EFFECTS ON REGULATORY T CELL SUBSETS IN SCHISTOSOMA MANSONI AND PLASMODIUM FALCIPARUM CO-INFECTIONS IN PRE-TEEN SCHOOL CHILDREN IN ASEMBO RARIEDA DIVISION OF WESTERN KENYA

ERICK M.O. MUOK (BSC HONS)

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APRIL 2008
DECLARATION

I, Erick M. O. Muok, declare that the work presented herein is my original work and has not been presented for the award of any degree anywhere.

SIGNATURE: [Signature]

DATE: April 25th, 2008

Erick M. O. Muok (BSc)

We confirm that the candidate under our supervision performed the work presented in this thesis.

SIGNATURE: [Signature]

DATE: 29th April 2008

Dr. Michael M. Gicheru
Department of Zoological Sciences, Kenyatta University

SIGNATURE: [Signature]

DATE: 29th April 2008

Prof. Zipporah W. Ng’ang’a
Department of Medical Laboratory Sciences, JKUAT

SIGNATURE: [Signature]

DATE: April 25th, 2008

Dr. Diana M. S. Karanja
Center for Global Health Research (CGHR), Kenya Medical Research Institute, Kisumu, Kenya
DEDICATION

This work is dedicated to my daughter Perez T. Odhiambo, my wife Keziah A. Odhiambo and my mother Kerina O. Muok for their unrelenting support.
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xii</td>
</tr>
<tr>
<td>Abstract</td>
<td>xiii</td>
</tr>
</tbody>
</table>

CHAPTER ONE: INTRODUCTION

1.1 Background Information 1
1.2 Statement of problem 4
1.3 Research Question 5
1.4 Null Hypothesis 5
1.5 Objectives of the study 6
1.5.1 General Objective 6
1.5.2 Specific Objectives 6
1.6 Justification 7
### CHAPTER TWO: LITERATURE REVIEW

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Clinical manifestation of schistosomiasis mansoni and falciparum malaria</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>Natural T regulatory cells control tissue damage during parasitic infection</td>
<td>12</td>
</tr>
<tr>
<td>2.3</td>
<td>Regulatory T cells and Beneficial Relationship for The Host and the Parasite</td>
<td>13</td>
</tr>
<tr>
<td>2.4</td>
<td>Detrimental roles of natural Treg to the host during parasitic infection</td>
<td>13</td>
</tr>
<tr>
<td>2.5</td>
<td>Controlling regulatory functions to favor parasite control</td>
<td>14</td>
</tr>
<tr>
<td>2.6</td>
<td>Controlling T regulatory cells function to enhance memory responses</td>
<td>16</td>
</tr>
<tr>
<td>2.7</td>
<td>Controlling T regulatory cells function to prevent immunopathology</td>
<td>16</td>
</tr>
</tbody>
</table>

### CHAPTER THREE: MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Study Area</td>
<td>18</td>
</tr>
<tr>
<td>3.2</td>
<td>Study Population</td>
<td></td>
</tr>
<tr>
<td>3.2.2</td>
<td>Inclusion criteria</td>
<td>19</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Exclusion criteria</td>
<td>19</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Sample Size</td>
<td>19</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Ethical Considerations</td>
<td>20</td>
</tr>
<tr>
<td>3.3</td>
<td>Experimental procedures</td>
<td>20</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Stool sample collection and quantification of eggs</td>
<td>20</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Parasitologic examination</td>
<td>21</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Collection of blood samples</td>
<td>22</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Hematological investigations</td>
<td>22</td>
</tr>
<tr>
<td>3.3.5</td>
<td>Immunophenotyping of blood</td>
<td>22</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Flow acquisition and cytometry analysis</td>
<td>23</td>
</tr>
</tbody>
</table>
CHAPTER FOUR: RESULTS

4.1 General characteristics of the study population based on *S. mansoni* intensity 25

4.2 General characteristics of the study population based on age 27

4.3 Cell surface staining and Flow cytometry analysis 29

4.4 T regulatory cells, Programmed Cell death-1 and *S. mansoni* egg intensities 31

4.4.1 T regulatory cells and intensity of *S. mansoni* infection 31

4.4.2 Programmed Cell Death -1 MFI expression and the intensity of infection 33

4.5 Relationships between T regulatory cells, Programmed Cell death-1 and Helminthic intensities 35

4.5.1 Percentage T regulatory cells proportions are not altered by the intensity of helminths in *S. mansoni* infection 35

4.5.2 Percentage CD25hi/PD1 MFI expression does not vary with different intensities of other helminthic co infection with *S. mansoni* 37

4.6 Relationships between Activated effector T cells (CD25high/HLA-DR+) and *S. mansoni* egg intensities (EPG) 39

4.7 Expression of T cell subsets in children with *S. mansoni* single infection and *S. mansoni* co infected with malaria 41

4.8 *Plasmodium falciparum* suppress T cell activation in Human schistosomiasis mansoni 43

4.9 *Plasmodium falciparum* infection dampens memory development
CHAPTER FIVE: DISCUSSION

5.1 Introduction to discussions

5.2 Prevalence of falciparum malaria in human schistosomiasis mansoni

5.3 T regulatory cells and PD-1 and variability in S. mansoni egg intensities (EPG) and other helminthic infections

5.4 Effects of Plasmodium falciparum Infection suppress activated T cells in Human Schistosomiasis mansoni

5.5 Effects of Plasmodium falciparum infection in human schistosomiasis mansoni slow memory development

CHAPTER SIX: CONCLUSIONS, RECOMMENDATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

6.1 Conclusions

6.2 Recommendations and Suggestions for future research

REFERENCES

APPENDIX I: Recipe for the flow cytometry reagents

APPENDIX II: Consent explanation and consent forms

APPENDIX IV: Flow cytometry experimental design

APPENDIX V: The map of the study area
LIST OF TABLES

Table 4.1: General characteristics of the study population based on Schistosoma mansoni egg intensity

Table 4.2: General characteristics of the study population based on age.

Table 4.3: Comparisons of expression of T cell surface markers among children with S. mansoni single infection and S. mansoni co-infected with malaria.
LIST OF FIGURES

Figure 2.1: Positive and negative roles of immunoregulation during parasitic infections 11

Figure 4.2A-D: Flow cytometry analysis of whole blood cells 30

Figure 4.3: T regulatory cell sub-population correlated with the different S. Mansoni egg intensities (EPG) - Low, medium and high 32

Figure 4.4: Programmed Cell death 1 (PD-1) Mean Fluorescent Intensity (MFI) correlated with different S. Mansoni egg intensities (EPG) - Low, medium and high 34

Figure 4.5: T regulatory cell proportions compared in different Helminthic intensities in S. Mansoni infection – Negative, Low and medium intensities 36

Figure 4.6: CD25hi/PD1 MFI expression compared in different Helminthic intensities in S. Mansoni – Negative, Low and medium intensities 38

Figure 4.7: Relationship between CD3+/CD4+/CD25^{med}/HLA-DR compared by S. mansoni egg intensity 40

Figure 4.8: Plasmodium falciparum infection suppress T cell activation in human schistosomiasis mansoni 44

Figure 4.9: Plasmodium falciparum infection suppress T cell activation in human schistosomiasis mansoni 46
### LIST OF DEFINITIONS, ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen Presenting cells</td>
</tr>
<tr>
<td>CD25</td>
<td>IL-2 receptor</td>
</tr>
<tr>
<td>CD3</td>
<td>Signaling part of T cell receptor complex</td>
</tr>
<tr>
<td>CD4</td>
<td>Antigen expressed on human T helper cells and thymocytes</td>
</tr>
<tr>
<td>CD45RO</td>
<td>Surface marker associated with memory.</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed Cell Death 1</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocynate</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells (CD3^+CD4^+CD25^{hi})</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Leucocyte Antigen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>R-phycoerythin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Activated Peridinin-chlorophyll-protein Complex</td>
</tr>
<tr>
<td>SEA</td>
<td>Soluble egg antigen</td>
</tr>
<tr>
<td>SWAP</td>
<td>Soluble worm antigen preparation</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
</tbody>
</table>
ABSTRACT

Studies of epidemiology and immune parameters of individuals co-infected with schistosomiasis and malaria have suggested strong support for involvement of both diseases in increasing morbidity and pathology of either disease. This has been attributed to shifts in the T regulatory cell (Treg) populations and Programmed Cell Death-1 (PD-1) expression on T cells, which are the indicators of immunoregulation in parasitic infections, leading to further dampening of the specific immune response to either of the disease. There is however a fundamental lack of understanding of the total mechanisms by which malaria infection interacts with the host immune system in *S. mansoni* infection. This study aimed at investigating the effects of falciparum malaria on levels of expression of T-regulatory subsets in pre-teen school children in Asembo Rarieda division infected with *Schistosoma mansoni*. The effects of intensity of infection with *Schistosoma mansoni* on expression of Treg and PD-1 subsets, and the differences in expression of T cell subsets - CD3, CD4 and CD25 in schistosomiasis mansoni single infection and schistosomiasis mansoni co-infection with *P. falciparum* were investigated. Four-color flow cytometric immunophenotyping of T-lymphocytes was performed using combinations of monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin (APC). The intensity of schistosome infection had no influence on expression of both Treg (p = 0.7629) and PD-1 (p = 0.2355), or effector T cell activation (CD3+/CD4+/CD25^{med}/HLADR^+) (r = -0.04524; p = 0.5787). There was also no significant difference in the mean percentage expression of CD3^+ T cells (73.7%) in *S. mansoni* single infection and *S. mansoni* and malaria co-infection (76.1%) (p=0.1572). Similarly, no significant statistical difference was found in
either the mean percentage expression of CD4^+ T cells in *S. mansoni* single infection (55.4%) and *S. mansoni* and malaria co-infection (55.4%) (p=0.9958) or in the Treg (CD3^+CD4^+CD25^hi). The mean percentage expression of CD3^+CD4^+CD25^hi was 1.57 in *S. mansoni* single infection and 1.67 in *S. mansoni* and malaria co-infection (p=0.5125). Children with *Schistosoma mansoni* and *P. falciparum* malaria however showed significantly reduced expression of activated T cells (CD3^+CD4^+CD25^{med}/HLA-DR+) (p=0.0173). Similarly, expression of Treg cell with memory (CD3^+CD4^+CD25^{hi}/CD45RO) was significantly lower in children with *S. mansoni* and *P. falciparum* double infections compared to those with *S. mansoni* infection alone (p=0.003). Thus schistosomiasis and malaria have deleterious effects on the host, both as single infections or double infections. These findings suggest that parasitic diseases such as schistosomiasis and malaria that often co-exist together have major effects on development of immunity to either of them and that control of schistosomiasis may have additional benefits with respect to the malaria epidemic in Sub-Saharan Africa. Hence integrated control of parasitic diseases is the way forward.
CHAPTER ONE: INTRODUCTION

1.1 Background information

Schistosomiasis and malaria are parasitic diseases that are co-endemic in regions of the world, where their devastating consequences are felt by communities least able to cope. The presence of these parasites results in high rates of co-infections (Petney and Andrews, 1998), which raises many questions as to the level of interactions, and their effects on morbidity of the affected populations.

Schistosomiasis and malaria are major parasitic diseases in developing countries whose epidemiological coexistence is frequently observed (Petney and Andrews, 1998; Nacher et al., 2000; Le Hesran et al., 2004; Sokhna et al., 2004; Briand et al., 2005; Lyke et al., 2005; Mwangi et al., 2006). Schistosomes are parasitic trematodes that infect over 200 million people worldwide (Engels et al., 2002). Malaria is one of the most prevalent parasitic diseases in tropical countries, with 500 million clinical cases reported yearly, resulting in 1 million deaths (WHO, 2000a). The pathology of malaria is caused by the asexual blood stages of the parasite, which have been the focus of many studies concerning protective immunity.

Mixed infection with these two parasites has been reported to have epidemiological impacts on either infections and may influence the clinical manifestation of either disease (Nacher, 2001; Druilhe and Tall, 2005) as well as development of acquired resistance to either or both parasites. Mixed infections may also have an important role on the regulation of inflammatory responses associated with the development of these infections.
and their respective morbidity (Mutapi et al., 2000; Nacher, 2001; Mwangi et al., 2006). Although major advances have been made towards the development of efficacious strategies for control of parasitic diseases such as malaria and schistosomiasis, there is still a great need for vaccines against parasitic infections. Currently, there are challenges and high costs associated with alternative control programs based on improvements in socio-economic conditions, sanitation, hygiene and vector control. Chemotherapy, although used to a great extent has the constant concern of drug resistance, and the need for repeated treatments.

One of the greatest drawbacks in developing a vaccine against parasitic diseases is the lack of complete understanding of the types of immune responses needed for protection. A better understanding of the immunological components involved in the development of protection against infection is needed to lay the foundation for the advancement of efficacious vaccines development (Todd and Colley 2002). There may be an absolute need to identify appropriate, protective antigens and mechanisms involved in protection against these complex organisms.

Chronic parasitic infection requires generation of controlled immune responses and failure to establish or to maintain homeostatic conditions usually lead to morbidity. Due to their dominant control of immune responses, appropriate T regulatory cells (T_{reg}) are thought to be important for suppression of immune responses, which is essential for the maintenance of efficacious defensive responses and the limitation of associated tissue damage due to excess inflammation.
Parasites have mechanisms of inducing regulatory responses normally associated with the termination of effector immune responses of the host. This is achievable by induction of host immune regulatory cytokines such as IL-10 and transforming growth factor β, which are produced by innate immune cells in response to pathogen-driven molecules or indirectly through the generation of regulatory cells (Mahanty et al., 1996; Plebanski et al., 1999).

Regulatory T cells (Treg) can be controlled to enhance memory responses that are of importance in vaccine development and enhancement of immune regulatory elements by parasites may contribute to failure of the memory development. Regulatory T cells can clearly control the intensity of secondary responses to infections (Mendez et al., 2004). Natural Treg are able to hamper the efficacy of vaccines against infectious agents (Kursar et al., 2002; Toka et al., 2004). Programmed Cell Death – 1 (PD-1) is an immunoregulatory co-receptor which can be expressed on activated T and B cells (Agata et al., 1996; Chen, 2004). Programmed Cell Death – 1 is a monomeric (Zhang et al., 2004) 55kDa membrane protein with a cytoplasmic tail that contains one immunotyrosine inhibitory motif (ITIM) (Shinohara et al., 1994). A regulatory role for PD-1 has been demonstrated by the development of PD-1 deficient mice, which develops life-threatening hyper-reactivity, lymphoproliferative/autoimmune diseases, including arthritis, lupus-like glomerulonephritis, diabetes, encephalomyelitis and cardiomyopathy (Nishimura and Honjo, 2001; Liang et al., 2003).
In schistosomiasis, it has recently been shown that up-regulated macrophage expression of PD-1, but not Programmed Cell Death Ligand -2 (PD-L2), can function to anergize T cells early in infection, through a contact-dependent mechanism (Smith et al., 2004). This worm-induced, F4/80+ macrophage-mediated induction of anergy via PD-1, independently of IL-4, IL-13, IL-10, TGF-B or NO (Smith et al., 2004), may explain earlier macrophage/adherent cell regulatory events in human schistosomiasis (Todd et al., 1979; Tweardy et al., 1987). It seems clear that PD-1, Programmed Cell Death Ligand – 1 (PD-L1) and PD-L2 – related mechanisms can play regulatory roles in both autoimmune and infectious diseases, including schistosomiasis and malaria.

1.2 Statement of the problem

Studies of co-infections between S. mansoni and P. falciparum have been carried out in other areas within and outside Kenya and have shown increased morbidity due to either of the disease as a result of the co-infection. For example, a recent study on S. mansoni and P. falciparum co-infections showed increased malaria infections in children with S. mansoni (Sokhna et al., 2004). It has also been shown that malaria may increase the level of morbidity in hepatosplenic schistosomiasis, and alter the host immune response towards schistosome antigens (Vernnervald and Dunne, 2004).

Although P. falciparum, S. mansoni and other Schistosome species are responsive to chemotherapy, most of the drugs are extremely expensive to most affected communities who are less able to cope, so most people do not get proper treatment. Therefore,
understanding of the immunoregulation of both or either of the disease is essential to ultimate design interventions such as vaccines.

1.3 Research questions

a) What are the effects of *P. falciparum* co-infection in *Schistosoma mansoni* infected children on T regulatory lymphocyte (CD3+/CD4+/CD25\textsuperscript{hi}) subpopulation and Programmed Cell Death – 1 (PD1) expression on Regulatory T cells compared to those with *S. mansoni* infection alone?

b) What are the effects of the presence of falciparum malaria in *S. mansoni* patients on the activation of T cells (CD3+/CD4+/CD25\textsuperscript{med}/HLA-DR)?

c) What are the effects of *P. falciparum* co-infection in *S. mansoni* infected children on memory T cell development?

1.4 Hypotheses

a) There are no differences in expression of T regulatory lymphocyte (CD3+/CD4+/CD25\textsuperscript{hi}) subpopulation and PD-1 expression on Regulatory T cells in schistosomiasis mansoni patients as a result of *P. falciparum* co-infection.

b) There is no alteration in the activation of T cells (CD3+/CD4+/CD25\textsuperscript{med}/HLA-DR) as result of *P. falciparum* co-infection in *S. mansoni* children aged 8-10 years.
c) Co-infection of Schistosomiasis mansoni and *Plasmodium* malaria has no effect on memory T cell development in children aged 8–10 years.

1.5 **Objectives of the Study**

1.5.1 **General objective**

To determine the effects of co-infection by *S. mansoni* and *P. falciparum* on expression of regulatory T cell subsets in pre-teen children.

1.5.2 **Specific objectives**

a) To determine the frequencies of natural Treg (defined by CD3⁺/CD4⁺/CD25^{hi}) cells as a percentage of total CD4⁺ cells in *S. mansoni* patients compared to *S. mansoni* and *P. falciparum* co-infected patients.

b) To determine the frequencies of cells expressing programmed cell death-1 (PD-1) receptors as a percentage of total CD4⁺ in *S. mansoni* and *P. falciparum* co-infected patients compared to those with *S. mansoni* single infection.

c) To determine the frequencies of activated T cells (CD3⁺/CD4⁺/CD25^{med}/HLA-DR⁺) as a percentage of Regulatory T cells resulting from *P. falciparum* co-infection in *S. mansoni* patients.

d) To determine the frequencies of memory T cells (CD45RO) as a percentage of regulatory T cells in children with dual infection of *S. mansoni* and *P. falciparum*.
1.6 Study Justification

Children who live in the tropics are exposed to parasitic diseases, and are particularly vulnerable to malaria and chronic helminthic infections. Studies have shown that children are more likely to suffer higher prevalence and/or particularly high intensity of helminthic infections compared to adults (Manson-Bahr and Apted, 2002). Recent studies show that co-infections of malaria and schistosomiasis impact on the outcomes of both diseases. It has been shown that malaria may increase the level of morbidity in hepatosplenic schistosomiasis, and alter the host immune response towards Schistosome antigens (Vernnervald and Dunne, 2004). It has also been reported that *S. mansoni* and *P. falciparum* co-infections leads to increased malaria attacks in children with *S. mansoni* (Sokhna *et al.*, 2004).

These studies suggest that *S. mansoni* and *P. falciparum* co-infection may cause shifts in the Treg populations and PD-1 expression on T cells as a percentage of total lymphocytes which may be indicators of immunoregulation in parasitic infections, further dampening of the specific immune response to either of the diseases. Studies on interaction of these two parasites (*S. mansoni* and *P. falciparum*) and their influence on host immunity is needed and will give valuable insights that may have far reaching public health implications in the management and control of these two infections.
CHAPTER TWO: LITERATURE REVIEW

2.1 Clinical manifestation of schistosomiasis mansoni and falciparum malaria diseases.

Schistosomiasis is a parasitic disease of a public health concern affecting approximately 200 million people in 74 tropical and subtropical countries worldwide (Chitsulo et al., 2000). Over 20,000 deaths are associated with severe consequences of infection, including bladder cancer or renal failure in *Schistosoma haematobium* and liver fibrosis and portal hypertension usually associated with *S. mansoni* (WHO, 2000b). Chronic infection has impact on health due to repeated infection and development of non-fatal but debilitating sequelae, such as granulomatous inflammation and fibrosis. Malaria is one of the most serious infections of human beings, with 600 million clinical cases and 2 million deaths each year (WHO, 2000a).

In most areas where malaria is endemic, helminthic infections are highly prevalent (Vernnervald and Dunne, 2004), and evolution of host immune responses to both types of parasites may have adapted to the presence of each other. Recent analyses of helminths (*Ascaris* and *Schistosoma* species) and *Plasmodium* co-infection in human beings shows an interaction between these parasites leading to modulation of immune responses to malaria (Hartgers and Yazdanbakhsh, 2006).

Chronic parasitic infection requires the generation of a controlled immune response that recognizes the invading pathogen and that limits the potentially harmful host response. The parasite avoids elimination by the host immune response, while at the same time
delaying or preventing host destruction. Thus, during parasitic infection, immune regulation can arise as the downstream effect of host response to the parasitic process and/or can be actively induced by the parasite as a defense strategy (Pearce et al., 1999).

The modulation of immune responses by a parasite is achieved through a complex adjustment of innate and acquired immune response of the host – a process that tends to maintain a ‘homeostatic’ environment. Parasites have developed strategies to do this; these include evasion of humoral and cellular immunity by antigenic variation, interference with antigen processing and presentation, and subversion of phagocytosis and killing by cells of the innate immune system (Sacks and Sher, 2002). A striking example of this phenomenon is the recent demonstration that *Leishmania major* actively induces Interleukin 10 (IL-10) – producing CD25+ T regulatory cells to prevent complete clearance of the parasites (Belkaid et al., 2001).

It has been recognized that T cells with suppressive or IL-10 producing T cells could be generated *in vivo* during parasitic infection (Mahanty et al., 1996; Plebanski et al., 1999), through decades of T suppressive studies, the concept of specialized subsets of regulatory T cells (Treg) contribute to this regulatory network has been shown. Several Treg types have been described based on their origin, generation and mechanism of action, with two main subsets identified i.e. ‘natural’ Foxp3+CD4+CD25hi Treg, which develop in the thymus and regulate self-reactive T cells in the periphery, and ‘inducible’ Treg (e.g. Th1 or Th3 cells), which can develop in the periphery from conventional
CD4+ T cells. Both types of Treg have been indicated to play a major role in the control of some of human parasitic infections.
Interactions between the parasite and the host that ranges from uncontrolled parasite growth to sterile elimination. Immunoregulatory activities like action of natural Treg can play a role at both extremes of the host parasite interaction. Immunoregulation can both control parasite induced pathology and at the same time lead to uncontrolled growth of the parasite and eventual death of the host. Immunoregulation can also be beneficial by controlling immunopathology. (Adapted from current opinions on immunology) (Belkaid et al., 2006)
2.2 Natural regulatory T cells control tissue damage during parasitic infection

Some studies on natural Treg emphasized that such cells control the extent of immune-mediated pathology in helminthic infections. Activated natural Treg efficiently control pathogenic T cells and innate responses in a model of murine colitis, allowing minimized collateral tissue damage (Liu et al., 2003; Powrie et al., 2003). Similar to the situation in gut tissue, during chronic parasitic infection the host must maintain constant immune pressure. Natural Treg are necessary to monitor this response and to prevent detrimental tissue damage especially in protection of sensitive tissues or tissues that have highly specialized functions such as liver that requires Treg-mediated control of immunopathology (Wilson et al., 2007).

Chronic infection with *S. mansoni*, which is associated with immunopathology can create extensive damage of liver. Only tight control of the egg-induced immune response allows survival of the infected host (Hoffman et al., 2000). It has been shown that immunosuppressive CD4+CD25+ Treg isolated from hepatic granulomas and from lymphoid tissues is a main producer of IL-10 in schistosome-infected mice (Hesse et al., 2004; Mckee and Pearce, 2004). In the non-healing model of *L. major* infection, pathology is controlled by natural Treg (Asseffa et al., 2002; Xu et al., 2003). *Leishmania amazonensis* infection in mice is characterized by the accumulation of natural Treg at sites of infection that transiently down-regulate immunopathology (Ji et al., 2005).
Another potential benefit of Treg responses to the host during parasitic infections is the consequence of their bystander effects. Recently, the concept of a hygiene hypothesis emerged; which stated that increasing rates of allergy and asthma in Western countries are a consequence of reduced infectious stressors during early childhood (Willis-karp et al., 2001). The mechanistic explanations appear to be associated with a ‘counter regulatory’ model that involves induction of various Treg populations during infections. Experimental work has lent strong support for this hypothesis (Maizels, 2005).

During gastrointestinal infection, helminth-driven natural Treg suppression of effector function is responsible for protection from airway inflammation (Wilson et al., 2005). Thus, microbial pressure in the gut or other peripheral tissues could lead to the maintenance of a pool of activated Treg (both natural and inducible) that would maintain host immune homeostasis and enhance the threshold required for immune responses.

2.3 Regulatory T cells and beneficial relationship for the host and the parasite

Even when natural Treg successfully preserve host homeostasis by controlling excessive immune responses, one consequence of such control is enhanced pathogen survival and, in some cases, long-term pathogen persistence. In a resistant murine model (Lishmania Major), mice remain chronically infected at the site of primary infection (Aebischer et al., 1993). Natural Treg accumulate at the site of infection and control, through IL-10-dependent and -independent mechanisms, the local function of effector cells. This ensures the long term survival of the parasite in the immune host (Belkaid et al., 2002).
Parasite persistence through immune suppression by natural Treg thus provides a major benefit to the host by enabling development of life-long immunity to reinfection. This model illustrates the fine balance that can be established between pathogens and hosts and how this equilibrium can become mutually beneficial.

2.4 Detrimental roles of natural regulatory T cells to the host during parasitic infection

Excessive regulatory control may allow parasite to expand in an uncontrolled manner and thus fail to secure host survival. In a murine model of malaria, for example, depletion of natural Treg protected mice from death caused by the lethal strain of *Plasmodium yoelii* by restoring a vigorous effector immune response, which eradicated the parasites (Hisaeda et al., 2004). Filaria nematode infection is associated with a profound downregulation of the host immune system. When mice are infected with *Brugia pahangi*, natural Treg expand and suppress excessive Th2 responses (Gillan and Devaney, 2005). In a model of murine filarial infection, the infection and subsequent immunosuppression is associated with accumulation of Treg in the thoracic cavity (Taylor et al., 2005).

Similarly, in humans infected with *P. falciparum*, removal of Treg enhances peripheral blood mononuclear cell proliferative and IFN-γ responses to malaria antigen (Walther et al., 2005). The non-healing lesions caused by a specific strain of *L. major* in mice were associated with enhanced IL-10 production and Treg presence at the site of infection (Anderson et al., 2005). Enhancement in the number of natural Treg in mice chronically infected with *L. major* was sufficient to trigger disease reactivation and to inhibit the
effector memory response (Mendez et al., 2004). Thus, over expression of Treg regulatory function, either from the endogenous pool or induced by the infection, can clearly become detrimental to the host by favoring excessive parasite expansion.

2.5 Controlling regulatory functions to favor parasite control

The capacity of a host to mount an effective immune response is limited by the pre-existence of counter regulatory elements. Targeting the molecules involved in regulatory cell activity in vivo such as cytotoxic T Lymphocyte-associated -4 (CTLA-4), transforming growth factor beta (TGF-b), or interleukin-10 (IL-10) alone or in combination has often proved effective to control a number of chronic infections (Gangappa et al., 1998; Murphy et al., 1998; Belkaid et al., 2001). Many mechanisms that boost immune responses and that favor the control of pathogens also abrogate Treg functions (Pasare and Medzhitov, 2003; Serra et al., 2003; Choi et al., 2004); the main target of this control appears to be activation of effector T cells that become unresponsive to natural Treg suppression. Far from being switched off by activation, natural Treg proliferate and their suppressive functions are boosted by encounters with activating signals (Caramalho et al., 2003; Yamazaki et al., 2003).

Strategies to manipulate natural Treg function or number clearly have high therapeutic potential. In a large number of infections in both mice and humans, depletion of natural Treg (based on expression of CD25) has resulted in enhanced effector immune responses (Suvas et al., 2003; Cabrera et al., 2004; Kinter et al., 2004; Mendez et al., 2004). Targeting CD25 might not always correlate with Treg neutralization. For example, some
natural Treg express the transcription factor Foxp3 but do not express CD25. Also, CD25 is transiently expressed on effector T cells. Targeting glucocorticoid-induced TNF family-related receptor (GITR), some of which is constitutively expressed by natural Treg and is induced on activated T cells in vivo, has also shown significant results (He et al., 2004; Taylor et al., 2005). Because Treg are central to the control of host homeostasis, systemic strategies might not be applicable in vivo in humans. Such strategies could bear the risk of triggering autoimmune disorders or uncontrolled pathological immune responses. Thus, there is clearly a need to develop strategies to control Treg function targeted at sites of infection.

2.6 Controlling regulatory T cell function to enhance memory responses

Currently no vaccines are available against human parasitic diseases. The enhancement of immune regulatory elements by parasites might contribute to this failure. Treg can clearly control the intensity of secondary responses to infections (Mendez et al., 2004). Likewise, natural Treg are able to hamper the efficacy of vaccines against infectious agents (Kursar et al., 2002; Toka et al., 2004). In a model of vaccination against murine malaria, depletion of natural Treg can induce a more durable immunity and better control of the parasite burden compared with vaccine alone (Moore et al., 2005).

Notably, such depletion also allowed an enhanced T cell response to subdominant epitopes (Moore et al., 2005). This may be particularly crucial for vaccines against parasitic infection, because some parasites usually have few, if any, dominant antigens.
2.7 Controlling regulatory T cell function to prevent immunopathology

Some pathologies are the consequence of uncontrolled immune responses. Induction or activation of regulatory elements represents a therapeutic objective when tissue damage is excessive. In a murine model of colitis, the transfer of natural Treg was sufficient to control established inflammatory disease (Mottet et al., 2003). Increasing natural Treg function or number could potentially be achieved by providing a cytokine milieu that favors natural Treg activity or survival, such as the presence of IL-2 or TGF-β. In a model of murine schistosome infection, retroviral transfer of the Foxp3 gene at the onset of granuloma formation enhances Foxp3 expression in the granuloma and strongly suppresses granuloma development (Sigh et al., 2005). A similar approach performed on antigen-specific cells might represent a powerful way to generate a large number of antigen-specific Treg that could target sites of infection and tissue damage.
CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

Schistosomiasis mansoni and Plasmodium malaria are both highly prevalent in western Kenya and particularly Rarieda division (Asembo) where this study was carried out 50 kms from Kisumu city (Appendix IV). Schools in this area, are those with over 50% prevalence of *S. mansoni*, generally within 3 km of the lake (Handzel *et al.*, 2003). Malaria prevalence in this study area is approximately 71.5% according to a Hospital-based Health and Demographic Surveillance study in Western Kenya which considered 5,409 sick children presenting to the Hospital (Adazu *et al.*, 2005). This area experiences some of the highest malaria transmission in the world (Bloland *et al.*, 1999). Rarieda Division (Asembo) covers an area of 178.4 km$^2$ and consists of approximately 79 villages with a total estimated population of 56,883 persons (GOK., 2000) with 99% of them being from the Luo ethnic group. Most of the people in this area are subsistence farmers who are also involved in fishing in Lake Victoria. Rainfall peaks two times a year with the long rains between March and June and the short rains between November-December (Bloland *et al.*, 1999).

3.2 Study population

School going pupils aged between 8 to 10 years (pre-teen age) in Asembo schools were recruited into the study following parental informed consent and assent by the minors. This age group was chosen because pre-teens have comparably higher prevalence of schistosomiasis than other age groups and are yet to undergo “age related resistance” to infection by *S. mansoni* (Manson-Bahr and Apted, 2002). Schools from which study
participants were selected generally within 1.5 km of the lake and had over 50% prevalence of *S. mansoni* as reported in an earlier study (Handzel *et al.*, 2003).

### 3.2.2 Inclusion criteria

Study participants were included in the study if they were permanent residents of the study area, aged between 8 and 10 years at the time of recruitment, were school going pupils, their guardian/parent gave consent, and they voluntarily gave assent.

### 3.2.3 Exclusion criteria

Study participants were excluded from the study if they were not residents of the study area, and younger or older than 8-10 years of age at the time of recruitment, were not in school, their guardian/parent did not give consent, and they were not willing to participate in the study or give assent.

### 3.2.1 Sample size

Study participants were recruited from 8 schools in Asembo Bay all of which were within 3 km of Lake Victoria. All parents of children aged 8 – 10 years were approached for consenting. The sample size for this study was based on an earlier reported prevalence of 50% (Handzel *et al.*, 2003). Upon parental consent, a total of four hundred and eighty five (485) pupils volunteered for parasite screening for both *S. mansoni* and *P. falciparum*, out of which one hundred and seventy nine (179) were positive for *S. mansoni* eggs. One hundred and fifty three (153) volunteered further to be included in the immunological assays out of which thirty-two (32) had both *S. mansoni* and *P.*
falciparum infections. Thus data analyzed herein is based on: 32 study participants positive for both schistosomiasis mansoni and Plasmodium malaria and 121 study participants’ positive for schistosomiasis mansoni only.

3.2.4 Ethical considerations

Approval of the study was obtained from the Scientific Steering Committee of the Kenya Medical Research Institute as well as the National/KEMRI Ethical Review Committee and Kenyatta University. Informed consent from the parents/guardians and assent from the children was obtained, and, permission from the schools and the community was obtained before the study participants were enrolled into the study. Consent forms and participation information was given in the language they best understood (English, Kiswahili, or Luo; Appendix II). A coded identification number was assigned to each participant and used for purposes of confidentiality, sample tracking and identification for treatment.

3.3 Experimental procedures

3.3.1 Stool sample collection and quantification of eggs per gram in stool.

Fresh stool samples were collected in stool cups (Elkay products, Inc. Shrewsburt, MA, USA) and transported to KEMRI – Kisian Schistosomiasis laboratory within the first three hours of collection. Quantitative S. mansoni egg production in stool was determined by Kato/Katz technique (Katz et al., 1972) as outlined in the bench Aids for the diagnosis of intestinal helminths (WHO, 1991) using the Helm Tec R kato/Katz kit (Helm Tec R p & D Pesquisa e Desenvolvimento Ltda. Belo horizonte, Brazil).
Using a wooden applicator stick, a small mound of the faecal sample was placed on a paper towel, and a small plastic screen (60-105 mesh) pressed on top so that some of the faecal material sieved through. The faeces accumulating on top of the screen was scrapped using a flat-sided plastic spatula. A hole made on a plastic plate of 41.7μ in the center was placed on the center of a microscope slide and the hole filled with faeces from the spatula before the template was removed.

The faecal material was then covered with a cellophane strip pre-soaked with 3% malachite green, 50% glycerol and 47% water, and the inverted microscope slide pressed firmly against a smooth surface to spread the faecal material evenly. Glycerol was allowed to clear the faeces for 20 minutes before hookworms screening, but *S. mansoni* eggs were determined at least 24 hours later after further clearing. The slides were examined systematically and the scored number of eggs multiplied by 24 to give the total number of eggs per gram (WHO, 1991). Ova of other helminths were also scored. In the current study, the three different groups based on egg intensities were grouped according to WHO standards of Low intensity 1–99, Medium intensity 100–399 and High intensity ≥ 400 (WHO, 1991).

3.3.2 Parasitologic examination of malaria parasites

Thick and thin blood smears were stained using 5% Giemsa solution and examined for malaria parasites. Parasite density per microliter (μl) of blood was calculated by counting the number of parasites per 300 white blood cells and multiplying by 40, assuming an average white blood cell count of 8000/μl.
3.3.3 Collection of blood samples

Following the microscopic confirmation of presence *S. mansoni* eggs, whole blood was collected into purple top EDTA tubes (Becton Dickinson, USA) by venipuncture and transported to the laboratory within three hours of collection.

3.3.4 Measurement hemoglobin

The hemoglobin measurements were carried out in blood at the collection site using a portable B-hemoglobin photometer (Hemocue AB Angelholm, Sweden).

3.3.5 Immunophenotyping of blood

Four-color flow cytometric immunophenotyping of T-lymphocyte was performed using combinations of monoclonal antibodies directly conjugated to flourescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) as described by Rodriguez *et al*, (1996). 100μL of the whole blood was pipetted into a labeled Falcon 2058 tubes and specific antibodies - PD-1 FITC, CD3 FITC, CD25 PE, CD4 PERCP, CD3 APC, HLA-DR APC, CD45RO APC (BD Biosciences Pharmigen, San Diego, A; e-Biosciences, San Diego, A) added then vortexed for about three seconds at low speed and tubes incubated at room temperature for 30 minutes with a brief mixing after fifteen minutes. Two millilitres of the diluted FACS lysing solution was added, inverted several times and incubated for another 10 minutes in the dark. This was followed with a 5 minutes centrifugation at 1500g at 20°C. The supernatants were then removed by aspiration and cells washed twice with 2-ml wash buffer followed by another 5 minutes centrifugation at 1500g. The cells were then fixed in 250 μL of a fixative reagent, vortexed and stored for
not longer than 24 hours at 2° - 8°C before acquisition. See appendix I for the recipes of the reagents, and appendix III for the flow cytometry experimental design.

3.3.6 Flow acquisition and cytometric analysis for regulatory T cells

T regulatory cell data was acquired on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer. The instrument was weekly checked using QC window beads (Flow Cytometry Standard, San Juan, PR). Forward scatter and side scatter measurements were made using linear amplifiers, whereas fluorescence measurements were made with logarithmic amplifiers. Flow cytometric dot plots and quadrant statistics were generated by CellQuestPRO software (Becton Dickinson Immunocytometry Systems). Analysis was performed after gating around a lymphocyte population on a forward scatter versus side scatter dot-plot. Analysis for T cell subsets was done gating on the CD3+ population.

3.4 Statistical Analysis

The proportion of the cells expressing the different surface markers was determined using CellQuestPRO software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Statistical analyses were performed using PRIZM software (Graph Pad Inc) and Microsoft Excel. One-way ANOVA with variance was used to examine possible correlations between EPG and Treg percentage, and EPG and PD-1 MFI percentage. Spearman's rank correlation test was used to examine the possible correlations between EPG and activated T cells. Two tailed t test was used to compare the expression of T cell surface markers, activated T cell, and memory T cells among children with S. mansoni.
single infection and *S. mansoni* co-infected with malaria. The level of significance was set at $p = 0.05$. 

Malaria prevalence was 29% in the low intensity group, 65.5% in the medium intensity group, and 89.5% in the high intensity group. These prevalence rates are comparable but there was significant variation among the groups with a mean (mean ± standard deviation) of 1.58 ± 3.58 for the medium intensity group, 5.00 ± 2.83 for the high intensity group, and 11.50 ± 8.64 for the low intensity group. Weight again was found to be significantly different among the groups with mean (mean ± standard deviation) of 30.15 ± 2.41 for low intensity group, 35.67 ± 2.77 for medium intensity group and 29.63 ± 4.22 for high intensity group.
CHAPTER FOUR: RESULTS

4.1 General characteristics of the study population based on *S. mansoni* intensity

The general characteristics of the children recruited into the study after a microscopic confirmation of presence *S. mansoni* eggs was summarized in three groups described in the experimental procedures: low intensity group with a mean egg per gram (EPG) (mean ± standard deviation) of 40.88 ± 26.54, medium intensity group with a mean EPG of 195.07 ± 85.08 and high intensity group with a mean EPG of 867.65 ± 448.97. The prevalence of other helminthes was 38.20% in the low intensity group, 43.90% in the medium intensity group and 43.48% in the high intensity group (Table 4.1).

Malaria prevalence was 29% in the low intensity group, 30.30% in the medium intensity group and 10.53% in the high intensity group showing that the low and medium intensity groups were comparable but there was low prevalence in the high intensity group. Hemoglobin levels in grams per deciliters (g/dl) was found to be comparable among the groups with a mean (mean ± standard deviation) of 11.2 ± 1.58 for low intensity group, 11.6 ± 1.39 for medium intensity group and 11.1 ± 2.06 for high intensity group. Participants height in centimeters was also found to be comparable among the groups with a mean (mean ± standard deviation) of 130.29 ± 8.56 for low intensity group, 131.54 ± 8.59 for medium intensity group and 133.74 ± 1.19 for high intensity group. Weight again was found to be comparable among the groups with a mean (mean ± standard deviation) of 27.75 ± 5.18 for low intensity group, 28.75 ± 5.37 for medium intensity group and 29.65 ± 4.22 for high intensity group (Table 4.1).
Table 4.1: General characteristics of the study population based on *Schistosoma mansoni* egg intensity

<table>
<thead>
<tr>
<th>Participant Condition</th>
<th>Low intensity <em>S. mansoni</em> (n = 89) (Mean ± SD)</th>
<th>Medium intensity <em>S. mansoni</em> (n = 41) (Mean ± SD)</th>
<th>High intensity <em>S. mansoni</em> (n = 23) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mansoni</em> Egg counts (EPG)</td>
<td>40.88 ± 26.54</td>
<td>195.07 ± 85.08</td>
<td>867.65 ± 448.97</td>
</tr>
<tr>
<td>Prevalence of other helminthes <em>(hookworm, Ascaris, Trichuris)</em></td>
<td>38.20%</td>
<td>43.90%</td>
<td>43.48%</td>
</tr>
<tr>
<td>Malaria prevalence</td>
<td>29%</td>
<td>30.3%</td>
<td>10.53%</td>
</tr>
<tr>
<td>Hemoglobin levels (g/dl)</td>
<td>11.2 ± 1.58</td>
<td>11.6 ± 1.39</td>
<td>11.1 ± 2.06</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>130.29 ± 8.56</td>
<td>131.54 ± 8.59</td>
<td>133.74 ± 1.19</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>27.75 ± 5.18</td>
<td>28.75 ± 5.37</td>
<td>29.65 ± 4.22</td>
</tr>
</tbody>
</table>

Values are arithmetic mean of the variables assessed in the three *S. mansoni* Egg intensity groups.
4.2 **General characteristics of the study population based on age**

The general characteristics of the children recruited into the study after a microscopic confirmation of presence *S. mansoni* eggs based on the differences in age was summarized in three groups based on age (8, 9 and 10 years old). The prevalence of other helminthes was 36.64% in the participants aged 8 years, 40% in the participants aged 9 years old, and 49.21% in the participants aged 10 years old showing a steady increase in prevalence with age indicative of more water contact in older children compared to younger ones (Table 4.2).

Malaria prevalence was 32% in the 8-year-old participants, 20.5% in 9-year olds and 27.6% in 10-year olds showing 9-year-olds having the lowest prevalence among the three groups (Table 4.2). Hemoglobin levels in grams per deciliters (g/dl) was found to be comparable among the groups with a mean (mean ± standard deviation) of 11.59 ± 1.03 in 8-year-old participants, 11.168 ± 1.737 in 9-year-old participants and 11.265 ± 1.758 in 10-year-old participants. Height in centimeters was also, as expected, continued in a steady increase with age having a mean (mean ± standard deviation) of 125.4 ± 8.406 in 8-year-old participants, 128.72 ± 8.425 in 9-year-old participants and 135.348 ± 6.655 in 10-year-old participants; the same trend followed by weight with a mean (mean ± standard deviation) of 25.3 ± 3.706 in 8-year-old participants, 26.74 ± 4.476 in 9-year-old participants, and 30.406 ± 4.16 in 10-year-old participants (Table 4.2).
Table 4.2: General characteristics of the study population based on age

<table>
<thead>
<tr>
<th>Study Participant status</th>
<th>8 years old</th>
<th>9 years old</th>
<th>Ten Years old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence of other helminthes</td>
<td>36.64%</td>
<td>40%</td>
<td>49.21%</td>
</tr>
<tr>
<td><em>(hookworm, Ascaris, Trichuris)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria prevalence</td>
<td>32%</td>
<td>20.5%</td>
<td>27.6%</td>
</tr>
<tr>
<td>Hemoglobin levels (g/dl)</td>
<td>$11.59 \pm 1.03$</td>
<td>$11.168 \pm 1.737$</td>
<td>$11.265 \pm 1.758$</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>$125.4 \pm 8.406$</td>
<td>$128.72 \pm 8.425$</td>
<td>$135.348 \pm 6.655$</td>
</tr>
<tr>
<td>Weight (Kgs)</td>
<td>$25.3 \pm 3.706$</td>
<td>$26.74 \pm 4.476$</td>
<td>$30.406 \pm 4.16$</td>
</tr>
</tbody>
</table>

Values are arithmetic mean of the variables assessed in the three Age groups.
4.3 Cell surface staining and flowcytometric analysis

Blood was collected by venipuncture into EDTA coated glass tubes (BD Vacutainer System, Franklin Lakes, NJ). To identify Treg in peripheral blood, anti-CD3 (FITC or APC conjugated, clone: UCHT1, e-Bioscience, San Diego, CA), anti-CD4 (FITC or PerCP conjugated, clone: OKT-4, e-Bioscience, San Diego, CA), and anti-CD25 (PE conjugated, clone: BC96, e-Bioscience, San Diego, CA) were used. Because anti-FOXP3 antibodies were not commercially available at the beginning of this study; Treg cells are defined as the CD3+/CD4+/CD25\textsuperscript{high} population. Gating to separate CD25\textsuperscript{neg} cells from CD25\textsuperscript{medium} cells was based on fluorescence minus one (FMO) stained samples. The gating used to distinguish CD25\textsuperscript{high} from CD25\textsuperscript{medium} cells was determined by examining the CD4+/CD25\textsuperscript{+} dot plots from all patients studied and setting thresholds that most often differentiated these subpopulations (CD25\textsuperscript{low}, CD25\textsuperscript{medium}, CD25\textsuperscript{high}). These thresholds (shown in Figure 4.3) were determined prior to any phenotypic analyses, were used for all analyses in this study, and are based on those used throughout the literature. To examine phenotypic subsets of Treg, the following antibodies were used: anti-PD-1 (CD279; FITC conjugated, clone: MIH4, e-Bioscience, San Diego, CA), anti-CD152 (APC conjugated, clone: BNI3, BD Pharmedgen, San Diego, CA), anti-CD45RO (APC conjugated, clone: UCHL1, BD Pharmedgen, San Diego, CA), anti-HLA-DR (APC conjugated, clone: LN3, e-Bioscience, San Diego, CA). FMO-stained samples were used to separate positive from all negative populations.
Figure 4.2a-d: Flow cytometry analysis of whole blood cells

Figure 4.2a showing the gating for all Lymphocyte, figure 4.2b showing gating for T cell (CD3), figure 4.2c showing the gating for Helper T cells (CD4), figure 4.2d a threshold was set between $CD25^{\text{medium}}$ and $CD25^{\text{high}}$ cells.

4.4.1 Treg and intensity of S. mansoni infection

Proportions of Treg in the peripheral blood of the children before treatment with praziquantel in relation to intensity of the infection was analyzed based on number of eggs per gram of feces (EPG) in the three different groups (low egg intensity n = 82, medium egg intensity n = 49 and high egg intensity n = 20). There was no significant statistical difference between intensity of infection and the percentage of Treg in circulation (Figure 4.3; p = 0.7629).
Fig. 4.3 Treg and intensity of *S. mansoni* infection

Figure 4.3: Percentages of natural Treg ($\%CD3^+/CD4^+/CD25^{hi}$) compared in different *S. mansoni* egg intensities. Low EPG, n = 82; Medium EPG n = 49; High EPG n = 20. No significant statistical difference between worm intensities and proportions of Treg.
4.4.2 Programmed cell death –1 expression on Treg and the intensity of infection.

Mean fluorescent intensities of PD-1 was also analyzed in the peripheral blood of the same patients before treatment with praziquantel in relation to intensity of the infection, based on the number of eggs per gram of feces (EPG) in the three different groups (low egg intensity n = 82, medium egg intensity n = 49 and high egg intensity n = 20). There was no significant statistical difference between intensity of infection and the percentage of PD-1 MFI in circulation (figure 4.4; p = 0.2355).
Fig. 4.4: Programmed cell Death –1 expression on Treg and the intensity of infection

Percentages of Programmed Cell death 1 (PD-1) expressed on Treg compared by different *S. mansoni* egg intensities. Low EPG, n = 82; Medium EPG n = 49; High EPG n = 20. *No significant statistical difference between PD-1 Mfi and proportions of Treg
4.5 Relationships between T regulatory cells, PD-1 MFI and Helmintic intensities

4.5.1 Percentage Treg proportions and the intensity of helminths in *S. mansoni* infection

Proportions (in percentages) of Treg in the peripheral blood of the children before treatment with praziquantel in relation to helminthic intensity of the infection was analyzed based on number of eggs recorded in the three different groups (negative patients for other helminths n = 82, low intensity n = 49 and medium intensity n = 20). There was no significant statistical relationship between intensity of helminthic infection and the percentage of Treg in circulation (Figure 4.5; \( p = 0.9485 \)).
Helminthic intensities in *S. mansoni* infection

**Fig. 4.5:** Percentage Treg proportions are not altered by the intensity of helminths in *S. mansoni* infection

Percentage CD3/CD4/CD25<sup>hi</sup> compared by *S. mansoni* infection alone (without other helminthic infection), with low intensity helminthic infection or with medium intensity helminthic infection. Low EPG, n = 82; Medium EPG n = 49; High EPG n = 20. *No significant statistical difference between presence of other helminthic infection and proportions of Treg*
4.5.2 CD3⁺/CD4⁺/CD25⁺/PD1 MFI expression in helminthic co-infection with *S. mansoni*

CD179, a marker for programmed cell death-1 (PD-1) expression was also analyzed in the peripheral blood of the children before treatment with praziquantel in relation to helminthic intensity. Based on the number of eggs of other helminths recorded in the three different groups (negative patients for other helminths n = 82, low intensity n = 49, and medium intensity n = 20), there was no significant statistical difference between intensity of helminthic infection intensity and the expression of CD3⁺/CD4⁺/CD25⁺/PD1 MFI in circulation in *S. mansoni* infected children (Figure 4.6; p = 0.4754).
Fig. 4.6: CD3+/CD4+/CD25^{hi}/PD-1 MFI expression in helminthic co-infection with *S. mansoni*

CD3+/CD4+/CD25^{hi}/PD-1 MFI compared by *S. mansoni* infection alone (without other helminthic infection), with low intensity helminthic infection or with medium intensity helminthic infection. Low EPG, n = 82; Medium EPG n = 49; High EPG n = 20. *No significant statistical difference between presence of other helminthic infection and proportions of Treg and PD-1 Mfi.*
4.6 Relationships between Activated T cells (CD25^{med}/HLA-DR^+ ) and *S. mansoni* egg intensities (EPG)

By gating CD4^+/CD25^+ based on the Fluorocrome Minus One (FMO) and examining all the patients’ data (*S. mansoni* positive at different intensities), effector T cells that were activated (CD25^{med}/HLADR+) were identified and were further analyzed by comparing their proportions in patients with different *S. mansoni* intensities (EPG). There was no significant statistical correlation between *S. mansoni* egg intensity (EPG) and CD25^{med}/HLADR+ (n = 153: r = -0.04524; p = 0.5787; Figure 4.7).
Fig. 4.7: Relationships between Activated T cells (CD25^{med}/HLA-DR^+) and S. mansoni egg intensities (EPG)

Percentage CD3^+/CD4^+/CD25^{med}/HLA-DR^+ compared by different S. mansoni egg intensity (EPG). (n = 153; p = 0.5787; r = 0.04524) No significant statistical correlation between Activated T cells (CD25^{med}/HLA-DR^+) and S. mansoni egg intensities (EPG)
4.7 Expression of T cells in children with *S. mansoni* single infection and *S. mansoni* co-infection with malaria.

Proportions of different subsets of T cells in the peripheral blood of the children with *S. mansoni* infection before treatment with praziquantel were analyzed in two groups, those with single *S. mansoni* infection and those with *S. mansoni* and malaria co-infection. The mean percentage expression of CD3 (T cells) was 73.74% in *S. mansoni* single infection and 76.05% in *S. mansoni* and malaria co-infection, but there was no significant statistical difference between the two groups (Table 4.3; \(p=0.1572\)).

Mean percentage expression of CD4+ T cells was 55.37% in *S. mansoni* single infection and 55.38% in *S. mansoni* and malaria co-infection, but there was no significant statistical difference between the two groups (\(p=0.9958\); Table 4.3). The mean percentage expression of activated helper T cells \((CD3^+/CD4^+/CD25_{med})\) was 13.54% in *S. mansoni* single infection and 11.22% in *S. mansoni* and malaria co-infection, but there was no significant statistical difference between the two groups (\(p=0.0575\); Table 4.3).

Proportions (in percentages) of Treg \((CD3^+/CD4^+/CD25_{hi})\) was also analyzed and the mean percentage expression of CD3+/CD4+/CD25_{hi} was 1.569% in *S. mansoni* single infection and 1.669% in *S. mansoni* and malaria co-infection, but there was no significant statistical difference between the two groups (\(p=0.5125\); Table 4.3).
Table 4.3: Comparisons of expression of T cell surface markers among children with *S. mansoni* single infection and *S. mansoni* co infected with malaria.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Phenotype</th>
<th><em>S. mansoni</em> single infection</th>
<th><em>S. mansoni</em> co-infected with malaria</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N = 90 Mean ± SEM</td>
<td>n = 32 Mean ± SEM</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>CD3+</td>
<td>(73.74 ± 0.8625)</td>
<td>(76.05 ± 1.211)</td>
<td>0.1572</td>
</tr>
<tr>
<td>Helper T cells</td>
<td>CD4+</td>
<td>(55.37 ± 0.9473)</td>
<td>(55.38 ± 1.736)</td>
<td>0.9958</td>
</tr>
<tr>
<td>Activated Helper T cells</td>
<td>CD3+/CD4+/CD25\textsuperscript{med}</td>
<td>(13.54 ± 0.5719)</td>
<td>(11.22 ± 0.7076)</td>
<td>0.0575</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>CD3/CD4/CD25\textsuperscript{hi}</td>
<td>(1.569 ± 0.07195)</td>
<td>(1.669 ± 0.1564)</td>
<td>0.5125</td>
</tr>
</tbody>
</table>

Table 4.3: T cell subsets expressed as percentage of cell in the CD3 gate. Differences in the means between the children with *S. mansoni* single infection and *S. mansoni* co-infected with malaria were assed using student T test. Standard Error of Mean (SEM) defined variation in each group.
4.8 *Plasmodium falciparum* infection and T cell activation in human *schistosomiasis mansoni*

In order to examine the effects of *P. falciparum* infection in human schistosomiasis mansoni on the proportion of Treg that express activation, the proportion of activated T cells (% CD3⁺/CD4⁺/CD25med/HLA-DR⁺) in children with *S. mansoni* single infection and those with both *Schistosoma mansoni* and falciparum malaria was analyzed. There was a significant statistical difference in the proportions of activated T cells (CD3⁺/CD4⁺/CD25med/HLA-DR⁺) in *S. mansoni* single infection compared to *S. mansoni* and malaria dual infections (p=0.0173; Figure 4.8).
Figure 4.8: *Plasmodium falciparum* infection suppress T cell activation in human *Schistosomiasis mansoni*

CD3⁺/CD4⁺/CD25<median>/HLA-DR⁺ T cell subsets expressed as percentage of CD3 gate. Malaria negative (n = 90; malaria positive n = 32; p = 0.0173) ** Significant statistical difference in the proportions of activated T cells in *S. mansoni* single infection compared to *S. mansoni* and malaria co-infection

4.9 *Plasmodium falciparum* infection and expression of memory Treg in human *Schistosomiasis mansoni*
To examine the effects of *P. falciparum* co-infection in schistosomiasis on Treg memory, proportion of Treg that express CD45RO, which is a maker for memory, was analyzed. The percentage of memory Treg (% CD3⁺/CD4⁺/CD25^{high}/CD45RO⁺) in children with *S. mansoni* single infection was compared to those with *S. mansoni* and *P. falciparum* double infections. There was a significant statistical difference in the proportions of memory Treg in patients with *S. mansoni* single infection compared to patients with *Plasmodium* malaria infection in human schistosomiasis mansoni (Malaria negative n = 90; malaria positive n = 32; p = 0.0030); Figure 4.9).
Fig. 4.9: *Plasmodium falciparum* infection suppress T cell activation in human schistosomiasis mansoni

Percentage CD3/CD4+/CD25hi/CD45RO T cell subsets expressed as percentage of cell in the CD3 gate. Malaria negative n = 90; malaria positive n = 32; p = 0.0173, ** Significant statistical difference in the proportions of memory Treg in patients with *S. mansoni* single infection compared to patients with *Plasmodium* malaria infection in human schistosomiasis mansoni
CHAPTER FIVE: DISCUSSION

5.1 Introduction to the discussions

Various studies have addressed immune responses to schistosomiasis using different age groups to correlate the acquisition of immunity. Immunity has been shown to develop during the second decade of life and resistance to re-infection after chemotherapy has been associated with older children and adults (Butterworth et al., 1985; Roberts et al., 1993). Co-infection of schistosomiasis and malaria has been reported to have epidemiological impacts on either infection and may influence the clinical manifestation of either disease. Development of acquired resistance to infection of either or both parasites may also have an important role on the regulation of inflammatory factors associated with the development of these infections and their respective morbidity (Mutapi et al., 2000).

In this study, the interaction between schistosomiasis and malaria in school going children aged between 8 years and 10 years is evaluated in an area where there is high prevalence of both infections in Asembo Rarieda division (Handzel et al., 2003; Adazu et al., 2005). The overall goals of this study were to determine whether P. falciparum infection in human schistosomiasis cause immunoregulatory differences in pre-teen school going children who have schistosomiasis mansoni. It was appreciated that the study area is also endemic for diseases other than schistosomiasis and malaria, notably other helminthes, hence the study attempted to determine whether the presence of these infections could influence the immune responses in human schistosomiasis.
5.2 Prevalence of falciparum malaria in human schistosomiasis mansoni.

This study has shown falciparum malaria prevalence was 29% in children with low *S. mansoni* infection (1-99/gram of faeces) and 30.30% in children with medium *S. mansoni* infection (100-399/gram of faeces). Generally this is a very high prevalence in both the groups and is consistent with earlier reports that showed increased malaria attacks in children with *S. mansoni* (Sokhna *et al.*, 2004). Even when natural Treg successfully preserve host homeostasis by controlling excessive immune responses, one consequence of such control is enhanced pathogen survival and, in some cases, long-term pathogen persistence which this current data possibly suggests. In a *L. major* resistant murine model, mice remain chronically infected at the site of primary infection (Aebischer *et al.*, 1993). Natural Treg accumulate at the site of infection and control, through IL-10-dependent and -independent mechanisms, the local function of effector cells. This ensures the long term survival of the parasite in the immune host (Belkaid *et al.*, 2002).

This study looked into the different groups based on intensity of *S. mansoni* eggs (EPG) and the striking observation was that low malaria prevalence in patients with high *S. mansoni* EPG (≥400/gram of faeces) was 10.53% - three fold below the low and medium intensity groups. It is unclear why there was low prevalence of malaria in heavy infections with schistosomiasis mansoni as this study hypothesis is based on earlier studies that suggested the activation of Treg cells that contributes to immune suppression during malaria infection, and helps malaria parasite to escape from host immune responses (Hisaeda *et al.*, 2004). Several investigators have provided evidence that cell mediated immunity and humoral immunity act in concert or sequentially to control and
clear blood-stage malaria infection (Langhorne, 1989; Stevenson and Tam, 1993; Taylor-
- Robinson et al., 1993). It is possible that concurrent schistosomiasis infection may alter
or enhance these immune responses since in response to helminthic infections at chronic
stages the host induces predominantly T helper 2 responses while in malaria infection the
host produces predominantly T helper 1 cytokines (Sokhna et al., 2004). Helminthic
infections have also been shown to shift immune responses towards the production of
non-cytophilic antibody subclasses (ImmunoglobulinG2 (IgG2), IgG4 and IgM), whereas
protection against malaria is associated with the presence of IgG1 and IgG3 cytophilic
antibody subclasses (Bouharoum-Tayoun and Druihe, 1992; Polley, 2003; Sigh et al.,
2004).

The observation in this study suggests that it may be necessary to consider intensity of
infection when dealing with dual infections, which the previous studies did not take into
account. This could further go along in unlocking the continuing controversy on the
immune modulation during malaria and helminthic infections as resent analyses indicate
interactions between Plasmodium malaria and helminths, findings for malaria-parasite
loads being controversial (Hartgers and Yazdanbakhsh, 2006). Some studies have shown
that co-infection increases parasitemia and clinical malaria, whereas others have shown
co-infection to have the opposite effect.

This study suggests that light or moderate S. mansoni intensity in schistosomiasis
mansoni patients increases parasitemia and clinical malaria, whereas heavy S. mansoni
intensity in schistosomiasis mansoni patients is parallels decreases in parasitemia and clinical malaria as previously observed by different investigators.

5.3 T reg and PD-1 expression in S. mansoni and other helminthic infections

Natural T regulatory cells have a major role in the control of immune responses in multiple settings, including thymic development, autoimmunity, atopic allergy, transplantation, and infectious diseases (Sakaguchi et al., 1995; Baecher-Allan et al., 2001; Jonuleit et al., 2001); (Levings et al., 2006). In human paracoccidiodomycosis, CD4⁺CD25⁺ cells (defined as Treg) accumulate in characteristic granulomatous lesions and exert strong suppressive activity on effector cells, implying that Treg might contribute to the regulation of these lesions. In murine experimental schistosomiasis, Treg responses control both Th1 and Th2 responses in an IL-10 independent manner (Baumgart et al., 2006; Taylor et al., 2006), and are associated with regulation of granuloma formation in chronic infections (Sigh et al., 2005).

In the current study, there was no significant difference in the Treg subpopulations and PD-1 expression among the three different groups (low intensity, medium intensity and high intensity) based on S. mansoni intensities (EPG). These observations suggests that in the acute stages of S. mansoni infections, once the female adult Schistosome starts laying eggs which are excreted in the feces it does not matter whether they are may or few but the immune responses remain the same at least for the regulatory factors. Similar observations were made in another unrelated study in our laboratory with occupationally exposed adult males, which showed that Treg percentages did not correlate with the
intensity of infection (Watanabe et al., 2007). This was said to have been confounded by the extremely high intensities of infection observed in the occupationally exposed cohort which is the same in this study where a good number of children had heavy intensity, far above the WHO number of ≥400 EPG classification of heavy infection.

The differences in Treg subpopulations and expression of PD-1 on T cell on persons with schistosomiasis mansoni and other helminth infection (commonly hookworm and Ascaris) compared with persons that had schistosomiasis mansoni single infection. There was no significant difference in the expression of PD-1 on T cells and Treg subpopulations of patients with the other helminthes in S. mansoni infection compared to those that had both schistosomiasis mansoni and the other soil transmitted helminthes. Studies have looked at helminthes as a group with the view of trying to investigate their interactions with other infections like malaria and HIV (Mwinzi et al., 2001). Analyses of helminth (commonly Ascaris and Schistosoma species) and Plasmodium malaria co-infection in human showed an interaction between these two parasites (Hartgers and Yazdanbakhsh, 2006). Interactions have also been observed in animal models where it has been shown in mice co-infected with Litomosoides sigmodontis and P. berghei to have cross-tolerance and Treg responses due to high circulating concentrations of helminth induced interleukin 10, which is essential in prevention of cerebral malaria (Specht S, unpublished data).

The observations in these studies therefore suggests that once a patient with schistosomiasis mansoni gets to acute stages characterized by excretion of S. mansoni
eggs, immune responses markers responsible for immunoregulation remain unaltered by presence of many worms/eggs. Helminthic infections other than schistosomiasis (commonly *Ascaris* and *Hookworm*) modifies the immune system in the same manner as *S. mansoni* particularly their dual infection does not affect the immunoregulatory factors like PD-1 expression on T cells and Treg subpopulations.

5.4 Effects of *Plasmodium falciparum* infection on activated T cells in human Schistosomiasis mansoni patients

To determine if falciparum malaria in human schistosomiasis mansoni patients would have effects on T cell activation, percentage proportion of effector T cells (% CD3⁺/CD4⁺/CD25med/HLA-DR⁺) in children with *S. mansoni* single infection and those with both schistosomiasis mansoni and falciparum malaria was analyzed. There was a significant statistical difference in the proportions of activated T cells (CD3⁺/CD4⁺/CD25med/HLA-DR⁺) in *S. mansoni* single infection compared to *S. mansoni* and malaria dual infections. In the current study, it was hypothesized that there would be an increase in the proportions of Treg due to the dual infection. Though there was no significant difference in the Treg populations in the two groups, their regulatory activity was observed to be more pronounced in the dual infection with schistosomiasis mansoni and falciparum malaria compared to the *S. mansoni* single infection. The mechanistic explanations for this appear to be associated with a ‘counter regulatory’ model that involves induction of various Treg populations during parasitic infections. Experimental work has lent strong support for this hypothesis (Maizels, 2005).
The current study suggests that *P. falciparum* infection in human schistosomiasis mansoni down regulates further the immune system as earlier reported by several investigators (Gangappa *et al.*, 1998; Murphy *et al.*, 1998; Belkaid *et al.*, 2001). The parasitic infections dampens the immune system by suppressing T cell activation, which is consistent with earlier reports that the capacity of a host to mount an effective immune response is limited by the pre-existence of counter regulatory elements where Treg is one of them (Belkaid *et al.*, 2006). This means that targeting the molecules involved in regulatory cell activity *in vivo* such as CTLA-4, TGF-b or IL-10 alone or in combination can effective in controlling chronic infections as was previously reported (Gangappa *et al.*, 1998; Murphy *et al.*, 1998; Belkaid *et al.*, 2001).

Many mechanisms that boost immune responses and that favor the control of pathogens also abrogate Treg functions (Pasare and Medzhitov, 2003; Serra *et al.*, 2003; Choi *et al.*, 2004); the main target of this control appears to be activation of effector T cells that become unresponsive to natural Treg suppression. Far from being switched off by activation, natural Treg proliferate and their suppressive functions are boosted by encounters with activating signals (Caramalho *et al.*, 2003; Yamazaki *et al.*, 2003).

Data in this study suggests that concomitant parasitic infections, in this case schistosomiasis mansoni and *falciparum* malaria leads to heightened down-regulatory cell activity, which is associated with counter regulation that has been shown in *vivo* to be controlled by molecules such as CTLA-4, TGF-b or IL-10 alone or in combination.
5.5 Effects of *Plasmodium falciparum* infection in human schistosomiasis mansoni on level of memory Treg

The differences in expressions of CD45RO, a marker for memory development as a percentage (\( \% \text{CD}3^+\text{CD}4^+\text{CD}25^{\text{high}}\text{CD}45\text{RO}^+ \)) in children with *S. mansoni* single infection was compared to those with schistosomiasis mansoni and *P. falciparum* double infections. There was a significant statistical difference in the proportions of expression of memory Treg in patients with *S. mansoni* single infection compared to patients with *Plasmodium* malaria infection in human schistosomiasis mansoni (\( p=0.0030 \)).

This observation is consistent with other investigators work that has shown that Treg dampens the immune system. Studies done in murine filarial infection, the infection and subsequent immunosuppression was found to be associated with accumulation of Treg in the thoracic cavity (Taylor *et al.*, 2005). Similarly, in humans infected with *P. falciparum*, removal of Treg enhanced peripheral blood mononuclear cell proliferation and IFN-\( \gamma \) responses to malaria antigen (Walther *et al.*, 2005). The non-healing lesions caused by a specific strain of *L. major* in mice were associated with enhanced IL-10 production and Treg presence at the site of infection (Anderson *et al.*, 2005). Enhancement in the number of natural Treg in mice chronically infected with *L. major* was sufficient to trigger disease reactivation and to inhibit the effector memory response (Mendez *et al.*, 2004).

Thus data in the current study suggests that falciparum malaria and schistosomiasis mansoni double infection can cause over expression of Treg regulatory functions, either
from the endogenous pool or induced by the infection. This could be a contributing factor to the delay in vaccines development against parasitic diseases. The enhancement of immune regulatory elements by parasites might contribute to this failure. Moreover, Treg can clearly control the intensity of secondary responses to infections (Mendez et al., 2004). Likewise, natural Treg are able to hamper the efficacy of vaccines against infectious agents (Kursar et al., 2002; Toka et al., 2004). In a model of vaccination against murine malaria, depletion of natural Treg can induced a more durable immunity and better control of the parasite burden compared with vaccine alone (Moore et al., 2005).

The current study suggests that while it is true that Treg alters development of immunity in parasitic infections including human schistosomiasis, it is more pronounced in double infections especially in falciparum malaria and schistosomiasis mansoni infection.
CHAPTER SIX: CONCLUSIONS, RECOMMENDATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

6.1 Conclusions

The following conclusions can be drawn from this study:

Expression of CD3⁺/CD4⁺/CD25^{high}/CD45RO on T cells indicative of memory development is reduced in children with *Plasmodium falciparum* co-infection with *Schistosoma mansoni* compared to children with *S. mansoni* alone.

Expression of CD3⁺/CD4⁺/CD25^{med}/HLA-DR on T cells indicative of T cell activation is reduced in children with *Plasmodium falciparum* co-infection with *Schistosoma mansoni* compared to children with *S. mansoni* alone.

Helminthic infection loads, and different *S. mansoni* infection egg loads does not alter Treg, HLA-DR expression and PD-1 expression in human schistosomiasis mansoni infection.

*Plasmodium falciparum* prevalence is similar in children with light and moderate intensities of *S. mansoni* infection but lower in children with heavy *S. mansoni* egg intensities in human schistosomiasis mansoni.

Falciparum malaria infection in human schistosomiasis mansoni does not alter the proportions of T cell subsets (CD3, CD4 and CD25).
6.2 Recommendations and suggestions for future research

This study has reported that malaria reduces the number of T cells expressing memory in human schistosomiasis. This study only looked at the expression of the T cell markers. Other aspects of the immune system warrant further analysis of this co-infection especially looking at the cytokine profiles.

Previous studies have not narrowed down to immune responses in humans to most of other helminthes other than *S. mansoni* whose effects on the proportion of Treg were investigated in this study. Though the difference was insignificant, these other helminthes might sometimes be found in isolation on particular hosts and it is worth studying to know their immune responses in isolation.

T regulatory cells in this thesis are described, as $CD3^+/CD4^+/CD25^{\text{high}}$, which is a partial definition of regulatory cells and may not be exclusive. At the time when the main study of which this study was part of, FOXP3, an intracellular molecule that further defines Treg wasn’t accessible to us. Future studies should involve more markers that have since been identified as markers for Treg.

This study has reported reduced malaria infection with high Schistosome intensity. This was not conclusive because of low sample size and may require further investigation with a bigger sample size and other varied age groups.
REFERENCES


Baumgart, M., Tomkins, F., Leng, J. and Hesse, M. (2006). Naturally occurring CD4+Foxp3+ regulatory T cell are essential, IL-10-independent part of the immunoregulatory network in *Schistosoma mansoni* egg-induced inflamation. *Journal of Immunology* 176, 5374-5387.


APPENDIX I: Recipe for the reagents

1. Wash Buffer
PBS, 0.1% sodium Azide, 1% FBS or BSA

4 Lysing Buffer
8.29g NH₄CL (0.15M), 1gKHCO₃ (10.0mM), 37.2mg Na₂EDTA (0.1mM), 800ml DH₂O (PH 7.2-7.4 with 1N HCL) adjust to 1 lt. And Filter sterilized.

5 Fixative
1% paraformaldehyde, PH 7.4

6 FACS lysing solution
BD FACs Lysing solution diluted 1:10 in dH₂O
Appendix II: Consent explanation and Consent forms

**TITLE OF THE STUDY:** Immune Responses In *Schistosoma Mansoni* And *Plasmodium Falciparum* Co-Infections In Pre-Teen School Going Pupils In Western Kenya.

**INSTITUTIONS**
Kenya Medical Research Institute, Center for Vector Biology and Control Research (KEMRI-CGHR), Kisumu Kenya.
School of Biological sciences, department of Zoological Sciences Kenyatta University, Nairobi, Kenya.

**STUDENT:**
MR. ERICK M.O. MUOK (BSc)
156/5735/2003
Department of Zoological Sciences, Kenyatta University, Nairobi, Kenya.

**SUPERVISORS:**
1. Prof. ZIPPORAH W. NG’ANG’A
Department Medical Laboratory Sciences, Jomo Kenyatta University, Nairobi, Kenya.
2. DR. MICHAEL M. GICHERU
Department of Zoological Sciences, Kenyatta University, Nairobi, Kenya.
3. DR. DIANA M.S. KARANJA
Center for Vector Biology and Control research (CVBCR), KEMRI, Kisumu, Kenya.
**Participation Information**

Your child is being asked to take part in a medical research performed by Kenya Medical Research Institute (KEMRI) and Kenyatta University. It’s of importance that you understand that-your child’s participation is entirely voluntary, you upon decision can withdrew your child from this study without any penalty and after reading about this study you can ask questions from the start or thereafter during the study for the betterment of your understanding.

**Introduction:**

Intestinal Schistosomiasis caused by *S. mansoni* and Malaria caused by *P. falciparum*, are both highly prevalent in western Kenya. The vectors are snails and Anopheline mosquitoes respectively. Persons whose activities cause them to come in contact with water infested with snails so there may be transmission going on are at risk of contacting the Schistosomiasis disease as well as those who have contact to infected anopheles mosquitoes are likely to get Malaria. Schistosomiasis can sometimes be serious and even fatal if not diagnosed and treated properly. Malaria on the other hand is responsible for the highest number of deaths in the tropics mostly in children and pregnant women. Earlier studies have shown very high prevalence of Schistomiasis around the lake. This study is designed to investigate the immune interactions in *P. falciparum* and *S. mansoni* co-infections and how immune responses to either of the individual parasite are influenced by the presence of the other parasite.
Purpose of the study

We will test for Schistosomiasis, malaria and other Helminths; to do this we will need to take some of your child's feaces and finger prick blood. In addition we will also draw about 3mls of blood from your child for Immunology assays. If they have Schistosomiasis, malaria and other Helminths they will be offered treatment.

Risks, Hazards and Discomforts of study participation:

There is minimal risk or hazard to your child if you agree to let he/she participate in this study. There will be minimal discomfort of having their finger pricked and blood drawn by a qualified/well-trained staff. There will be very high sterility observed during finger pricking and blood drawl to minimize risks and Hazards.

Benefits

Your child will benefit on the fact that we will diagnose him/her for Schistosomiasis, malaria and other Helminthes. If your child is found with any of these diseases or conditions, we will offer them free treatment.

Procedures to be followed:

If you agree for your child to participate in this study, we will ask your child to give out stool and urine that will be examined for schistosomiasis eggs and other helminthes. We will also request for your child’s blood to be drawn for Malaria testing and other Immunological assays.

Please put your name, your child’s name, your village name and the name of your child’s
school on the form below and sign the form if you agree for your child to participate in this study. All study participants enrolled in this study will be assigned a study number that will be used as reference for treatment and even publication purposes. All information's about the study participants and their medical records will be kept highly confidential.
INFORMED CONSENT AGREEMENT

Subjects under 18 years

I, Mr./Mrs./Miss ..................................................., being an adult and being lawful guardian of:

Msr/Miss (Child’s name) ........................................... Age ..............

School name. .................................................. Villages.................................

do hereby give permission to Prof/Dr./Mr./Miss ................................................

To include him/her in the intended research study as detailed in the protocol that has been explained to and understood by me. I have also understood the implications and benefits of the tests and treatments to Msr/Miss (Child’s name)..............................................

I accept the tests to be carried out and treatments to be offered to him or her incase the tests are positive since all the above conditions have been explained to me in the.

.............................................Languages in which I best understand.

Parent’s/Guardian’s signature ..................................................

Date .................................................................

Place .................................................................

Name of Person Obtaining Consent ................................................

Witness .................................................................
**Assent for children**

You are being asked to give out stool and urine that will be examined for schistosomiasis eggs and other helminthes. We are also requesting that your blood be drawn for Malaria testing and other immunological assays. In the event we find that you are suffering from any of these diseases, we will offer you treatment. You don’t have to do this if you don’t want to, but there is no danger of doing it.

Do you agree to give out stool and Urine that will be examined for schistosomiasis eggs and other helminthes?

**YES** _______________ **NO** _______________

Do you agree that we draw 3mls of blood from you for Malaria testing and other immunological assays?

**YES** _______________ **NO** _______________

Child’s name ________________________________________

Child’s Signature or Thumbprint___________________________________________

Person Obtaining the Consent_______________________________

Signature_________________________________________

Witness_________________________________________

Witness signature_________________________________________
Appendix III: Flow cytometry experimental design

Staining of CD3⁺/CD4⁺ regulatory T cells:

1) No stain  2) CD4 FITC  3) CD4 PE  4) CD4 PERCP  5) CD4 APC

<table>
<thead>
<tr>
<th>6)</th>
<th>PD-1 FITC</th>
<th>CD25 PE</th>
<th>CD4 PERCP</th>
<th>CD3 APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>7)</td>
<td>CD3 FITC</td>
<td>CD25 PE</td>
<td>CD4 PERCP</td>
<td>CD152 APC (CTLA-4)</td>
</tr>
<tr>
<td>8)</td>
<td>CD3 FITC</td>
<td>CD25 PE</td>
<td>CD4 PERCP</td>
<td>CD45RO APC</td>
</tr>
<tr>
<td>9)</td>
<td>CD3 FITC</td>
<td>CD25 PE</td>
<td>CD4 PERCP</td>
<td>HLA-DR APC</td>
</tr>
<tr>
<td>10)*</td>
<td>CD3 FITC</td>
<td>No PE antibody</td>
<td>CD4 PERCP</td>
<td>HLA-DR APC</td>
</tr>
<tr>
<td>11)*</td>
<td>No FITC antibody</td>
<td>CD25 PE</td>
<td>CD4 PERCP</td>
<td>CD3 APC</td>
</tr>
<tr>
<td>12)*</td>
<td>CD3 FITC</td>
<td>CD25 PE</td>
<td>CD4 PERCP</td>
<td>No APC antibody</td>
</tr>
</tbody>
</table>

*-Fluorescence minus one control

This table gives the design of how samples were treated. The table gives the different molecules and their corresponding fluorochrome conjugate and isotype of the monoclonal antibody.
Appendix IV: Map of the study area.

Adapted from KEMRI/CDC GIS-mapping department.