overview

This study followed up successfully a total of 113 eight to ten-year old school children for a period of two years. The children were assigned to two parallel groups designated arm A and arm B. Children in study arm A were kept as worm free as possible by carrying out follow-ups every four months for diagnosis and treatment. Out of the 68 children followed up in this group, 10 remained un-infected throughout the study period following treatment at the start of the study. In the study arm B, the children were only treated at the beginning of the study and at the end of the study period for those who were reinfected. In this group, 26 children were not re-infected throughout the study period. Overall, results from the study indicated that treatment carried out for the children in the two study arms was very successful in reducing infection intensity from a mean of 217.8 eggs per gram of faeces (EPG) at baseline to a mean of 43.22 EPG of faeces. The study did not find any correlation between cytokine levels and infection intensity in egg per gram of faeces.

5.2 Relationship between infection intensity and cytokine production

Majority of the children in this study (56.6%) had low infection intensity, that is, less than 100 epg of faeces. Those who had high infection intensity (400 epg of faeces and above) were 15.9% while the rest had moderate infection intensity (100-399 epg of faeces). This pattern of infection intensity is consistent with results from an earlier study done on school children in the same area in which 62.7% of children had light infection (Handzel et al., 2003).
The cytokines IFN-γ, IL-5, IL-10 and IL-13 were all produced *in vitro* following culture of whole blood samples from the children with SEA and SWAP. These four cytokines are among the key mediators of immune responses in human schistosomiasis. Very weak correlations were obtained between levels of these cytokines and infection intensity for all the three infection intensity groups, that is, low, medium and high infection intensity groups.

IFN-γ is the only T<sub>H1</sub> cytokine considered in this study. It is important in the regulation and amplification of T<sub>H1</sub> responses. Very low levels of this cytokine were produced in response to both SEA and SWAP. In fact no detectable cytokine was produced by the medium and high infection intensity group in the last follow-up. These two groups however had very few subjects, with the medium infection group and high infection group having a total of 7 children and 4 children respectively. Production of IFN-γ in response to both SEA and SWAP was not influenced by the level of infection in all the three infection intensity groups. Similar results were obtained in another study involving children and adults in a fishing community in Uganda (Joseph *et al.*, 2004). The low levels of this cytokine were probably as a result of down-regulatory effects of T<sub>H2</sub> cytokines, most notably IL-10 whose levels were fairly elevated in all infection intensity groups.

Three T<sub>H2</sub> cytokines were considered in this study. These were IL-5, IL-10 and IL-13. Each of these cytokines is important in the immune response to schistosomiasis. IL-5 regulates eosinophils whose levels in humans are associated with resistance. IL-10 is important in immune regulation, with studies in man indicating that it down-regulates T<sub>H1</sub> responses. IL-13 on the other hand has been implicated in development of fibrosis and is also involved in regulation of IgE (Joseph *et al.*, 2004). None of these cytokines correlated significantly with infection intensity except IL-13 SEA for the low infection intensity group, last follow-up. This however contrasts partly with a report about immune responses during acute and chronic
phases of human *Schistosoma mansoni* infection (Caldas *et al.*, 2008). No association was found between intensity of infection and production of IL-13 (Caldas *et al.*, 2008), IL-5 and IL-10 (Joseph *et al.*, 2004). A study in Zimbabwe involving individuals living in a *S. Haematobium* endemic area did however report a significant correlation between IL-10 production and infection intensity (Mutapi *et al.*, 2007).

### 5.3 Effect of treatment of *Schistosoma mansoni* infection with Praziquantel

Praziquantel is currently the most widely used drug for the treatment of schistosomiasis. It is safe to use and has been demonstrated to be effective in not only decreasing some of the schistosomiasis-associated morbidities, but can also resolve and delay resurgence of these morbidities after subsequent reinfection (Hatz *et al.*, 1998; Vennervald *et al.*, 2005). There is further evidence that treatment with this drug induces a change in the immune responses that are associated with resistance to reinfection (Lederman *et al.*, 1984; Booth *et al.*, 2004; Oliveira *et al.*, 2006). The drug kills adult worms and mature eggs causing a release of antigens from the two stages of the life cycle of the parasite. Immature stages of the parasite are however refractory to the drug (Harnett and Kusel 1986; Giboda and Smith 1994; Vereecken *et al.*, 2007).

At the beginning of this study, children who tested positive for the presence of *S. mansoni* eggs in their stool were all treated with Praziquantel. One group of children designated arm A were diagnosed and treated with Praziquantel if found positive and bled every four month throughout the two year study period. A parallel group of children were followed up for diagnosis, treatment if reinfected and bled at the end of the two year study period. In the multiple follow-up group (Arm A), 10 children were not reinfected throughout the study period following the initial treatment. At each follow-up time in this group, there was always
a certain number of children who tested positive for the presence of *S. mansoni* eggs in their stool, meaning that they had most likely been reinfected. By the end of the two year study period, 51 children out of a total 68 that were followed-up throughout this period were reinfected at least once following the initial treatment. Out of 45 children who were diagnosed and treated at the beginning and at the end of the study in the single follow-up group (Arm B), 19 were not reinfected throughout the study period. Treatment with Praziquantel therefore helped to reduce prevalence of *Schistosoma mansoni* in the two study groups. It also helped to reduce infection intensity from as high as 2088 and a mean of 217.8 eggs per gram of faeces at baseline to high of 828 and mean of 43.22 eggs per gram of faeces at the end of the study for all the children in the two study arms. These results are in line with the World Health Assembly resolution 54.19 (WHA 54.19) which recommends regular periodic treatment of all school age children in schistosomiasis endemic areas with Praziquantel. This is to help reduce morbidity associated with the disease, infection intensity, and prevalence of the disease.

While there was an overall reduction in prevalence and infection intensity in children in the two parallel study groups, children who were reinfected in the multiple follow-up group had much lower infection intensities compared to the single follow-up group. The multiple follow-up group had the advantage of receiving treatment fairly promptly after reinfection since diagnosis was done every four months. This way, the reinfection was cleared before worm burden could reach levels that can cause serious morbidity. The number of children reinfected by the last follow-up were however more in the multiple follow-up group, 58 out of 68 compared to 19 out of 45 for the single follow-up group. It was assumed that the level of water contact for children in both study groups remained as had been established at the beginning of the study. This is because the children were recruited from schools around the
lake and randomly assigned to the two study groups. Those who were followed up throughout the study period maintained residence in the area.

More re-infections were observed in the multiple follow-up group, contrary to results obtained from adult studies. Continued susceptibility to re-infection in this group despite repeated treatment following re-infections might be due to augmented responses of blocking antibodies. Several studies have reported an association between elevated levels of IgM and IgG4 antibodies and susceptibility to infections (Khalife et al., 2000).

Follow-up and retreatment after two years is the most compatible period with the WHA 54.19. While the resolution calls for the regular periodic treatment of all school age children in schistosomiasis endemic areas with appropriate drugs, it does not however specify the interval between subsequent re-treatments. Results from the current study seem to favour a two year interval between retreatments rather than four months. This follows the observation from the study that there were less reinfections in the single follow-up group compared to the multiple follow-up group. Nsowah-Nuamah et al. (2004) carried out a study on urinary schistosomiasis in Ghana to predict the optimum time for the second chemotherapy (Praziquantel). They predicted an interval of 11.8 months for the timing of the second chemotherapy in an area where there are no other transmission control measures. The longest predicted period (13.8 months) for the second chemotherapy was in an area where chemotherapy was combined with public education of residents on how to avoid infection with the parasite. The two time intervals of four months and twenty four months used in the current study are very different from those periods predicted for second chemotherapy in the Ghana study. While the four months interval is most appropriate in morbidity reduction, it’s expensive to implement especially for developing countries. Two years on the other hand will
be too long an interval that those reinfected soon after first treatment will re-establish infection intensities almost equal to pre-treatment levels.

5.4 Change in cytokine levels with each successive treatment

Fifty eight out of 68 children in study arm A were reinfected at least once during the two year study period. Diagnosis, treatment if re-infected and collection of venous blood was done for children in this study arm after about every four months. Cytokine levels in these children were determined from whole blood samples collected at each bleed. Levels of IFN-γ from SEA samples were generally very low, in some cases too low to be detected. In order to determine changes in cytokine levels after each successive treatment, the children were categorised into single, two treatment, three treatment and 3-5 treatment groups. Comparison was made between baseline cytokine levels and levels after each successive treatment. None of the treatment groups had significant differences between baseline and post-treatment IFN-γ levels for both SEA and SWAP samples. There was a marginal drop in levels of the cytokine after treatment in children who had only one treatment. This is the group considered resistant to reinfection since the children in the group remained un-infected throughout the study period. This is despite their continued residence in a schistosomiasis endemic area with active reinfections. Children in the two or more treatment groups had a marginal increase in cytokine levels especially following the second or third treatment. A study involving subjects of mixed ages in Uganda similarly did not report any significant change in cytokine levels after treatment with Praziquantel (Joseph et al., 2004).

Production of low amounts of IFN-γ during schistosomiasis infection is associated with chronic infection and is probably caused by down-regulation by T_{h}2 cytokines. It is only during the acute phase of the disease and in endemic normal individuals that IFN-γ levels are
elevated. Endemic normal refers to a group of individuals living in schistosomiasis endemic areas who remain schistosomiasis egg-negative in despite coming in contact with water bearing the infectious cercariae. Resistance to infection in these individuals is attributed to their high schistosomiasis antigen specific IFN-γ (Correa-Oliveira et al., 1997 and Caldas et al., 2008). This implies that the mechanisms of resistance in endemic normal individuals and individuals who develop resistance following chemotherapeutic cure are different. Resistance to re-infection in the later is associated predominantly with antigen-specific PBMC production of IL-5 and IL-4, IgE and peripheral blood eosinophilia (Walter et al., 2006).

This study did not find any significant effect of the number of treatments with Praziquantel on IFN-γ production by whole blood samples from the children in response to SEA and SWAP. This observation is similar to findings from another study done Gabon children infected with Schistosoma haematobium. This study found no significant difference in levels of IFN-γ produced by children with no treatment, those with a single treatment and others who had multiple treatments in a period of 24 months. It was further reported that IFN-γ levels were lowest in the group of children who were repeatedly treated in that same period although the same group had the highest increase in T_{H2} cytokines. The lower IFN-γ levels in this group were attributed to down-regulation by the increased T_{H2} cytokines (Biggelar van den et al., 2002). This study does not however report a corresponding increase in T_{H2} cytokines as in the Gabon study.

There was no significant change in levels of IL-5 from SEA samples after treatment in all treatment groups. The levels only increased marginally after treatment in the groups that had one treatment and the one that had two treatments. There was however a marginal decline after the first treatment in children who had three or more treatments. IL-5 levels for SWAP samples similarly declined after the first treatment, with the drop being significant in the
group which had two treatments. Levels of the cytokine were slightly higher for SWAP samples compared to SEA samples. This mirrors to a small extent findings from the Uganda study involving subjects of all ages from a cohort living near Lake Albert. The study reported that Th2 cytokines were mainly produced in response to worm antigens and that the greatest increase in these cytokines was only seen in response to SWAP and tegument antigens. In the same study, a similar trend was observed for all the Th2 cytokines studied, that is, IL-4, IL-5, IL-10 and IL-13 (Joseph et al., 2004). While this study also focused on these cytokines except IL-4, no such general trend was observed about their levels before and after treatment.

IL-10 is the only cytokine whose levels changed significantly after treatment for both SEA and SWAP samples in the current study. Levels of the cytokine for SEA samples increased significantly after the first treatment in the group of children who had three treatments. There was a significant decline however in levels of the same cytokine after the first treatment for SWAP samples in children with three treatments. In a Ugandan mixed cohort study, IL-10 is also the only cytokine produced in comparably higher amounts in response to both SEA and SWAP. The same study also reported a significant increase in IL-10 levels after treatment for both SEA and SWAP samples (Joseph et al., 2004). A slightly different scenario was seen in a study that sought to determine the effect of multiple treatments on cytokine levels in *Schistosoma haematobium* infected children. While the study reported significant elevation in production of IL-5 and IL-13 in response to SWAP in children treated multiple times, the increase in IL-10 was less profound (Biggelar van den et al., 2002).

There were no significant changes in the levels of IL-13 produced from SEA samples for all treatment groups in this study. There were also no significant differences between SEA baseline and last follow-up comparisons for all treatment groups. This implies that repeated
treatment did not affect production of IL-13 from SEA samples. A different picture was however seen with IL-13 from SWAP samples. Levels of the cytokine declined after the first treatment for all treatment groups although only the group that had three treatments recorded a significant drop. For baseline and 24 months comparisons, the group that received two treatments had a significantly higher last follow-up IL-13 median for SWAP samples. Biggelaar van den et al. (2002) reported contrasting findings on changes in levels of this cytokine in *S. haematobium* infected children following repeated treatment. Mean levels of the cytokine increased significantly in children treated repeatedly. Joseph et al. (2004) also reported significant increase in levels IL-13 along with other T<sub>H</sub>2 cytokines in response to SWAP after treatment in a cohort of *Schistosoma mansoni* infected individuals of different ages.

IL-13 is an important mediator of T<sub>H</sub>2-mediated inflammation which is responsible fibrosis associated with Schistosome eggs. It also plays a role eliciting IgE responses to Schistosome egg antigens (Chiaramonte et al., 1999). Levels of the cytokine initially declined following treatment possibly due to immunoregulatory changes that are thought to occur after treatment and which result in morbidity control. This same mechanism could be responsible for lack of morbidity in a group of individuals who remain asymptomatic despite harboring the infection for a long period of time even without treatment.

### 5.5 Effect of multiple treatments on cytokine levels

Comparison of cytokine levels was made between the two study arms: the multiple treatment group (Arm A) and the single treatment group (Arm B). Only 58 out of the initial 68 children in study arm A had more than one treatment and were therefore included in the comparison with the single treatment group of study arm B. There was no significance difference in mean
and median IFN-γ levels from both SEA and SWAP stimulated samples between the two study arms. This partly agrees with findings by Biggelar van den et al. (2002) in a study to determine effect of repeated treatment on immunity to *Schistosoma haematobium*. This study did not report any significant change in IFN-γ levels following treatment. Two cytokines were however reported to be significantly elevated in the multiple treatment group, that is IL-5 and IL-10 produced in response to adult worm antigen stimulation. In our study, IL-13 from SWAP stimulated samples was the only T_{H2} cytokine that was produced in significantly higher amounts from the multiple treatment group compared to the single treatment group.

In a study to determine the effect of treatment of *Schistosoma mansoni* on immunity to reinfection, Roberts et al. (1993) reported elevated IL-5 levels after treatment in all age groups. The increase was however more pronounced in adults than in children and was associated with resistance to reinfection in adults. Many other studies involving adults have reported raised levels of T_{H2} cytokines IL-4, IL-5 and IL-13 together with Ig E and eosinophilia in response to adult worm antigen preparation (Walter et al., 2006, Roberts et al., 1993 and Dunne et al., 1992). All these are responses that correlate with resistance to reinfection in adults. The responses are brought about by increased exposure to antigens released, most probably in abundance, from dying worms following chemotherapy. Praziquantel is thought to disrupt the worms’ outer tegument thus exposing the worms underlying antigens which may boost response to adult worm antigens (Walter et al., 2006).

There is widespread consensus that some adults develop resistance to reinfection following repeated rounds of treatments after reinfection. In a parallel study to this one, some adult car washers occupationally exposed to schistosomiasis became increasingly resistant to reinfecion after each successive treatment following reinfection (Karanja et al., 2002). In this study however, no such pattern emerged among children in the multiple treatment. A few children
remained uninfected both in the single follow-up and multiple follow-up groups. There was also no difference in the cytokine profiles of the children in the single treatment and multiple treatment groups.

Susceptibility to reinfection remains in children even after several rounds of treatment following reinfection. This is not due to an inability of their PBMCs to respond to *S. mansoni* antigens as noted by Roberts *et al.* (1993). It is hypothesised that their cellular responses are either ineffective at mediating protection or are inhibited from doing so. The inhibition is thought to be due to the elevated levels of blocking antibodies; IgM and IgG2 specific for worm antigens. While it is true that our study did not find any significant elevation in any of the cytokines assayed following repeated rounds of treatment, there were a few significant increases in cytokine levels after treatment. There is also the possibility of cross-regulation between T\(sub H1\) and T\(sub H2\) cytokines.
CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

(a) There were no significant correlations between cytokine levels and infection intensity in eggs per gram (epg) of faeces for all the cytokines. The only exception was for IL-13 from soluble egg antigen (SEA) stimulated samples from the low infection intensity group, last follow-up. Generally, IFN-γ levels were very low and in some instances were below detection levels from both SEA and SWAP stimulated whole blood samples. For IL-5 and IL-10, very week correlations were obtained between levels of these cytokines and infection intensity in epg of faeces. Some of the correlations were negative.

(b) There were no progressive changes in cytokine levels after successive treatments. For IFN-γ, no consistent changes in levels of the cytokine were observed following treatment for both the SEA and SWAP stimulated samples. Interleukin-5 produced in response to egg antigens did not change significantly after subsequent treatments. The only significant change in IL-5 was a drop following first treatment from adult worm antigen stimulated samples for children who had two treatments. Results for IL-10 fluctuated following successive treatments. Levels of IL-13 from both SEA and SWAP stimulated samples were also not significantly affected by number of treatments. Repeated cycles of treatment following reinfection in 8 to 10 year-old children do not therefore result in elevation of cytokine levels, especially those associated with resistance to reinfection.

iii. Comparison between cytokine levels of children treated once at the beginning of the study and those treated multiple times did not yield significant differences. Levels of IFN-γ, IL-5, and IL-10 from both SEA and SWAP stimulated samples were not
significantly different between the single and multiple treatment groups. It is only IL-13 from SWAP-stimulated samples for the multiple treatment group that was significantly higher than that for the single treatment group. From these results, it appears that repeated cycles of treatment following reinfection do not result in protective immune responses as measured by cytokine levels. Based on these results, the null hypotheses “There are no changes in cytokine profiles of 8 to 10 year old school children upon treatment of *S. mansoni* infection” and “There are no differences in cytokine profiles between children who have had multiple rounds of treatment, reinfection and retreatment of *S. mansoni* infection and those treated once at the beginning of the study during a two year study period” are accepted.

6.2 Recommendations

6.2.1 Applications of the findings

a) Other immunological parameters such as Ig E and B cells should be used to assess possible immunity development in children following multiple treatments after reinfection. The children should also be followed-up for a longer period than two years.

b) The observation that reinfections continue to occur after treatment in endemic areas calls for use of a combination of several disease control measures such as public education and provision of safe water and sanitation facilities such as latrines in addition to treatment programs.

c) Public health ministries of countries where schistosomiasis is endemic should adopt a follow-up retreatment period that is reasonably long while implementing the World
Health Assembly Resolution 54.19. Findings from this study support a follow-up retreatment period that is longer than 3 months but less than two years.

### 6.2.2 Study gaps and future studies

Within two years of the study, no consistent cytokine profile could be established from whole blood samples from the children in response to Schistosome egg and worm antigens. Findings from many studies done in schistosomiasis endemic areas report that immunity to the disease tends to develop with age and that children are more susceptible to infection than adults. More studies are therefore needed to follow-up these children to establish at what age immunity begins to develop. Other immune correlates of resistance should also be studied such as antibody responses to both parasite egg and worm antigens. While this study used two different follow-up periods of two years and four months, studies should be done to determine the most appropriate follow-up time for retreatment of especially during mass treatment programmes. This is because the 2 year period is too long as new reinfections will have been re-established to near the pre-treatment intensities. Four months follow-up period is too short and will ultimately be very expensive to carry out especially when implementing mass treatment programmes as recommended by the World Health Assembly resolution 54.19 (WHA 54.19).

More work needs to be done to further characterize the immunological profile and genetic polymorphisms of individuals living in schistosomiasis endemic areas but who remain uninfected. These individuals who have been referred to as ‘endemic normals’ have natural immunity to schistosomiasis which keeps them worm free despite being exposed to infection. It would also be interesting to determine if there are naturally resistant children and to
determine their immunological responses to parasite antigens. Such information would be valuable to ongoing efforts to develop an antischistosome vaccine.
REFERENCES


Karanja D., Hightower A., Colley D., Mwinzi P., Galil K., Andove J. and Secor W. (2002). Resistance to reinfection with Schistosoma mansoni in occupationally exposed adults and


APPENDIX I

Laboratory protocols

a) ELISA Reagents

Preparation of IL-5/IL-10/IL-13 Reagent Diluent (200ml total volume), (store at 4°C)

- 2g BSA
- 160ml 1X PBS
- Adjust pH to 7.2-7.4
- Bring volume to 200ml with 1X PBS
- Filter sterilize

Preparation of IL-4/IFN-γ Reagent Diluent (200ml total volume), (Store at 4°C)

- 0.48g Trizma base
- 1.75g NaCl
- 0.2g BSA
- 180 ml water
- Adjust pH to 7.2 to 7.4
- Add 100 µl Tween 20
- Bring volume to 200ml with water
- Filter sterilize
- Wash Buffer, Store at room temperature

3L 1X PBS
1.5ml Tween20
Mix well

Preparation of 2N H₂SO₄, Store at room temperature

- 86ml Distilled H2O
- 14ml Sulfuric Acid (H₂SO₄), which is normally 14N
**Preparation of 1 liter 10X PBS**

80g Sodium Chloride (NaCl)

2g Potassium Chloride (KCl)

14.4g Sodium phosphate Dibasic (Na$_2$HPO$_4$)

21.12g Sodium phosphate Monobasic (NaH$_2$PO$_4$)**

Dissolve in 800ml dH$_2$O and adjust pH to 7.4

Bring volume to 1 liter

Autoclave and store at room temperature up to 6 months.

**Conversely, you can add 2.4g Potassium Phosphate (KH$_2$PO$_4$)**

**Note:**

**Bring all reagents to Room Temperature Before Use.**

**b) Whole blood culture**

*(using heparinized venous blood)*

Bring sterile heparinized whole blood into the sterile biosafety cabinet and split it into two portions of 4ml each into sterile 5ml falcon tubes:

**4 ml for Whole Blood Cultures for Cytokine production** (IL-4, IL-5, IL-13, IL-10, IFN-γ,) by ELISA

Begin by labeling the sterile 10ml Falcon tubes well arranged in a rack. Write the bleed date, the patient number, the antigen being added and the date of harvest.

Gently add 1.42ml of complete media (without NHS) to each tube using P1000 –avoid introduction of bubbles.

Gently add 330ul of blood to each tube. *Be sure to swirl or pipette up and down before and in between removing each 330 microliter volume to ensure the 330ul aliquot is uniform from culture-to-culture)*

Then add the appropriate antigen preparation to the appropriate tube- you now have in each tube:

Media (-no NHS -see recipe below): (1.35Mls) **1420ul**
Diluted antigen (as per RO1 protocol working dilutions): **250ul** Whole heparinized sterile blood: **330ul**

**This brings the total volume in each tube to 2ml and a 1:6 (1 part: 5parts) dilution of the whole blood.**

If adding anti IL-10 to wells, add 5µl in addition to the 250µl appropriate antigen and mix up and down. The resulting concentration of anti-IL-10 in the well is 2.5µg/ml. Add 5µl of control antibody to appropriate tubes, as necessary (final concentration is also 2.5µg/ml)

Place tubes in incubator for 5 days.

Harvest supernatants on day 5 as follows:
( I) Centrifuge the whole blood culture tubes

(II) Harvest the supernatant fluids from the cultures

**One 1.2ml for cytokine ELISA**

**Label cryotubes as follows:**

*Patient Number, Study Arm (A or B), Antigen, Start date of culture.*

Order of Preference for Culture Set-up:

c) **Complete Media for whole blood culture - Store at 4°C**

(Do not add NHS)

(Prepare in upper chamber of 500ml filter flask)

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>2ml</td>
<td>L-Glutamine</td>
</tr>
<tr>
<td>2ml</td>
<td>Pen-Strep</td>
</tr>
<tr>
<td>156ml</td>
<td>RPMI</td>
</tr>
</tbody>
</table>

Filter Sterilize and store at 4°C
c) Antigen Preparation- Store at –20°C

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Starting Concentration (Changes depending on antigen lot – adjust accordingly!!)</th>
<th>Final Concentration (ug/ml)</th>
<th>Working Concentration (ug/ml)</th>
<th>Amount Ag Added (ul)</th>
<th>Amount RPMI Added (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWAP</td>
<td>4/7/03, 2.28mg/ml</td>
<td>10</td>
<td>100</td>
<td>219</td>
<td>4.781</td>
</tr>
<tr>
<td>SEA</td>
<td>4/7/03, 4.014mg/ml</td>
<td>5</td>
<td>50</td>
<td>62.3</td>
<td>4.938</td>
</tr>
<tr>
<td>PHA</td>
<td>2mg/ml</td>
<td>20</td>
<td>200</td>
<td>500</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*** Add 5ml RPMI to each tube, then remove the amount to be added. Ex. Remove 219ul from 5ml RPMI for SWAP dilution. Then add 219ul of SWAP.

Equation: \((\text{Starting concentration})(x) = (\text{Working Concentration})(5\text{ml})\)

Example: CDC2 SWAP \((3.474\text{mg/ml}\times1000\text{mg/ug})(x)=(100\text{ug/ml})(5\text{ml})\)

\(x = 0.144\text{ml} = 144\text{ul}\)

2ml whole blood Culture Set-up

330µl whole blood

250µl Antigen preparation at the appropriate working concentration

1420µl Complete Media
d) Instructions for Cytokine ELISAs, 2 assays at a time (total of 10 plates)

DAY ONE

1. Label 10 plates, 2 for each cytokine (IL-4,5,10,13 and IFN-g). Also label with KID ID for each set.

2. Thaw one aliquot of Capture Antibody just prior to coating, and quickly microfuge tube to pull all liquid together. Dilute the Capture Antibody in sterile, 20μm filtered 1X PBS to working concentration and immediately coat a 96-well Immulon 2HB ELISA plate with 100μl per well using repeat pipettor. Seal each plate individually and incubate overnight at room temperature.

DAY TWO

3. Remove reagent diluents from the 4C and allow to attain room temperature.

4. Wash each plate 3 times. Empty contents of plate by inverting over the sink, add wash buffer with manual plate washer, tap plate with hands and empty again. That is one wash. After third wash, blot the plate dry on paper towels or gauze. Ensure the plate is completely empty of all liquid and also of any bubbles in the wells.

5. Block each plate with 300μl of respective reagent diluent using either repeat pipettor or multichannel pipette and reagent reservoir. Incubate 1 hour at room temperature.

While plates are blocking, remove samples from the freezer to thaw, quickly microfuge the tubes to pull all the liquid together and prepare standard dilutions. Once samples are thawed, shake each vial to mix contents. Write out the hard copy plate template.

Label 8 tubes per cytokine A-H. Fill tube A with 1000μl of respective reagent diluent and tubes B-H with 500μl reagent diluent. Add standards as per calculations to make the recommended working concentration in tube A. Vortex tube A and transfer 500μl from A to tube B, repeating this from subsequent tubes thus making serial dilutions starting from A(highest concentration) to H (lowest concentration).

6. After blocking for one hour, wash each plate 3 times.

7. Once the plates are blotted, first add the standards. Use the same tip for tubes A-H, but work from the lowest concentration tube to the highest.

Once the standards are added, proceed quickly to add the samples according to the template set up.

After adding standards and samples, cover plates and incubate at room temperature for two hours.
8. After one hour incubation with samples/stds, remove detection antibody aliquots from the -20C freezer to thaw, and quickly microfuge tubes to pull all liquid together. Prepare the secondary antibody to working concentrations in respective reagent diluents. Add 2% heat inactivated normal goat serum to IL-13 and IFN-gamma.

9. After the second hour with stds and samples, wash plates 3 times. Blot dry and add secondary antibody dilutions with repeat pipettor. Incubate 1 hour at room temperature.

15 minutes prior to the end of the incubation with secondary antibody, prepare dilutions of Streptavidin-HRP in the respective diluents.

10. Wash plates three times and add dilutions of Streptavidin-HRP, 100μl/well using repeat pipettor. Stack plates, cover top plate and cover stack to keep out of direct light. Incubate at room temperature for 20 minutes.

During this time, remove TMB substrate from the 4C to come to room temperature. Ensure you have enough stop solution to finish the assay (at least 60ml). Turn on the plate reader and set up sofmax files for the day.

15 minutes after incubation, prepare TMB by mixing 50ml of component A with 50ml component B.

11. After 20 minutes, wash plates 3 times. Blot dry and add 100μl/well substrate solution using multichannel pipette. Add substrate to plates IL-10 and IL-13 first as they develop the slowest. Then add to IL-5, IL-4 and IFN-gamma. Let the plates develop until a deep blue color appears in A1 and B1 wells (5-10 minutes, depending on the cytokine). Once that has happened, add 50μl stop solution to the plates using a multi-channel pipette.

Once plates have been stopped, they must be read immediately. They will quickly start to precipitate and thus skew the readings on the plate reader.

12. Before reading the plates, use kimwipes to thoroughly clean the bottoms and inspect the wells for bubbles.
APPENDIX II

INFORMED CONSENT AGREEMENT

a) SUBJECTS UNDER 18 YEARS

I, Mr./Mrs./Miss__________________________________________, being a person aged 18 years and over and being the lawful/legal guardian of:

Msr/Miss (Child’s name)__________________________________ Age________

School name___________________________________________ House Number___________________

dohereby give permission to Prof/Dr./Mr./Mrs./Miss.________________________________________

to include her/him in the intended research study as detailed in the protocol, which has been explained to and understood by me. I have also been made to understand the implications and benefits of the tests and treatments to Msr/Miss__________________________________. I accept the tests to be carried out. I understand that I may withdraw him/her from the research at any time, for any reason, without any penalty or harm. All the above conditions have been explained to me in the ____________________ language in which I am fluent.

Guardian’s signature _____________________________________

Date _____________________________________

Place _____________________________________

Person Obtaining Consent ________________________________

Witness ____________________________________________
b) Treatment Consent

If your child has the infection, he/she can be treated for it by trained people from KEMRI. If he/she has other infections caused by worms, he/she can be treated for those also. The treatments are free. Is it okay for your child to receive treatment if he/she has a worm infection?

Yes____ No____

Parent’s/guardian’s signature_______________________________________
c) Assent for children:
You are being asked to provide urine, stool and blood samples so that we can check to see if you have any worms living in your body. If we find worms, you will get some medicine to make you better. You don’t have to do this if you don’t want to but there is no danger if you do. It might help you. Do you agree to give us some urine, stool and blood to check for the worms?

Yes____ No____

Child’s Name_________________________________________

Person Obtaining Consent_____________________________________

Signature_____________________________________________________

Witness_______________________________________________________

Witness Signature_____________________________________________

OFFICIAL STAMP.
d) CONSENT FORMS IN DHOLUO
OTAS YIERUOK GI JOGO MAN E BWO HIGNI APAR GI ABORO (18)

An migosi/moluor/mikaye/nyadendi______________________________

Maja higni apar gi aboro (18) kadhi nyime kendo bedo janyol/jarit ayanga mar
______________________________________________________________

Nying nyathi en ________________________________

hike gin___________________________________________

Skul mosome _____________________________________________

andiko mar ot en________________________________________

Akawo thuoloni mar miyo Prof/Laktar/Migosi/Moluor/Mikaye/Nyadendi
___________________________________________________________

thuolo mar bedo gi ________________________________ enonroni. Kaka oler
malongo e oboke man malono, ma oseler tiende kendo a sewinjo maber. Ma osemiya winjo
ayanga maber ber mar pim to gi thiethne. Ayie mondo otim pim. An gingeyo ni anyalo gole
enonro saa asaya kanitie gima omiyo to kum kata hinyruok moro amora onge. Weche man
malogo osepimna gi dholuo ma awinjo maber.

Seyi mar Janyuol/Jarit______________________________________________

Tarik___________________________________________________________

Gweng__________________________________________________________

Ngatno moyudo obokeni__________________________________________

Janeno__________________________________________________________
**OBOKE MAR THIETH**

Ka nyathini nigi tuo inyalo thiedhe gijogo motiegi moa KEMRI. Ka en gituo moro mokel gi njofni inyalo thiedhe ne mago bende. Thieth itimo nono. Bende yot mondo nyathini othiedhi ka en gituo mar njofni.

Seyi mar Janyuol/Jarit_________________________________________________

**DUOKO MAO KUOM NYATHI**

Ikwayi mondo ichiw oko matin (lach) kod maduong’ mondo wangi wane kanitie kute mag njofni modak ei dendi. Kawayudo njofni to ibiro miyi yath mondo idhi maber, ok ochuno ni nyakaitim ka ok idwar to onge rach moro. Onyalo konyi. Bende iyie mar miyowa lach kod oko maduong’ mondo wange kanitie njofni.

Ee ________________________Ooyo ____________________________

Nying nyathi _______________________________________________________

Ng’at mikawo kuome duoko _________________________________________

Seyi_____________________________________________________________

Janeno ___________________________________________________________

Seyi mar Janeno__________________________________________________
APPENDIX III

Life Cycle of Schistosomes

Life cycle of three Schistosome species: *S. mansoni*, *S. haematobium* and *S. japonicum*. (Adapted from Ross *et al.*, 2002)
G: 6-year-old boy with gross reactive hepatosplenomegaly. H: 19-year-old man with symptoms of chronic fibrotic hepatic schistosomiasis—splenomegaly, external varices, ascites, and growth retardation (Adapted from Gryseels et al., 2006)