EXPRESSION OF CD23 BY *SCHISTOSOMA MANSONI* ANTIGEN-ACTIVATED B CELLS OF HIGHLY EXPOSED ADULT MALES ON THE SHORES OF LAKE VICTORIA, WESTERN KENYA

BY

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DECLARATION

I certify that this thesis is my original work and has not been previously submitted for the award of a degree in any other university.

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DEDICATION

The late

Prof. Ayub Victor Okoth Opiyo Ofulla….

…..may your soul find eternal peace……

Abstract

Increased B cell membrane CD23 (low affinity IgE receptor) and its soluble cleavage product (sCD23) is related to the development of resistance to re-infection with *Schistosoma mansoni*. CD23 occurs as two isoforms (CD23a and CD23b), whose expression by B cells responding to *S. mansoni* has not been investigated. On the other hand, most studies on IgE role in immunity have mainly revolved around soluble IgE, and rarely on cell surface receptor-bound IgE, although both are now known to play distinct roles. Furthermore, growing evidence indicates that toll-like receptor 4 (TLR4) influences the development of adaptive immunity to helmintic infections, although its relationship with CD23 expression and IgE production remains unclear. While there is evidence of CD23, IgE and TLR4 involvement in immunity to *S. mansoni*, it is unclear how these molecules are co-expressed, in addition to the limited information on the preferential expression of CD23 isoforms following schistosome antigen challenge. This study specifically sought to establish the predominantly expressed CD23 isoform by B cells of individuals infected by *S. mansoni*. The study also determined the relationship between the level of B cell surface CD23 expression and IgE levels. Furthermore, the study established the relationship between CD23 expression and B cell activation, as well as the relationship between the level of CD23 and TLR4 expression by B cells. Study participants were 170 randomly selected adult male sand harvesters and fishermen from three beaches on Lake Victoria and 5 donors from North America. Stool samples were used to diagnose *S. mansoni* infection using Kato-Katz technique. Venous blood was collected for culture B cell isolation, RNA extraction, and for CD23, CD69, CD40 and TLR4 expression determination by flow cytometry. The extracted RNA was used for reverse transcriptase PCR to determine CD23 isoform mRNA expression. Whole blood was used to determine B cell-bound surface IgE. Plasma and culture supernatants were used to run ELISA for IgE and sCD23. This study found CD23b mRNA was predominantly expressed in B cells from *S. mansoni* infected individuals. Circulating B cells had high levels of surface-bound IgE, which positively correlated to B cell surface CD23 expression. B cell expression of TLR4 was strongly associated with CD23 ($p = 0.0285$), and CD69 ($p < 0.0001$) expression. This study concludes that CD23, TLR4 and IgE are all important dusing B cell responses to *S. mansoni*, and are all involved from the time of B cell activation. These findings are important since they boost the understanding on CD23 expression dynamics, revealing its plausible role on B cells in immunity against *S. mansoni*. This study recommends further investigation into B cell CD23 isoform expression, the role of co-expression of surface CD23 and TLR4, following *S. mansoni* infection.

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ACCRONYMS AND ABBREVIATIONS

aCD40	anti-CD40 monoclonal antibodies
BD	Becton-Dickinson
BSA	bovine serum albumin
CD	cluster of differentiation
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
DC-SIGN	dendritic cell-specific ICAM-3-grabbing non-integrin (CD209)
EPG	eggs per gram (of stool)
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
IFN-γ	interferon-gamma
IgE	immunoglobulin-E
IL-	interleukin
KEMRI	Kenya Medical Research Institute
mCD23	CD23 expressed on cell surface
NHS	normal human serum
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PHA	phytohaemagglutinin
PWM	poke weed mitogen
rIL-4	recombinant interleukin-4
RPMI 1640	Rosswell Park Memorial Institute-1640

sCD23	soluble CD23
SEA	soluble egg antigen
SWAP	soluble worm antigen preparation
Th	helper T cell
TNF-α	tumour necrosis factor-alpha

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Schistosomiasis is an intravascular metazoan parasitic disease caused by *Schistosoma* species that affects at least 200 million people globally (Brooker and Smith, 2013). *S. mansoni, S. haematobium* and *S. japonicum* account for most of the cases of the disease contracted from waterborne larvae that penetrate the skin and enter the bloodstream, where they develop, pair, and reach sexual maturity (Gryseels *et al.*, 2006). Adult pairs of *S. mansoni* reside in the mesenteric veins of the intestine, where each female lays up to 300 eggs per day (Fitzsimmons *et al.*, 2004). The most common ways of through which people make contact with infective cercariae, thereby getting schistosomiasis, in developing countries is by wading or swimming in lakes, ponds and other water bodies which are infested with the snails (usually of the genera *Biomphalaria*, *Bulinus*, or *Oncomelania*; in the case of *S. mansoni*, it is *Biomphalaria spp.*) that is the natural reservoir of the pathogen (Odiere *et al.*, 2011; Opisa *et al.*, 2011; Brooker and Smith, 2013).

Lake Victoria is the largest fresh water lake in the region and supports livelihoods of the majority living around it mainly through fishing, although may also practice sand harvesting, car washing, and an array of domestic chores, including laundry (Mutapi, 2001; Fitzsimmons *et al.*, 2004). This makes the community predisposed as they are likely to make a lot of contact with the infesed water, raising their chances of getting cercarial infection.

One of the critical areas of schistosomiasis research under focus presently is whether substantial acquired immunity develops following initial infection. Studies in western Kenya have shown a tendency to develop resistance to re-infection, but the patterns observed differ depending largely on the exposure profiles of the person (Karanja *et al.*, 2002a; Mwinzi *et al.*, 2009). For example, data from parts of Kisumu in Western Kenya indicate that occupationally employed car washers have re-infection intervals with *S. mansoni* different from those of commercial sand harvesters. These patterns change over time, and a possible explanation for this difference could be based on different exposure patterns and duration of exposure since childhood (Mwinzi *et al.*, 2009; Black *et al.*, 2010).

The process by which immunity to re-infection by any parasite is conferred is complex and may involve many cells, cellular and soluble products. In helminthic infections, the immunoglobulin IgE plays a central role in the destruction of the worm, and all antibodies are produced by mature and properly activated B cells. B cells upon appropriate antigenic stimulation proliferate and differentiate, under the control of mainly T cell-derived factors, into immunoglobulin-secreting plasma cells (Tangye *et al.*, 2002). Although many factors influence the immune response to antigens, the nature of antibody response primarily depends on the nature of the antigen, while the amount of response lies with the quantity and quality of Th help received (Chung *et al.*, 2002). In schistosomal infections, high titres of parasite-specific serum IgE are always achieved, but the exact mechanisms for this class of antibody is not well understood in these infections (Mutapi, 2001; Fitzsimmons *et al.*, 2004). However, studies have pointed to the tendency of individuals with high IgE titres to resist re-infection with *S. mansoni* when exposure level is

similar to those with lower parasite-specific serum IgE titres (Nutman *et al.*, 1984; Rihet *et al.*, 1991; Karanja *et al.*, 2002a).

The frequency of water exposure, total duration of exposure, HIV-1 infection, CD4 T cell numbers, and age are some of the factors that have been studied as predisposing to schistosomal infection and/or regulators of resistance development. In one such study involving actively working men, decreased CD4 T-cell counts in HIV-1-positive individuals corresponded to increased susceptibility to *S. mansoni* re-infection (Karanja *et al.*, 2002a). Following treatment, the interaction of praziquantel with schistosomes results in qualitative and quantitative alteration of host-parasite-specific immune responses, including modifications in the cell proliferative responses and modifications in the levels and types of antibody and cytokine responses (Mutapi, 2001).

IgE most likely exerts its functions through its receptors which include the high affinity receptor (FcεRI) and the low affinity receptor (FcεRII or CD23), also known as the human leukocyte differentiation antigen (Hibbert *et al.*, 2005b). CD23 is a 45 kD type II membrane glycoprotein expressed on numerous hematopoietic lineage cells and functions as the low affinity receptor for IgE (White *et al.*, 1997). There are two forms of the 45-kD CD23; CD23a and CD23b, which differ only in their cytosolic domains. The former is constitutively expressed by B cells while the latter induced by exposure of cells to many factors, notably IL-4 (Yokota *et al.*, 1988; Hibbert *et al.*, 2005b).

Membrane bound CD23 undergoes proteolysis giving rise to soluble CD23 (sCD23) molecules of molecular weights 37 kD, 33 kD, 29 kD, 25 kD and 16 kD. All these fragments contain the lectin binding domain, and retain the ability to bind IgE, except the 16 kD one (Hibbert *et al.*, 2005b; McCloskey *et al.*, 2007). Many cytokine activities have been attributed to sCD23, especially the 25 kD fragment (McCloskey *et al.*, 2007), including being an autocrine growth factor for some Epstein-Barr virus-transformed mature B cell lines, a differentiation factor for prothymocytes, and also in prevention of apoptosis of germinal center centrocytes (White *et al.*, 1997). Membrane-bound CD23 has roles in IgE binding, regulation of IgE synthesis, cell adhesion and antigen presentation (White *et al.*, 1997; McCloskey *et al.*, 2007). The expression of CD23 on B cells has been shown to be related to resistance to re-infection in a cohort repeatedly treated using praziquantel (Mwinzi *et al.*, 2009). The regulation of production of IgE may be regulated through this receptor. While the role of CD23 as a receptor for IgE is clear (Gould H *et al.*, 1991; Kilmon *et al.*, 2001; Kijimoto-Ochiai, 2002), the exact factors influencing the patterns of CD23 expression and sCD23 release, and correlations with IgE synthesis, also remain less understood (Kicza *et al.*, 1989; McCloskey *et al.*, 2007), particularly in helminthic diseases.

Invading microorganisms are controlled by the innate immune mechanisms which recognize pathogens by rearranged high affinity receptors, also called pettern recognition receptors (PRRs) and adaptive (mediated by B and T lymphocytes) immune mechanisms (Heine and Lien, 2003; Takeda and Akira, 2003). The family of Toll-like receptors (TLRs) are part of the PRRs, and are activated by specific components of microbes and certain host molecules (Werling and Jungi, 2003). TLRs have been reported to influence the development of adaptive immune responses, by

activating antigen-presenting cells (Dabbagh and Lewis, 2003; Schwarz *et al.*, 2003). TLR activation leads to the induction of inflammatory responses as well as the development of antigen-specific adaptive immunity (Takeda *et al.*, 2003).

TLRs are also expressed by T and B lymphocytes, and their respective ligands activate processes that modulate their function (Fillatreau and Manz, 2006; Kabelitz, 2007; LaRosa *et al.*, 2008; van Maren *et al.*, 2008). The activation of naïve B cells requires the sequential integration of signals provided by antigen-receptor cross-linking and by antigen presentation to specific helper T (Th) cells through immune synapse, although it also seems to be heavily dependent on innate stimuli acting on TLRs expressed by B cells, or indirectly via cytokines provided by TLR-activated DCs (Fillatreau and Manz, 2006; Ruprecht and Lanzavecchia, 2006). Binding of TLR in B cells has also been illustrated to stimulate activation (Ruprecht and Lanzavecchia, 2006), proliferation, the release of immunoglobulins, and the production of chemokines (Bernasconi *et al.*, 2003; Bourke *et al.*, 2003). Toll-like receptor 4 (TLR4) is essential for LPS-induced activation of follicular B cells, leading to cell division and isotype switching (Chow *et al.*, 1999; Hoshino *et al.*, 1999; Ogata *et al.*, 2000; Hayashi *et al.*, 2005; Gerondakis *et al.*, 2007), a function that has also been reported to be mediated by the cleavage products of CD23 (Kikutani *et al.*, 1986; McCloskey *et al.*, 2007; Montero-Vega and de Andres, 2009). Furthermore, in a study conducted in Kenya, human schistosomiasis was found to be associated with TLR4 expression by B cells (Onguru *et al.*, 2011b).

It is thus evident that B cell expression of CD23 isoforms has not been exhaustively described, especially in the context of *Schistosoma mansoni* infections, in order to understand which of the

two may be more involved in immune responses to *S. mansoni*. Similarly, there is not adequate information on the relationship between B cell surface CD23 expression and IgE levels in schistosomiasis patients, particularly with regard to the proportion of IgE that may remain bound to the surface of B cells. Furthermore, the expression of CD23 has not been investigated in relation to the expression of toll-like receptors, notably TLR4, by B cells.

1.2 Statement of the Problem

While it is becoming clear that CD23 is related to resistance to re-infection with *S. mansoni*, the factors involved in its expression by B cells, or those controlling its function remain unclear. There is limited information on the preferential expression of the CD23 isoforms (CD23a and CD23b), as well as the mechanisms involved in its cleavage from cell surfaces. CD23 binds to IgE with a lower affinity than FcεRI, but the factors influencing the dynamics of this binding are not described in human schistosomiasis. The relationship between CD23 expression and the expression of markers of B cell activation (CD69 and CD40) have not been adequately investigated in schistosomal infections. The role of TLR4 in antihelmintic immunity is rapidly unraveling, but how this molecule is important in the activation of B cells responding to schistosome antigens is poorly understood, as well as its interaction with B cell receptors (CD23, CD40 and CD69). To unravel part of this compound problem, a mechanistic approach to describe the levels and associations of the stated parameters will form basis for more specific and rigorous tests that will provide more details of the dynamics of CD23/IgE involvement in immune responses to *S. mansoni* infection.

1.3 Objectives

1.3.1 General Objective

To investigate the relationship between B cell CD23 expression and IgE production and binding to B cells during *S. mansoni* infections among highly exposed persons, and how CD23 expression is related to TLR4 expression by B cell followin *S. mansoni* infections.

1.3.2 Specific Objectives

1. To establish the predominantly expressed CD23 isoform by B cells of individuals infected by *S. mansoni*.
2. To determine the relationship between the level of B cell surface CD23 expression and IgE levels in individuals infected by *S. mansoni.*
3. To establish the relationship between CD23 expression and B cell activation (CD69 and CD40 expression) in peripheral blood B cells from individuals infected by *S. mansoni.*
4. To determine the relationship between the level of CD23 expression and pattern-recognition receptor TLR4 expression among individuals infected by *S. mansoni.*

1.4 Null Hypotheses

1. There is no difference in the level of CD23 isoform expression by B cells of individuals infected by *S. mansoni*.
2. There is no relationship between the level of B cell surface CD23 expression and IgE levels in individuals infected by *S. mansoni.*
3. CD23 expression is not related to B cell activation (CD69 and CD40 expression) in peripheral blood B cells from individuals infected by *S. mansoni.*

4. There is no relationship between the level of CD23 expression and pattern-recognition receptor TLR4 expression among individuals infected by *S. mansoni.*

1.5 Justification

Human schistosomiasis has remained one of the world's most important helminthic diseases, especially given its pathogenesis and the fact that it has remained among the most prominent neglected tropical diseases in the region. In helminthic infections, IgE is thought to play a central role in the destruction of the worm (Getahun *et al.*, 2005). Infection by *S. mansoni* is associated with a T_H2-type response, characterized by high IgE levels, circulating and tissue eosinophilia, and a harmful fibrotic response to schistosome ova, leading to hepatic fibrosis (Ganley-Leal *et al.*, 2006). Whereas increased CD23 expression on B cells has been strongly linked to resistance to *S. mansoni* re-infection (Mwinzi *et al.*, 2009), very little has been elucidated on the mechanisms involved. In a recent study, the expression of CD23 on B cells has been found to be proportional to sCD23 levels, directly related to IgE levels but unrelated to *S. mansoni* egg counts in stool (Onguru *et al.*, 2011a). B cells interact with other immune cells, and antigens, through receptors and cytokines either produced by B cells themselves or other cells (principally activated T cells). The B cell markers of interest in this study included the early activation marker CD69, and the accessory molecule CD40, the latter being useful in antigen presentation to T cells (von Bergwelt-Baildon *et al.*, 2004; Liebig *et al.*, 2009). In addition, TLR4 has been reported to influence the development of adaptive immune responses, possibly by activating antigen-presenting cells (Dabbagh and Lewis, 2003; Schwarz *et al.*, 2003). It has also been shown to be raised during *S. mansoni* (Onguru *et al.*, 2011b).

It was thus vital to investigate B cell behaviour, especially in IgE synthesis and regulation, as this isotype is crucial in anti-helminthic responses. Particularly, the expression and role of CD23 (and the specific roles of the two isoforms; CD23a and CD23b) in the regulation of IgE synthesis, as well as interactions with toll-like receptor 4 (TLR4), and so the defense against schistosomiasis was investigated in this study.

1.6 Significance

The findings of this study raise our understanding of the involvement of B cells in anti-schistosome immunity development. First, it is the first study reporting the preferential expression of CD23 isoforms during schistosomiasis, providing novel knowledge on CD23 involvement in immunity to *S. mansoni*. The study equally reveals the the existence of high levels of IgE bound to CD23 on the surface of B cells, confirming the role of CD23 in IgE-mediated responses to *S. mansoni*. Finally, the expression of TLR4 has been compared to CD23 expression by B cells responding to *S. mansoni*, revealing the possible interaction of the the two markers in the activation of B cells following schistosome antigen challenge. The study therefore provides grounds for further studies on CD23, especially those that would seek to develop a therepuetic intervention by manipulating B cell responses to *S. mansoni.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Biology and Epidemiology of Schistosomiasis

Schistosomiasis, also known as bilharziasis (or simply bilharzia), is a parasitic disease caused by trematode flatworms (*blood flukes*) of the genus *Schistosoma*. Human infection follows exposure to larval forms (cercariae) in water harbouring infected snails (Gryseels *et al.,* 2006). The cercariae, released by freshwater snails, penetrate the skin of people who get into contact the infested water, often causing a transient dermatitis called *swimmer's itch* (Fitzsimmons *et al.*, 2004). In the body, the larvae develop into adult schistosomes, which live in the blood vessels, where they develop, pair and reach sexual maturity. Adult pairs of *S. mansoni* reside in the mesenteric veins of the intestine where each female lays up to 300 eggs per day (Fitzsimmons *et al.*, 2004), some of which are passed out of the body in feces while others are trapped in body tissues, causing an immune reaction (Gryseels *et al.*, 2006). In urinary schistosomiasis, there is progressive damage to the bladder, ureters and kidneys. In intestinal schistosomiasis, there is progressive enlargement of the liver and spleen, intestinal damage, and hypertension of the abdominal blood vessels (Fitzsimmons *et al.*, 2004). The life cycle human schistosomes is illustrated in Figure 2.1.

Control of schistosomiasis is based on drug treatment, snail control, improved sanitation and health education (Engels *et al.*, 2002). Schistosome infection is widespread with relatively low mortality rate, but high morbidity rate, causing severe debilitating illness in millions of people (Gryseels *et al.,* 2006). The disease is often associated with water resource development projects,

like dams and irrigation schemes, where the snail intermediate hosts of the parasite breed (Chitsulo *et al.*, 2000; Steinmann *et al.*, 2006; Brooker *et al.*, 2009; Stothard *et al.*, 2009).

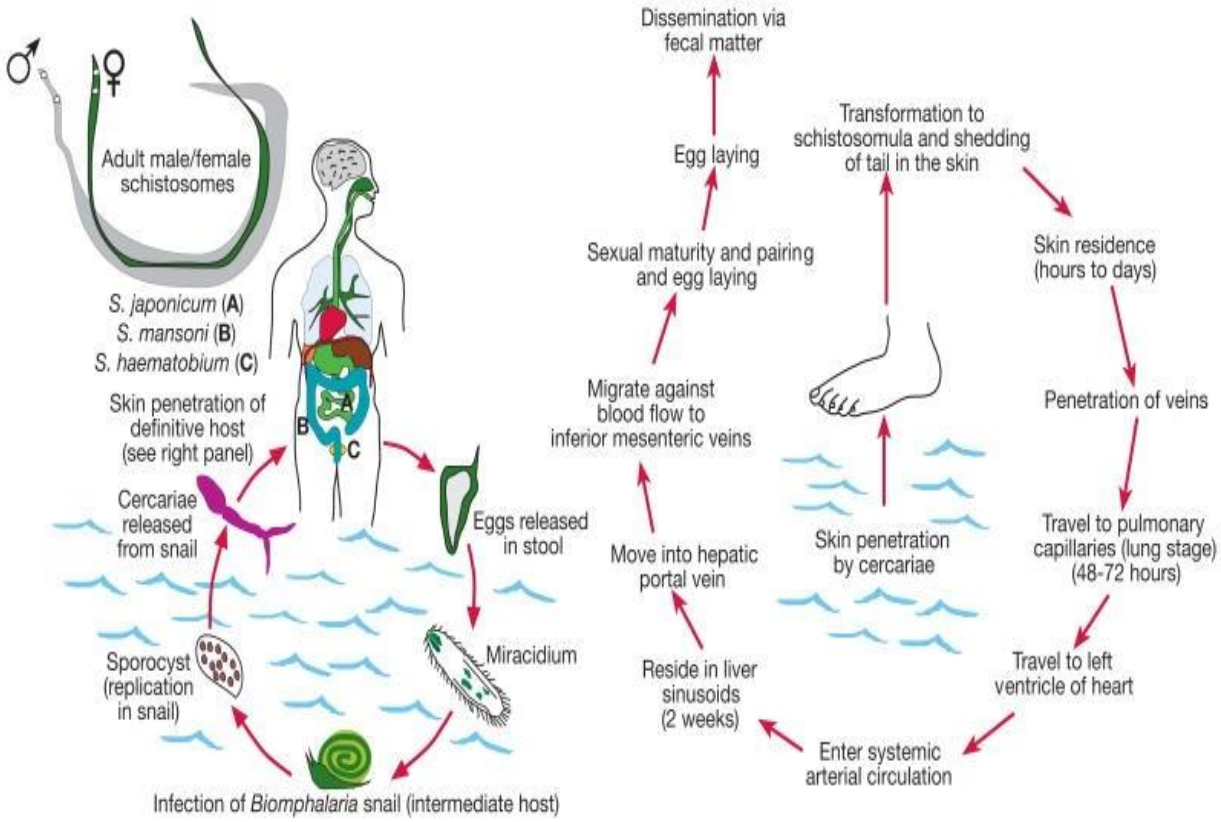

Figure 2.1. Life cycle of schistosomes (http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book; *last accessed July 2016*)

Schistosomiasis is found in tropical countries in Africa, Caribbean, eastern South America, east Asia and in the Middle East (Brooker and Smith, 2013). *S. mansoni* is found in parts of South America and the Caribbean, Africa, and the Middle East; *S. haematobium* in Africa and the Middle East; and *S. japonicum* in the Far East. *S. mekongi* and *S. intercalatum* are found focally in Southeast Asia and central West Africa, respectively (Chitsulo *et al.*, 2000; Brooker and Smith, 2013).

An estimated 207 million people have the disease, 120 million being symptomatic (Brooker *et al.*, 2009). A few countries, with the support of the WHO, have eradicated the disease, and many more are working towards its eradication (Mutapi, 2001). In some cases, urbanization, pollution, and the consequent destruction of snail habitat has reduced exposure, with a subsequent decrease in new infections (Mutapi, 2001; Fitzsimmons *et al.*, 2004; Brooker and Smith, 2013). Schistosomiasis is mostly contracted in developing countries by people wading or swimming in lakes, ponds and other bodies of water which are infested with the snails (usually of the genera *Biomphalaria*, *Bulinus*, or *Oncomelania*; in the case of *S. mansoni*, it is *Biomphalaria spp.*) that is the natural reservoir of the pathogen (Brooker *et al.*, 2009).

2.2 Immune Responses in Schistosomiasis

The immune response and immunopathologic manifestations in schistosomiasis are largely dependent on antigen-specific $CD4^+$ Th cells (Araujo *et al.*, 1996). In turn, the stimulatory and regulatory function of the $CD4^+$ Th cells is dependent on signals emanating from accessory cells (Hernandez *et al.*, 1997; von Bergwelt-Baildon *et al.*, 2004; Liebig *et al.*, 2009). B cells are capable of functioning as accessory cells (Hernandez *et al.*, 1997). Infection by *S. mansoni* is leads to a powerful T_H2-type response (Mbow *et al.*, 2013), characterized by high plasma IgE levels, significant circulating and tissue eosinnophilia, and a harmful fibrotic response to schistosome egg, leading to hepatic fibrosis, as well as the other tissue destructive responses (Araujo *et al.*, 1996; Mbow *et al.*, 2013).

The process by which immunity to re-infection by any given parasite is conferred is complex and may involve many cells and cellular products. In helminthic infections, IgE plays a central role

in the destruction of the worms (Nutman *et al.*, 1984). All antibodies are produced by mature and properly activated B cells. Upon appropriate antigenic stimulation, B cells proliferate and differentiate, under the control of mainly T cell-derived factors, into immunoglobulin-secreting plasma cells. The nature of antibody response primarily depends on the nature of the antigen, while the amount of response lies with the quantity and quality of Th help received (Janeway *et al.*, 2001). In schistosomal infections, high titres of parasite-specific serum IgE are always achieved, but the exact role for this class of antibody is not well understood in these infections. However, certain studies have pointed to tendency of individuals with high titres to resist re-infection with *S. mansoni* when exposure index is similar to those with lower parasite-specific serum IgE titres (Nutman *et al.*, 1984; Hagan, 1993; Fitzsimmons *et al.*, 2014).

2.3 B cell Activation

B cell differentiation is thought of as a decision tree, where a sequence of signals is needed first for progression of the resting B cell into an activated state, followed by isotype switching, and then for development into either memory or short-lived or long-lived plasma cells (Tangye and Hodgkin, 2004). IL-4, IL-5, IL-6, and IL-10 are known to stimulate antibody production by B cells while IL-13, IL-21 IFN-γ and TGF-β promote isotype switching, the release of switched immunoglobulin, and the induction of molecular intermediates associated with switching in concert with the first group. IL-12 is a potent proinflammatory cytokine that promotes Th1 activities (Banchereau and Rousset, 1992; Tangye and Hodgkin, 2004).

A number of biochemical changes are elicited in antigen-stimulated B cells, including an increase in transmembrane ion flux, a resulting depolarization of the membrane, production of

certain intermediates in the phosphatidylinositol pathway, protein kinase C activation, and an increase in intracellular free Ca^{2+} (Wright and McMaster, 2002; Hoek *et al.*, 2006). In addition, regulatory proteins such as NF-κB are translocated to the nucleus to participate in elevated transcription of mRNA for immunoglobulins (Janeway *et al.*, 2001). Dramatic morphological changes accompany B lymphocyte stimulation. Resting B cells are only 8-10μm in diameter when viewed on smears and have very small amounts of pale-staining cytoplasm. There is little endoplasmic reticulum, and ribosomes are free in cytoplasm. When stimulated in culture by mitogens (e.g. pokeweed mitogen or staphylococcus organisms), B cells convert into proliferating lymphoblasts, which are larger, have much more cytoplasm, with clusters of ribosomes and a conspicuous golgi apparatus (Banchereau and Rousset, 1992). Depending on the nature of the stimulus, some of the replicating B cells will be destined to become memory cells (Banchereau and Rousset, 1992; Sims *et al.*, 2005).

Primary B cell development takes place in the bone marrow, where immature B cells must generate a functional B cell receptor (BCR) and overcome negative selection induced by reactivity with autoantibodies (Sims *et al.*, 2005). Transitional immature B cells lie developmentally between bone marrow immature and peripheral mature B cells, and represent recent bone marrow émigrés with phenotypic characteristics distinct from mature B cells (Melchers *et al.*, 1989). As with late stage bone marrow immature B cells, they are heat-stable antigen (HSA) high ($CD24^{high}$), and surface IgM high ($sIgM^{high}$). In a manner similar to $HSA^{high}/sIgM^{high}$ bone marrow immature B cells, they respond to *in vitro* antigen receptor cross-linking by apoptosis instead of proliferation (Chung *et al.*, 2002).

Contrary to traditional views, CD23 status does not exclusively mark the mature B cells, but characterizes a significant subset of the transitional B cells (Melchers *et al.*, 1989; Chung *et al.*, 2002; Kobayashi *et al.*, 2002). CD23 status represents a continuum in the maturation process. In addition to CD23, other B cell surface proteins including IgD, CD21, CD22 and B220 (CD45/Ly-5/T200), are regulated during development from bone marrow immature B cells to peripheral mature B cells. There exists a progressive increase in surface expression levels of CD21, CD22 and B220 from $CD23^-$ to $CD23^+$ transitional B cells and then to mature B cells (Melchers *et al.*, 1989; MacLennan, 1998).

2.4 CD23 Expression

The human leukocyte differentiation antigen, CD23, is a 45 kD type II membrane glycoprotein expressed on numerous hematopoietic lineage cells and functions as the low affinity receptor for IgE (White *et al.*, 1997). It is unique among Fc receptors in that it shares homology to C-type (calcium-dependent) lectins and is also homologous to, and genetically linked on human chromosome 19, to DC-SIGN (Hibbert *et al.*, 2005b). CD23 contains an α-helical coiled-coil stalk region between the extracellular lectin domain and the transmembrane region, believed to facilitate oligomerization of membrane-bound CD23 to trimers (White *et al.*, 1997; McCloskey *et al.*, 2007). Membrane-bound CD23 has roles in IgE binding, regulation of IgE synthesis, cell adhesion and antigen presentation (White *et al.*, 1997).

There exist two isoforms of the 45-kD CD23; CD23a and CD23b, which differ only in their cytosolic domains. The former is constitutively expressed by B cells while the latter induced by exposure of cells to IL-4 (Yokota *et al.*, 1988). Membrane bound CD23 undergoes proteolysis

giving rise to soluble CD23 molecules of molecular weights 37 kD, 33 kD, 29 kD, 25 kD and 16 kD. All these fragments contain the lectin binding domain, and retain the ability to bind IgE, except the 16 kD one (McCloskey *et al.*, 2007). Many cytokine activities have been attributed to soluble CD23 (especially the 25 kD fragment), including being an autocrine growth factor for some Epstein-Barr virus-transformed mature B cell lines, a differentiation factor for prothymocytes, and also in prevention of apoptosis of germinal center centrocytes (White *et al.*, 1997).

CD23 has many receptors including CD21, CD11b and CD11c. CD23 has potential in many cell systems, and in B cells, the most likely receptor is CD21, which is not expressed until the late pre-B cell stage. This may imply that other as yet unidentified receptors for CD23 might exist in the B cell precursor compartment (White *et al.*, 1997). The existence of CD23 has been shown on B cells, T lymphocytes, monocytes, eosinophils, platelets, and epidermal Langerhans' cells (Kicza *et al.*, 1989). Kikutani and colleagues (1986) described CD23 as an early B-cell marker, while sCD23 has been shown to possess B cell growth activity, this being exerted mainly on B cells in a defined state of preactivation (Gould H *et al.*, 1991; Kijimoto-Ochiai, 2002; Hibbert *et al.*, 2005a). $CD23^{+}$ cells and sCD23 act synergistically on the proliferation of CD23 B cell populations (McCloskey *et al.*, 2007).

The CD23/sCD23 system has been shown to be involved in IgE regulation and IgE-mediated allergic responses (Gordon *et al.*, 1986; Kikutani *et al.*, 1986; Kicza *et al.*, 1989). CD23 binds both IgE and CD21 at its lectin domain and, through these interactions, regulates the synthesis of IgE (Aubry *et al.*, 1992). Furthermore, paradoxical activities have been confirmed to be

produced by different fragments of CD23 following cleavage by endogenous proteases at different points. Generally, soluble CD23 monomers (derCD23) inhibit while oligomers (the trimeric lzCD23) stimulate IgE synthesis; exCD23 is also monomeric although it has not shown any significant influence on IgE synthesis (McCloskey *et al.*, 2007). Since sCD23 is released from B cells and monocytes, a regulatory circuit via sCD23 might be possible. It has been in deed shown that sCD23 leads to proliferation of $CD23^+$ B cells, suggesting that receptors for the soluble form of CD23 are not necessarily combined to the $CD23^+$ population (Callard *et al.*, 1988).

2.5 CD23 Regulation

CD23 plays a role in IgE production (McCloskey *et al.*, 2007), and cytokines involved in IgE synthesis also modulate CD23 expression on B cells, the best known of which are IL-4 and IFN-γ, the latter being important in immunoglobuline class switching to IgG_{2a} (Neva *et al.*, 1998). CD23 expression is likely a dynamic process that depends on the ratio between synthesis and proteolysis. This process could as such be influenced in part by the profile and concentration of produced cytokines that modulate CD23 expression and by the levels of IgE, as it has been established that IgE induces CD23 stabilization on cellular membrane (Corominas *et al.*, 1998; Neva *et al.*, 1998). In similar but unrelated studies among atopic donors, however, the use of IFN-γ as stimulus achieved little modulation of CD23 expression by PBMC-derived B cells (up to 1.2%). IFN-γ leads to a pronounced release of sCD23 into the culture supernatants (Kicza *et al.*, 1989). It is also noteworthy that IFN-γ is generally known to inhibit IgE synthesis (Mutapi, 2001) by suppressing IL-4 induction and germline ε transcripts, and Th2 differentiation (Kobayashi *et al.*, 2009).

2.6 Markers for B Cell Activation

2.6.1 CD69

This is an early marker of leukocyte activation (Hara *et al.*, 1986; Cosulich *et al.*, 1987; Cebrian *et al.*, 1988; Testi *et al.*, 1994; Sancho *et al.*, 1999) found in small subsets of lymphocytes in secondary lymphoid tissues of healthy persons (Sanchez-Mateos *et al.*, 1989). CD69 is persistently expressed by leukocytes in infiltrates of certain chronic inflammatory diseases (Garcia-Monzon *et al.*, 1990; Laffon *et al.*, 1991). While its exact role or mechanism of action is unclear, there are indications that it is involved in the downregulation of proinflammatory processes through the synthsesis of TGF-β (Sancho *et al.*, 2003). Several reports have linked CD69 to *in vitro* activation of bone marrow-derived cells, but these have not been replicated *in vivo* (Cosulich *et al.*, 1987; Cebrian *et al.*, 1988; Testi *et al.*, 1989). CD69 is also linked to thymocyte development and migration within lymphoid tissues (Feng *et al.*, 2002; Nakayama *et al.*, 2002).

2.6.2 CD40

Activation of CD40 dramatically enhances antigen presentation by both normal and malignaant B cells and these CD40-activated B cells (CD40-B cells) play an important role in the expansion of antigen-specific CD4+ and CD8+ T cells, as well as the priming of naïve T cells (Kondo *et al.*, 2002; von Bergwelt-Baildon *et al.*, 2002; Lapointe *et al.*, 2003; von Bergwelt-Baildon *et al.*, 2004). In cytotoxic T cell function, B cells have been shown to play an inductive role as APCs, following CD40 activation (von Bergwelt-Baildon *et al.*, 2002), an observation that has also been observed when tumour-specific antigens are involved in B cell activation (Lapointe *et al.*, 2003; von Bergwelt-Baildon *et al.*, 2004). The interaction of B cells with T cells through CD40-CD40L

(CD154) allows such T cells to provide signals to B cells that induce proliferation (Lane *et al.*, 1992), immunoglobulin switching (Fuleihan *et al.*, 1993), antibody secretion and rescue from apoptosis (Fang *et al.*, 1997) at different times during the life cycle of a B cell. The signal also plays a key role in the development of germinal centers and the survival of memory B cells (Gray *et al.*, 1996; Lee *et al.*, 2003; Wykes, 2003; Zarnegar *et al.*, 2004).

2.7 B cell Activation and IgE Production

Although helminth parasites induce the production of high levels of IgE antibodies, the immunoregulatory mechanisms determining this are generally poorly understood (Nutman *et al.*, 1984). The regulatory events controlling the synthesis of IgE have been mainly studied in rodent models using both *in vivo* and *in vitro* systems. Studies have shown the overall T cell-dependence of IgE antibody production and the homeostatic role of T helper and T suppressor cells (Saxon *et al.*, 1980; Turner *et al.*, 1981; Allman *et al.*, 1993). Success with these systems have been documented both in patients with high serum IgE, in atopic patients, and normal donors (Aubry *et al.*, 1992; Corominas *et al.*, 1998). Some workers have succeeded in enhancing IgE production by pokeweed mitogen (PWM) in cells from normal donors (Saxon *et al.*, 1980; Kimata *et al.*, 1983).

The specific importance of IgE response in parasitic diseases remains unclear though in some cases IgE production has correlated with resistance to helminth infection , cellular killing of parasites *in vitro* and the pathological processes associated with some clinical disorders (Nutman *et al.*, 1984; Redvall *et al.*, 2008). Although there are many reports of *in vitro* production of IgE by human peripheral blood cells, the conditions causing its induction or inhibition remain

debatable to-date. For example, most investigators have established that PWM does not induce IgE synthesis in PBMC from individuals without helmintic infections, while the mitogen enhances significant IgE production by PBMC of individuals with different helminth infections (Kimata *et al.*, 1983; Nutman *et al.*, 1984).

Parasite antigens do not induce either blastogenic responses or *in vitro* synthesis of IgG, IgM, or IgE in cell cultures from normal and atopic patients, but it is possible that the persistent antigenic stimulation or subtle T cell imbalances associated with helminth infection may contribute to the activation seen in patients (Mutapi, 2001). Whatever the cause, it is probable that cultured PBMC from patients exposed to these antigens are already maximally stimulated *in vivo* to produce IgE and thus signals triggered by PWM are not expected to provide additional augmentation, but rather inhibition to IgE synthesis (Turner *et al.*, 1981; Nutman *et al.*, 1984).

IgE exerts its effector functions through receptors expressed on cell surface (FcεRI and FcεRII), and, therefore, the levels of free IgE molecules do not reflect the concentrations of somatic IgE since most of IgE is bound by the high affinity receptors, FcεRI, on tissue mast cells (Liang and Ganley-Leal, 2009). The expression of IgE receptors may be altered by the cellular activation status of the cell, itself influenced by multiple immunological factors (Mawhorter *et al.*, 1996; Ganley-Leal *et al.*, 2006). The engagement of IgE by its cellular receptors is likely to confound measurements of IgE, interfering with the interpretation of the role of IgE in immunity (Liang and Ganley-Leal, 2009).

2.8 Factors Involved in IgE Synthesis

The processes of IgE production by B cells are involved in germline ε transcript expression, IgE class switching, clonal expansion of B cells, and differentiation into IgE-secreting plasma cells. All of these activities are controlled by a variety of cytokines and direct cell-cell contact between B cells and Th cells (Kobayashi *et al.*, 2002). In addition to IL-13, IL-4 is an important cytokine that promotes the expression of germline ε transcripts. CD40 is a member of the TNF receptor family whose stimulation is also necessary for IgE synthesis and probably for inducing switch recombinases (Xu and Rothman, 1994).

The functions of IL-10 in IgE synthesis by B cells are debatable, and in one study, IL-10 was found to facilitate IgE production by both human PBMC and highly purified B cells, enhance B cell proliferation, and promote the generation of plasma cells, although it did not enhance the expression of germline ε transcripts (Kobayashi *et al.*, 2002). Several other cytokines, including IL-6, IL-7 and IL-9, enhance both IgE and IgG4 production. In contrast, IFN-γ inhibits IgE synthesis by suppressing IL-4 induction and germline ε transcripts and Th2 differentiation (Xu and Rothman, 1994; Yssel *et al.*, 1998; Kobayashi *et al.*, 2009).

IgE antiboby responses in most individuals are tightly regulated both quantitatively and qualitatively, but the presence of helminth infections appear to overcome such regulatory mechanisms and high levels of IgE are consistently produced *in vivo*. The immunoregulatory mechanisms determining this IgE biosynthesis are poorly understood (Nutman *et al.*, 1984; Del Prete *et al.*, 1989; Ishizaka, 1989; King and Nutman, 1993; Arinola *et al.*, 2015).

2.9 B cell expression of TLR4

Humans interact with parasites in a complex and variable manner, which differs depending on the parasite, the environment, and the individual (Takeda and Akira, 2003). Invading pathogens are controlled by the innate (which recognize pathogens by rearranged high affinity receptors) and adaptive (mediated by B and T lymphocytes) immune mechanisms (Heine and Lien, 2003; Takeda and Akira, 2003). The establishment of adaptive immunity involves cell proliferation, gene activation and protein synthesis, which is often not rapid enough to eradicate microorganisms as opposed to innate immunity, which recognizes pathogens by germ-line-encoded pattern recognition receptors (PRR) and thus provides more rapid defense mechanisms (Akira and Hemmi, 2003; Heine and Lien, 2003). The family of Toll-like receptors (TLRs) comprises PRRs, that are activated by specific components of microbes and certain host molecules (Werling and Jungi, 2003), and constitute the first line of defense against many pathogens, playing a crucial role in innate immunity (Akira and Hemmi, 2003).

About 13 TLR family members have been identified in mammals, which recognize specific conserved components of microorganisms (Takeda and Akira, 2003). TLRs have been reported to influence the development of adaptive immune responses, possibly by activating antigen-presenting cells (Dabbagh and Lewis, 2003; Schwarz *et al.*, 2003). TLR activation leads to the induction of inflammatory responses as well as the development of antigen-specific adaptive immunity (Takeda *et al.*, 2003).

TLRs are also expressed by T and B lymphocytes, and their respective ligands activate processes that modulate their function (Fillatreau and Manz, 2006; Kabelitz, 2007; LaRosa *et al.*, 2008; van

Maren *et al.*, 2008). TLRs expressed on T cells seem to enhance cell proliferation, adhesion, and survival, while also modulating cytokine production (Kabelitz, 2007). Activation of naïve B cells requires the sequential integration of signals provided by antigen-receptor cross-linking and by antigen presentation to specific helper T (Th) cells through immune synapse, although it also seems to be heavily dependent on innate stimuli acting on TLRs expressed by B cells, or indirectly via cytokines provided by TLR-activated DCs (Fillatreau and Manz, 2006; Ruprecht and Lanzavecchia, 2006). Binding of TLR on B cells has also been illustrated to stimulate proliferation, the release of immunoglobulins, and the production of chemokines (Bernasconi *et al.*, 2003; Bourke *et al.*, 2003). Apart from TLRs, many other receptors are involved in innate recognition of microbes, and strong interactions occur between signaling through these receptors and signaling through TLR (Chow *et al.*, 1999; Heine and Lien, 2003; Underhill, 2003; Ruprecht and Lanzavecchia, 2006). It is known that TLR and BCR signaling can exhibit synergy in the activation of mature B cells (Viglianti *et al.*, 2003).

TLR4 is essential for LPS-induced activation of follicular B cells, leading to cell division and isotype switching (Chow *et al.*, 1999; Hoshino *et al.*, 1999; Ogata *et al.*, 2000; Gerondakis *et al.*, 2007). TLR4 has been shown to recognize the LPS present in the walls of Gram-negative bacteria, which activates mature B cells to proliferation and immunoglobulin secretion (Hayashi *et al.*, 2005). In the absence of mitogenic signals, mature peripheral B cells are found in a quiescent state having a small size, high nuclear to cytoplasmic volume, and low metabolic activity. Following TLR4 engagement, B cells exit G0 and enter the G1 phase of the cell cycle, where they increase in size and undertake an approximate doubling of RNA and protein levels, the majority of which is dedicated to new ribosome synthesis necessary for cellular replication

(Gerondakis *et al.*, 2007). Hayashi *et al.* (2005) described the role of TLR4 as promoting B cell maturation, while another study revealed the role of TLR4 in helminthic infections (Montero-Vega and de Andres, 2009). Furthermore, TLR4 expression on B cells has been shown to be associated with human schistosomiasis (Onguru *et al.*, 2011b), while it is also involved in the regulation (Nadiri *et al.*, 2015) and cleavage (Jackson *et al.*, 2009) of B cell surface CD23.

This study hypothesized that TLR4 expression is enhanced during *S. mansoni* infections, leading to B cell activation and raised CD23 expression, contributing to resistance to reinfection.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This study adopted a prospective longitudinal design, in which study participants were enrolled, and samples taken at baseline, then followed for 18 months, during which stool samples were collected every 2 months while blood samples were taken every 6 months. The overall study design is summarized in Figure 3.1

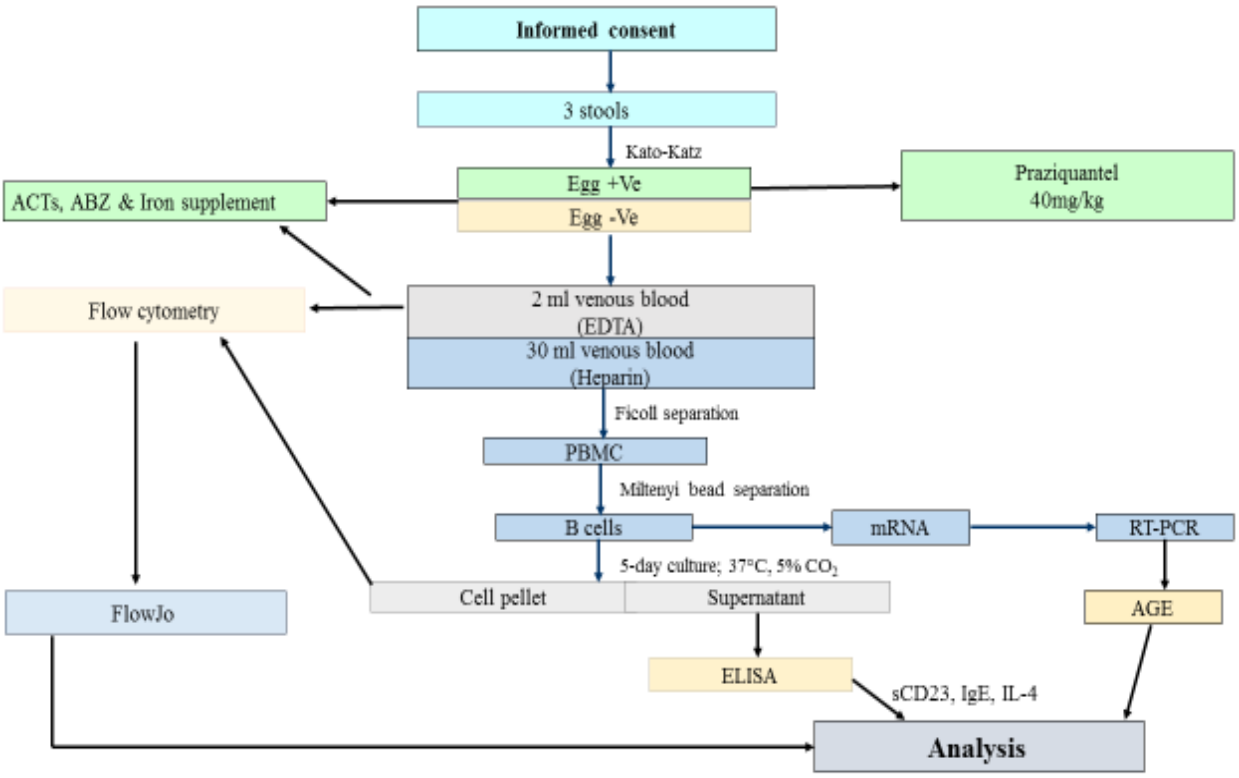

Figure 3.1. Schematic representation of the study design

3.2 Study Sites

Samples for this study were collected from individuals at three different beaches along the shores of Lake Victoria (Appendix IV). The first was the *sand harvesters* site, located within Kisumu Municipality in Kisumu County, while the other two were f*ishermen* sites, located in Uyoma

peninsula in Rarieda Sub County (Siaya County), about 80 Km from Kisumu City. All the sites were situated on the shores of L. Victoria, Western Kenya, an area endemic for human schistosomiasis (Karanja *et al.*, 2002a). In these sites, sand harvesters and fishermen are occupationally hyperexposed to infective *S. mansoni* cercariae, by entering and standing in the water for long hours as they work to earn a living, especially given most of these activities concentrate from mid-morning till evening, a period suitable for cercarial invasion (Lee and Lewis, 1977).

3.3 Study Population

According to the 2009 national census report, there were projected populations of 4,400 (Usoma), 3,700 (Madundu) and 5,300 (Luanda-Kotieno) people beaches. Many of the sand harvesters were drawn from areas where schiostosomiasis research had been conducted by the CGHR-KEMRI Schistosomiasis Research laboratory since the year 2003, and it was as such easy to enroll individuals who had received different rounds of praziquantel (PZQ) treatment for schistosomiasis. Some of these men had in previous studies been established to show resistance to re-infection with *S. mansoni*, while others remain highly susceptible following praziquantel treatment (Karanja *et al.*, 2002a; Mwinzi *et al.*, 2009). New enrolees, mostly from the fishing and sand harvester consortia, formed a prospective cohort that was followed for two years. This group of participants included persons who consented and enrolled into the study for the very first time and whose treatment and re-infection episodes were monitored for a period of three years, with follow-up blood collection done after every six months for immunologic assays. The participant exposure to infection was measured by the number of hours per day that they spent in the lake, recorded daily on a preformed water contact book. In addition, unexposed, healthy

donors from Boston, USA, without history of travel to *S. mansoni*-endemic areas and visiting Kisumu for the first time, were considered for inclusion as negative controls for CD23 isoform expression. Each person had access (or the right to refuse) to praziquantel, or treatment for other geohelminthic and parasitic diseases including hookworms and malaria (as detailed in Appendix I).

3.4 Sample Size Determination

Prevalence of schistosomiasis in occupationally exposed, previously untreated, newly diagnosed individuals in the study area is about 95% (Karanja *et al.*, 2002b). Elevated CD19+/CD23+ B cell levels have been found in a section of chronic schistosomiasis patients, who tend to exhibit resistance to re-infection with the fluke (Mwinzi *et al.*, 2009). For this study, a new and current group of volunteers with schistosomiasis was progressively enrolled and longitudinally followed.

To calculate sample size, previous data comparing cytokine (IL-4, IL-5, IL-10, IL-13, and IFN-γ) production in response to adult worm antigens for susceptible and resistant subjects was used, plus a statistical model comparing estimated means of the two groups over time. Individuals susceptible to reinfection have shown a mean value of 122 ± 146 pg/ml of blood; for the group that had developed resistance, the mean values was 20 ± 71 pg/ml (Mwinzi *et al.*, 2009). In sample size calculation using PS-Power and Sample Size Calculation software (Dupont and Plummer, 1990), a normal distribution of data was assumed; seven measurements over three years, a change of 122 pg/ml to 20 pg/ml for the resistant group, a more or less constant level of 120 pg/ml for the susceptible group, a constant standard deviation over time of 90 pg/ml for both groups, within subject correlation of 0.80, power of 80%, and an alpha of 0.01. Using a 1:1 ratio

of resistant to susceptible individuals, the study would need 39 persons per group, or 78 total enrollees. To correct for likely non-normal distribution of the data, the Pitman Asymptotic Relative Efficiency value of 0.864 was used (Dupont and Plummer, 1990). Thus, four groups of participants were enrolled: 1) approximately 45 (39/0.864) individuals who would be in the "developing resistance" group, who are susceptible to reinfection at enrollment and develop longer times to reinfection as the study went on; 2) 45 individuals who would remain susceptible to reinfection throughout the study were needed; 3) 45 who would maintain resistance during the study; 4) 40 donors (including 5 from Boston-USA and 35 males sampled from the community, who had mainly grown up away from the local community, and had not had exposure to the cercariae through domestic or economic activity) were included for comparison ($p < 0.05$). This brought the total participant number was 175.

The study participants were grouped into two: ***Untreated*** referred individuals without any history of praziquantel use, either because they were newly recruited into a study involving the drug, or had not been treated using the same in hospital. The group further included all who remained uninfected throughout the follow-up period for this study. On the other hand, the ***Treated*** group comprised of individuals previously treated repeatedly with praziquantel for intestinal schistosomiasis. Part of these had been participants in previous or ongoing studies, but who were recruited afresh into this study. This group later included all individuals who received multiple treatement following enrollemnt.

3.4.1 Sample Size Justification

It was estimated that over 300 participants (sand harvesters and fishermen) would be available during the proposed study period. Using this estimate, all eligible persons were invited to participate; with an acceptance rate of 80% from previous experience at the car wash and sand harvesting consortia (here estimated at 240 persons), the stated goal of 175 participants was achieved, and the sudty enrolled the requisite 170 respondents form the 3 sites, plus 5 from USA, totaling to 175.

3.5 Inclusion and Exclusion Criteria

Study participants were included in the study if they were members of the sand harvesting consortium in Kisumu, and the fishing consortium in Uyoma (operating within Luanda Kotieno and Madundu beaches). They were also eligible if they were 18 years or older (confirmed by physically presented national identification card or relevant valid document, like birth certificate, passport, driver's license or voter identification card), and had given written informed consent, and, they were willing to voluntarily participate in the study as explained to them. The potential participants must have been either sand harversters of fishermen to enroll. Individuals were excluded from the study if they were aged below 18 years (or lacked a valid identification document), were neithr sand harvesters nor fishermen, did not wish to participate in the proposed study, or, who declined to sign the Informed Consent form. Furthermore, any individual who was deemed not suitable to have blood drawn from, following clinical assessment by the study nurse was also not enrolled into the study.

3.6 Ethical Considerations

Clearance to conduct the study was sought from the School of Graduate Studies, Maseno University. This study was run within the Schistosomiasis Program at the Center for Global Health Research, KEMRI, and was duly reviewed and authorized by the KEMRI Scientific Steering Committee and the KEMRI Ethical Review Committee (Study Number 1611). The study recruited adult males aged over 18 years and above (confirmed by a valid national identification document, or any other legally acceptable document) occupationally exposed as sand harvesters fishermen along the shores of Lake Victoria. The sand harvester study cohorts were initiated in 2003 and have been maintained by the KEMRI to date, while the fishermen consortium was newly established in November 2010 for this particular study. These groups were of choice since these individuals, particularly males, spend long hours in the schistosome-infested lake water, thus are at increased risk of infection. Participant selection was non-random, and open to all eligible and willing candidates, so that all persons meeting the inclusion criteria (outlined under *Inclusion and Exclusion Criteria*) were asked to participate. Each prospective participant was explained to the importance of the proposed study and all procedures involved, including expected benefits and potential harm, and a written informed consent obtained prior to enrollment and sample collection (Appendix I). All prospective participants were approached to participate in the study by KEMRI staff supporting this study, who explained the study in English, Kiswahili and Dholuo (accordingly). Because there had not been any biomedical or health-related study along the selected fishing beaches, six such sessions were organized so that all prospective participants had adequate information on the study as at the time of enrollment for the fishermen sites, while only three sessions were required to achieve the same at the sand harvester site.

Private information that was obtained from individually identifiable persons was obtained using a short questionnaire at enrollment (including demographic information); blood and stool samples were collected before and after treatment for the cross-sectional group, and every two months for the prospective group to determine *S. mansoni*, soil-transmitted helminths and malaria (this done to increase benefiscence), as well as to evaluate cure following treatment. All those found to harbor these infections were offered appropriate treatment (PZQ, albendazole, Co-Artem). The only potential risks to study participants during specimen collection would be infections or injury during venous blood collection. Blood was however drawn using sterile techniques and sterile disposable materials by qualified KEMRI technologists to minimize risk of infection. The standardized questionnaire was not psychologically invasive and focused on information that only concerns schistosomiasis exposure habits and general demographic information.

To protect participant confidentiality, all data was collected and entered recorded using unique codes designed by the researcher, and only known to the technologists. Participants benefited by receiving free diagnosis and treatment for schistosomiasis, other helminthes and malaria. The field nurse and pharmaceutical technologist also offered free medical check-up and advice to the study participants, and where necessary, referred them accordingly for further treatment.

3.7 Potential Risks

To protect against the potential risks associated with obtaining venous blood specimens, like infection, blood was drawn under sterile conditions by qualified KEMRI laboratory technologists. To assure against any risks to the study participant's confidentiality, all data was

collected and entered using codes. Because the project nurse would treat participants for schistosomiasis, soil-transmitted helminthes and malaria when diagnosed, there was an arrangement with Jaramogi Oginga Odinga Teaching and Referral Hospital for medical referral, whenever need arose.

3.8 Potential Benefits

One of the direct benefits to the participants enrolled in this study was that they were followed closely (every 3 months) in regard to their occupational risk of acquiring *S. mansoni*, and treating them whenever they were found to be infected (re-infected). The participants were also examined for any of the most common soil-transmitted helminthic infections (hookworm, ascariasis, and trichuriasis) and malaria, and the study nurse treated them for those infections when they were found. All treatment was free-of-charge, and this interaction provided a good opportunity to educate the study community on health services available in their community.

3.9 Recruitment Procedures

The recruitment of participants was done at two fishing and one sand-harvesting beaches on the shores of Lake Victoria, Kenya. Fishermen and sand harvesters at the beaches were approached to participate in the studies by the researcher and/or study staff in English, Kiswahili, or Dholuo (the local language). The study was described in detail, especially indicating the longitudinal nature of the study, and the potential risks and benefits associated with participation in the study. Informed consent documents were provided to those expressing interest in participating, and these documents were explained as needed. The documents were provided in English, Kswahili, or Dholuo. Forty five (45) participants were recruited from each of the three beaches. On the

other hand, North American researchers (all from USA) without prior exposure to schistosomiasis-endemic regions and visiting KEMRI laboratories for the first time were approached and requested to donate blood to serve as negative controls for the cross-sectional survey of CD23 isoform mRNA expression. A total of 5 consented and gave the required amount of blood. Written informed consent was obtained prior to the inclusion of any individual into the study. Study participants (except the North American donors) were encouraged to remain in the study for at least 40 weeks so that any infected individuals could be fully monitored after treatment. However, any participant wishing to leave the study could do so at any time.

3.10 Confidentiality

Printed research data was kept in secured cabinets in a locked office at CGHR-KEMRI. Data obtained from a participant was assigned a random alphanumeric code, which was kept in the laboratory notebook of the persons technically performing the study. In the event of need to know a participant's identity, such as in the case of follow-up investigations, a separate set of records listing the blood donors' names and dates of donation were consulted. This code was kept with the patient's informed consent document. All procedures followed were in accordance with the Kenya Ministry of Health Policy, and were only done as outlined in the consent document (Appendix I).

3.11 Sample Collection

3.11.1 Stool

Special plastic containers of about 50 ml capacity with well fitting caps were given to each participant already labeled with his study ID and date. For three consecutive days, at enrollment

and every 2 months for up to 18 months, thee stool samples were requested and processed by the modified Kato-Katz technique (Vastergaard Frandsen), where each stool sample was processed on duplicate slides. These samples reached the laboratory within 4 hours to boost recovery and identification of other helmith (hookworm, ascaris and trichuiris) ova, alongside detecting *S. mansoni*). Any persons found to be infected with schistosomes or other soil-transmitted helminthes were notified directly, and treated accordingly using praziquantel and albendazole.

3.11.2 Blood

Following positive diagnosis of *S. mansoni* infection by the Kato-Katz technique, 30 ml of venous blood was collected in heparin-containing vaccutainer^{TM} tubes, and a further 2 ml in EDTA-containing tube from each participant for whole blood immunophenotyping. Blood samples were used to isolate and characterize B cell CD23 expression following infection with *S. mansoni*. CD23 isoform messenger RNA (mRNA) expression, B cell surface expression, and cleavage of CD23 from B cells were investigated. Also investigated was the level of CD23-bound IgE on B cell surface in individuals with *S. mansoni* infection. Each consenting participant was bled at enrollment then every six months for 18 months (4 bleeds in total).

3.12 Sample Storage

Samples collected from study participants including plasma, granulocytes and culture supernatants were stored at -20^{o}C until used in immunologic assays. It was anticipated that there would be some remaining samples after the relevant assays. These were stored frozen at -80^{o}C (cells stored in DMSO while culture supernatants stored as harvested) as space allowed, and as

was indicated in the Informed Consent forms, otherwise they were destroyed with permission from the Director, KEMRI.

3.13 Sample Processing

3.13.1 Identification of B Cell Sub-populations

From each sample drawn, 100 µl of fresh whole blood was stained with CD19 PE-Cy5, CD23 PE (BD Biosciences, USA), IgE-Biotin conjugate, Streptavidin-FITC (eBiosciences, USA), CD69 FITC, CD40 APC (BD Biosciences, USA) to identify B cell sub-populations. Cells were first allowed to stain for 30 minutes, lysed using 10% FACS lysis solution, then washed twice with 5 ml 1X FACS wash buffer, then centrifuged at 1500rpm for 5 minutes and supernatant carefully discarded. The cells were then fixed and and ran immediately, or kept at 4°C until acquisition (within 48 hours) on a four-colour FACSCalibur (Becton Dickinson, USA).

3.13.2 IgE Binding Assays

This technique, as modified by LiangGanley-Leal (2009) was used to quantify the amount of IgE carried on CD23 expressed on B cells. It was thus important in gauging the balance between cell-bound and free IgE, relative to CD23 expression as determined by ELISA; 100 µl of blood and re-suspended (cultured) whole blood and B cell pellet (after culture) was used. Briefly, for fresh whole blood and cultured whole blood, red blood cells were first lysed lysed using 10% FACS lysis solution, washed twice with 5 ml 1X FACS wash buffer then stained CD19 PE-Cy5, CD23 PE (BD Biosciences, USA), IgE-Biotin conjugate, Streptavidin-FITC (eBiosciences, USA). The cells were then incubated at 4°C for 30 minutes on a rotator, IgE-biotin conjugate added, washed with FACS wash buffer, and streptavidin-FITC added, incubated and washed once more with

FACS wash buffer. Cells were then fixed as above, and acquired similarly. Cultured B cells were treated as the whole blood, except there was no red cell lysis step.

3.13.3 B cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were separated from the heparinized blood by Ficoll Hypaque density gradient technique (Atlanta Biologicals, USA). Plasma and granulocyte compartments were extracted and frozen for later assays. Using magnetic beads, untouched B cells were isolated by negative selection using Miltenyi Kit II® (Miltenyi Biotech Inc., USA), according to manufacturer's instruction. Briefly, 100µl of heparin-preserved whole blood and B cell extract (at $4X10^5$ cells/well) were used for culture with different antigens for 5 days: in RPMI-1640 alone, and RPMI-1640 stimulated with soluble egg antigen (SEA), soluble worm antigen preparation (SWAP), recombinant IL-4 (rIL-4) and anti-CD40 mAb (in different combinations as shown on Tablle 3.1).

Table 3.1. Different antigens used in B cell culture

Stimulating antigens
RPMI-1640 alone
RPMI-1640 + rIL-4 + aCD40
RPMI-1640 + SWAP + rIL-4
RPMI-1640 + SWAP + aCD40
RPMI-1640 + SWAP + rIL-4 + aCD40
RPMI-1640 + SEA
RPMI-1640 + SEA + aCD40
RPMI-1640 + SEA + rIL-4+aCD40‡

3.13.4 RNA Extraction, RT PCR and Agarose Gel Electrophoresis

According to the manufacturer's instruction, total RNA was extracted from ~1 million B cells using RNeasy Mini Kit (QIAGEN^{TM}). The extracted RNA was further treated with DNase (1µl/50µl of extract) and heat at 37ºC for 30 minutes, then at 65ºC for 30 min, to remove any contaminant DNA. Reverse transcriptase polymerase chain reaction (RT-PCR) was run on the purified RNA extract using a MultiGene^{TM} II Personal Thermal Cycler (Labnet International Inc., NJ-USA) as per manufacturer's manual, to determine CD23a and CD23b mRNA expression, with β-actin mRNA as positive control. The PCR products were resolved by agarose gel electrophoresis on a 1% agarose, for 30 minutes, to identify bands reflecting the presence of CD23 mRNA. The DNA (3 µl) was labeled with 1 µl of EvaGreen^{TM} dye (Biotium Inc., California, USA), and carried in 1 µl 92% glycerine. Purity of the extracted RNA and detected DNA was confirmed by running RNase-free water as a control. The gel photos were captured by a camera reading from a UV transluminator. The level expression of CD23a and CD23b mRNA isoforms was then determined by densitometric analysis using ImageJ (NIH, USA). The primers used during the RNA amplification are summarized in Table 3.2.

Table 3.2. Primers used for RNA amplification

Primer	Sequence
CD23a forward	ATGGAGGAAGGTCAATATTC
CD23a reverse	TCCAGCTGTTTTAGACTCTG
CD23b forward	ATGAATCCTCCAAGCCAG
CD23b reverse	CACAGGAGAAGCAGAGTCAG
β-Actin forward	TCATGAAGTGTGACGTTGACATCCGT
β-Actin reverse	CCTAGAAGCATTTGCGGTGCACGATC

3.13.5 Enzyme-linked immunosorbent assays (ELISAs)

Plasma and culture supernatants from samples were stored frozen at -20°C for measuring free IgE and sCD23, using sandwich ELISA, using on a 96-well microplate. Using respective standard ELISA protocols, the listed factors were quantified according to manufacturer's instruction. Briefly, plates were coated (sCD23 ELISA plates were available pre-coated with anti-sCD23 monoclonal antibody) with the respective antigen, and incubated accordingly. The plates were then washed 3-5 times using 300 µl of the respective wash buffer per well, 300 µl of blocking buffer added per well and incubated for 1 hour at room temperature. Next, the plates were washed 3-5 times using 300 µl of the respective wash buffer per well, and 100 µl of blocking buffer added per well and incubated for 1 hour at room temperature. This was followed by different incubation ad wash procedures, the involved the addition of the respective enzyme conjugates, then substrates (Appendix II). After the final wash using 300 µl of the respective wash buffer per well, plates were immediately read using an electronic reader, Vmax (Molecular Devices, USA), at an absorbance of 450 nm.

3.14 Data Management

The data included participant sociodemographic information captured on the consent document and enrollment questionnaire, water contact hours, laboratory results of stool tests for schistosome diagnosis, immunological assay data, including flow cytometry, culture, ELISA, molecular biology data on mRNA expression, and treatment records for schistosomiasis. Data was stored in files in secured cabinets and password-protected databases (as applicable) and only accessible to the PI and mentors, a trained data clerk and a statistician. Databases were stored in accordance with the KEMRI ERC guidance and would only be destroyed with permission from the KEMRI Director.

3.15 Data Analysis and Reporting

Difference between the expression of CD23a and CD23b isoform mRNA by B cells was detected by student t-test following densitometric analyses by ImageJ®. Correlations between CD23 mRNA and surface expression and cleavage, level of bound and free IgE, and cytokines were statistically determined at baseline, and during follow-ups on *S. mansoni* re-infected persons using linear regression and correlation analyses. Specifically, statistical associations were checked between the free versus bound IgE and CD23, and mRNA expression. In addition, the study sought to establish correlation of fresh whole blood B cell surface expression of CD23, CD69 and TLR4 with worm intensity measured in eggs per gram (EPG) of stool. All statistical analyses were carried out using GraphPad Prism version 5 ($p < 0.05$).

CHAPTER FOUR

RESULTS

4.1 Introduction

This section presents the findings of the study, conducted on adult male sand harvesters and fishermen at two different sites (separated by approximately 80 Km) on the shores of Lake Victoria, in the Nyanza region, western Kenya. At the start of the study, 175 eligible individuals enrolled, with a mean age of 34.82 years (range: 18 to 75 years).

4.2 CD23 isoform mRNA expression

One of the objectives of this study was to characterize B cells from study participant on the basis of their CD23a and CD23b isoform mRNA expression. CD23b mRNA expression was more dominant than CD23a ($p < 0.001$). Figure 4.1 is an agarose gel electrophoresis photograph showing the comparison of CD23a and CD23b mRNA expression.

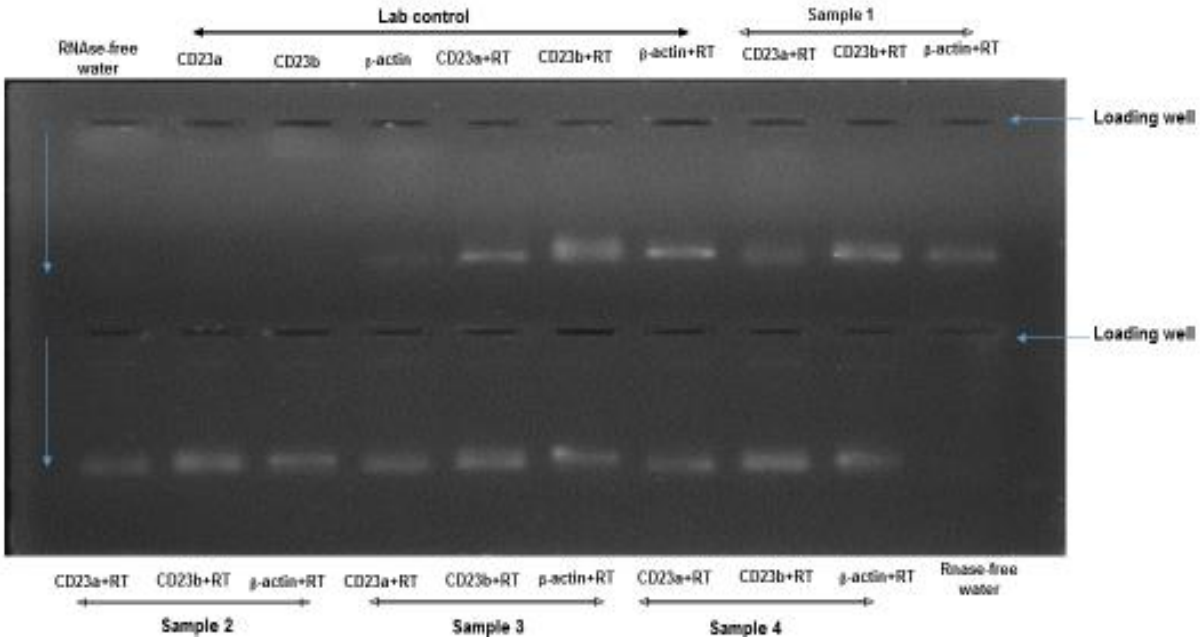

Figure 4.1. CD23a and CD23b mRNA expression by agarose gel electrophoresis. DNA was resolved in 1% agarose gel for 30 minutes. Figure 7B shows CD23 expression by B cells of one control and four *S. mansoni*-infected persons. CD23b was predominantly expressed over CD23a in all cases.

The exposed individuals had a higher CD23b/CD23a ratio than the unexposed-and-uninfected ones (t = 2.853; p < 0.0001), although this was not significantly different between the uninfected individuals in endemic areas and the hyperexposed persons (t = 0.0853; p = 0.679). IL-4 was found to significantly raise CD23b mRNA expression, unlike stimulation by either IgE alone or IgE (t = 2.378; p < 0. 001) plus IL-4 (t=1.617; p = 0.0314).

4.3 sCD23 and IgE in Plasma and Culture Supernatants

The amount of sCD23 detectable in plasma was positively correlated with the mean expression (MFI) of CD23 on B cells (r = 0.6137; *p* = 0.020), as indicated on Figure 4.2.

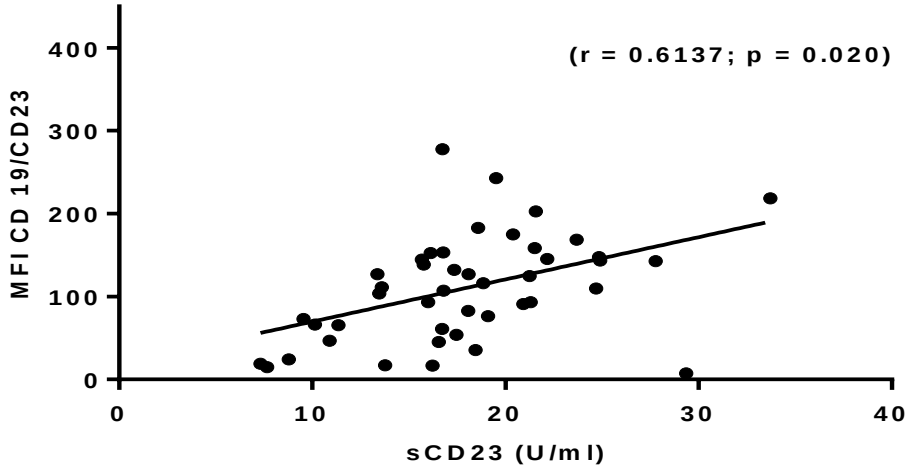

Figure 4.2. Correlation of sCD23 with CD23 MFI on whole blood B cells. The mean expression of CD23 on the surface of B cells.

The expression of CD23 on cultured B cells was positively correlated with plasma IgE levels (r = 0.4498; *p*=0.0001), as illustrated in Figure 4.3.

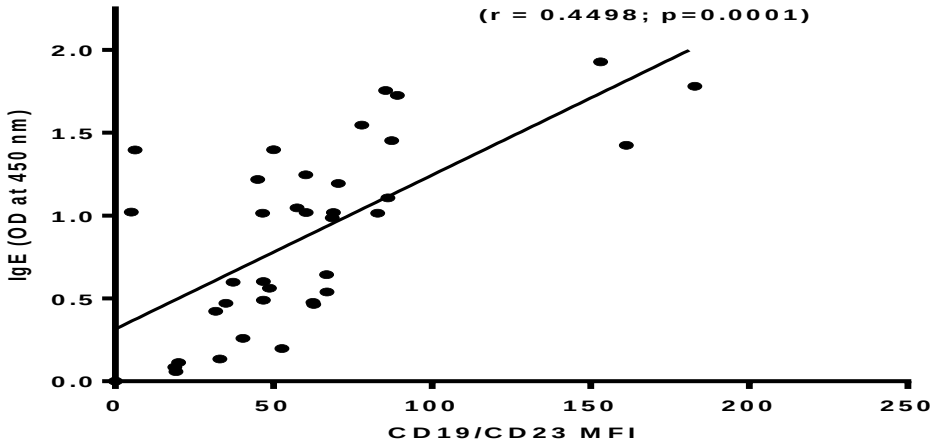

Figure 4.3. Correlation of culture IgE levels with CD23 expression on B cells. Increased mean expression of B cell surface CD23 correlated with increased IgE levels in culture supernatants.

Table 4.1 shows that the yields of IgE and sCD23, respectively, in culture supernatants were on average not significantly different from plasma levels, as deteremined by ANOVA, except in media stimulated with SWAP/rIL-4/aCD40 or SEA/rIL-4/aCD40 ($p < 0.001$). sCD23, and not IgE, was in addition significantly elevated ($p < 0.05$) in supernatants from wells cultured with either rIL-4 aCD40 (without SWAP or SEA). sCD23 levels were not raised in cultures stimulated with rIL-4, aCD40, SEA or SWAP as single antigens compared to RPMI ($p > 0.05$).

Table 4.1. Comparison of culture sCD23 and IgE levels

Stimulating antigens	***p* value**	
	sCD23	**IgE**
RPMI-1640 alone	> 0.05	> 0.05
RPMI-1640 + rIL-4 + aCD40‡	**< 0.001**	**< 0.01**
RPMI-1640 + SWAP + rIL-4	> 0.05	> 0.05
RPMI-1640 + SWAP + aCD40	> 0.05	> 0.05
RPMI-1640 + SWAP + rIL-4 + aCD40‡	**< 0.001**	**< 0.01**
RPMI-1640 + SEA + rIL-4	> 0.05	> 0.05
RPMI-1640 + SEA + aCD40	> 0.05	> 0.05
RPMI-1640 + SEA + rIL-4 + aCD40‡	**< 0.001**	**< 0.001**

‡ **Treatments with significant differences ($p<0.05$) are in bold**

4.4 Level of CD23-bound IgE on B cell surface

There was a significant amount of IgE bound to CD23 on the surface of B cells of fresh whole blood. The geometric mean expression of CD23 expressed on B cell surfaces was significantly higher ($t = 2.923$; $p = 0.0435$) on severally treated individuals relative to the praziquantel-naïve counterparts. On the other hand, the geometric mean expression of CD23-bound IgE expressed on B cell surfaces was significantly lower ($t = 4.994$; $p < 0.001$) on severally treated participants compared to those not previously treated with praziquantel (Figure 4.4).

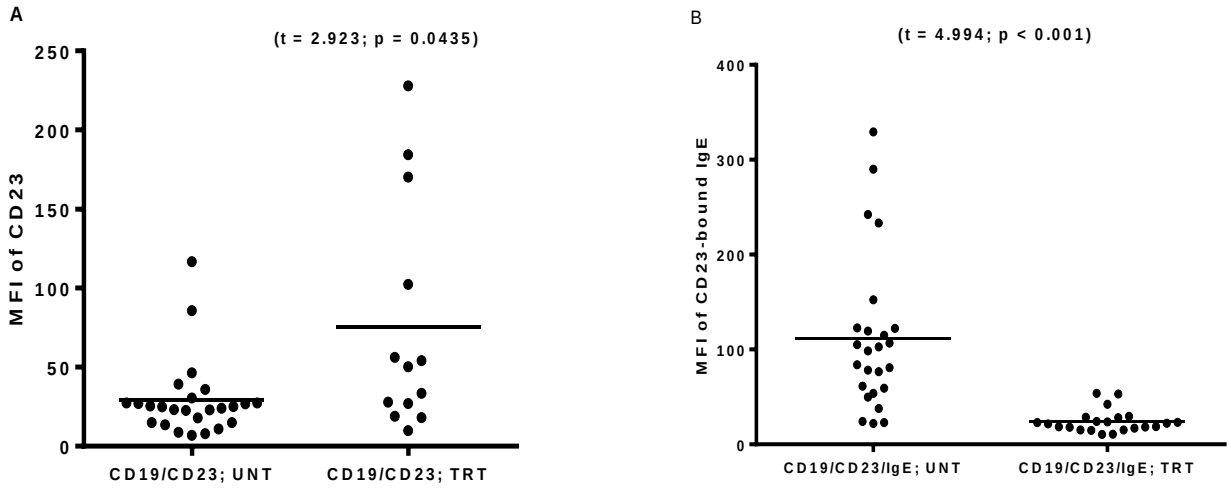

Figure 4.4. Geometric mean expression of CD23 and CD23-bound IgE on B cells. The mean fluorescence of CD23 (A) and CD23-bound IgE (B) differed between the treated (TRT) and untreated (UNT) groups.

4.5 B cell CD23, CD69, CD40 and TLR4 Expression

There was statistical correlation between CD40 and CD23 expression by B cells ($r = 0.2179$; $p = 0.0285$) as shown in Figure 4.5.

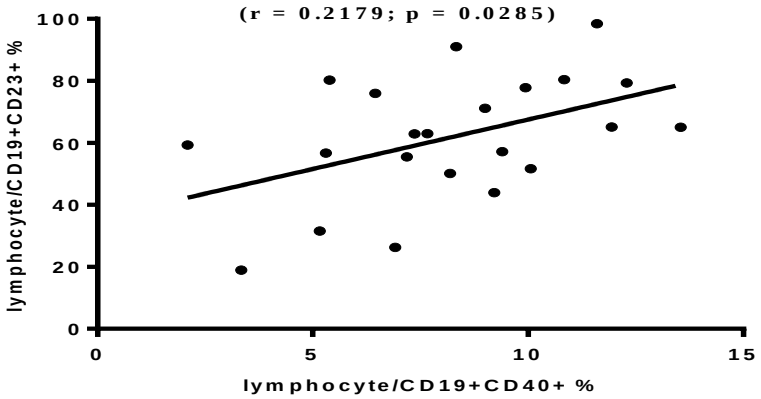

Figure 4.5. CD40 expression by whole blood B cells. There was a significant positive correlation between the percentage expression of CD40 and CD23 by B cells.

The study found no significant correlation of fresh whole blood B cell surface expression of CD23, CD69 and TLR4 with worm intensity measured in eggs per gram (EPG) of stool ($p > 0.05$). The percentage of TLR4-bearing B cells (CD19/TLR4%) positively correlated with both CD19/CD69% (r=0.6516; $p < 0.0001$) and CD19/CD23% (r=0.4819; p=0.0020). There was however no correlation between CD19/CD23% and CD19/CD69% (r=0.0613; p=0.2928). These findngs are presented on Figure 4.6.

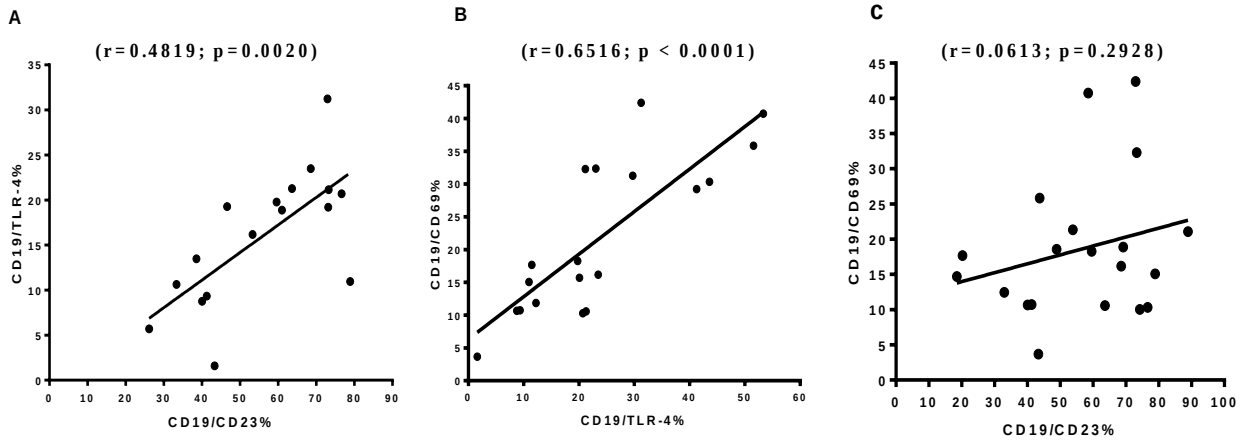

Figure 4.6. B cell surface CD23 expression relative to CD69 and TLR4 expression. There was a strong positive correlation between CD23% and TLR4% on B cells (A), and a strong positive correlation between TLR4% and CD69% on B cells (B), but no correlation between CD23% and CD69% on B cells (C).

When data on receptor expression was analyzed based on praziquantel treatment history, there was a strong correlation between the percentage expression of CD69 and TLR4 by B cells of praziquantel-naïve individuals ($p < 0.0001$; $r = 0.6247$), in praziquantel-treated individuals ($p < 0.0001$; $r = 0.3913$). The mean fluorescence same was equally positively correlated for both previously untreated ($p = 0.8391$; $r = 0.4876$) and treated ($p = 0.0106$, $r = 0.1983$) with praziquantel (Figure 4.7).

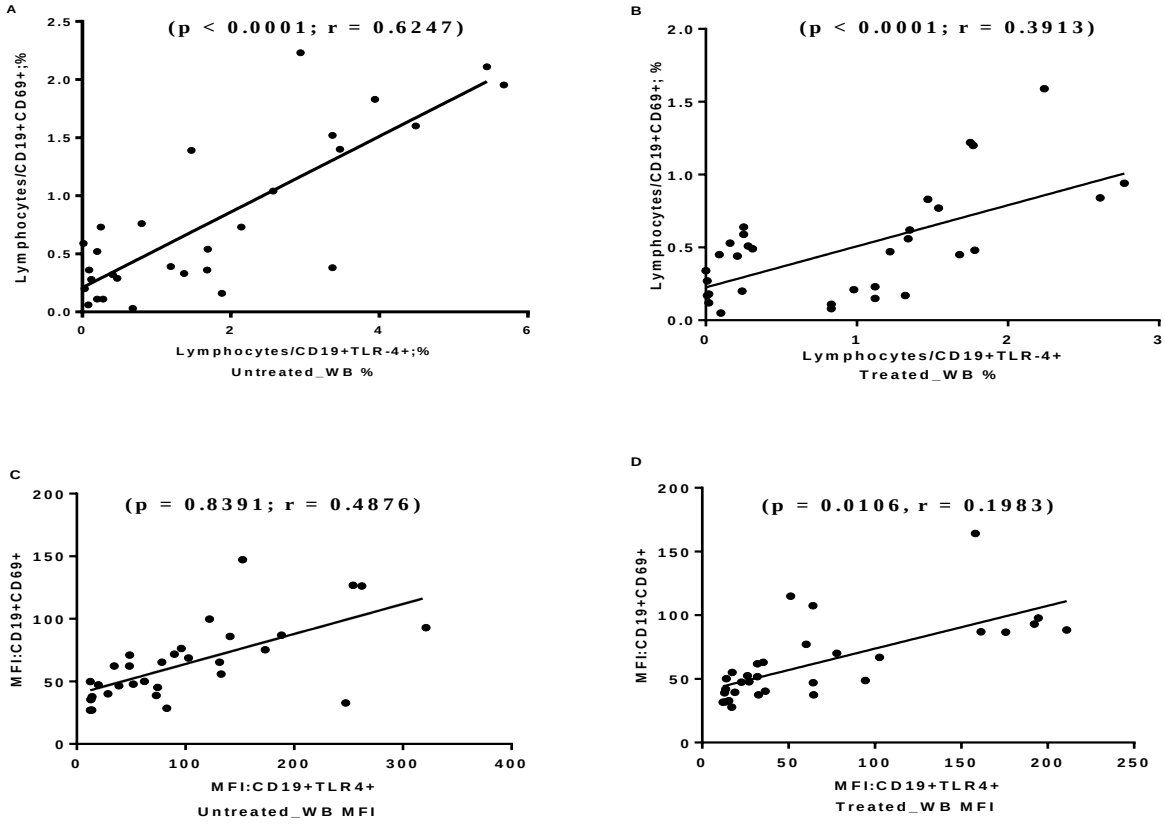

Figure 4.7. CD69 and TLR4-expression by fresh whole blood B cells. There was increased TLR4 expression (%) with CD69 expression (%) in both treated and untreated groups (A, B), similar to the mean TLR4 and CD69 expression by B cells (C, D).

4.6 Praziquantel Treatment History and Cultured Whole Blood B Cell Phenotypes

Upon 5 days culture, the proportion of B cells (CD19%) was not significantly different between media-only and PWM-stimulated B cells of individuals not previously treated with praziquantel (t=0.0681; *p*=0.1522). While CD19/TLR4% was lower on PWM-stimulated B cells of praziquantel-naïve participants (t=0.4963; *p*=0.0144), TLR4 MFI on B cells was not statistically different (t=0.1427; *p*=0.0628). However, CD19/CD69% was not different between the treated and untreated (t=0.2154; *p*=0.5071) groups. Similarly, the expression of CD69 on CD19/TLR4% was not statistically different between media-only and PWM-stimulated B cells (t=0.1849; *p*=0.0592). CD19/CD69% correlated with CD19/TLR4% in repeatedly treated participants (r=0.6725; *p*=0.0097) and not in untreated (r=0.0863; *p*=0.3236). CD69 and TLR4 MFI on CD19+ cells were not correlated in either treated (r=0.1358; *p*=0.6031) or untreated (r=0.1009; *p*=0.2905).

CHAPTER FIVE

DISCUSSION

5.1 Introduction

For communities living in areas endemic for water-related diseases like schistosomiasis, reducing the frequency and duration of contact with such infested waters may be crucial to reducing transmission of these diseases. The study population here lives generally close to the expansive Lake Victtoria, principally making it hyperexposed through regular domestic activities such as laundry, bathing, farming, and fishing (Steinmann *et al.*, 2006). This high exposure level poses a great challenge to attempts to control water-related health risks (Fenwick *et al.*, 2006; Fenwick and Webster, 2006; Brooker *et al.*, 2009).

During this study, the participants stated both the duration of time lived in the area and the duration of time they had been fishing. The mean and median duration lived in the area was about twice that of practising fishing, while there was a strong positive correlation between the duration of residence in the area and that of fishing. The observation was expected, since majority of the fishermen were natives of the local community. For those drawn from elsewhere, their primary reason for being around the beach was commercial fishing or sand harvesting (for persons participating in this study). The duration lived was also generally longer than the duration of fishing practice since the residents joined fishing at some age, perhaps after completing or when nearly completing primary education, approximately aged 12-15 years.

5.2 CD23 Isoform Expression by B Cells

CD23 expression on B cells has been shown to be related to resistance to re-infection with *Schistosoma mansoni* in a hyperendemic, repeatedly-treated cohort of adult males (Mwinzi *et al.*, 2009). Apart from IL-4, the other factors influencing CD23 expression and its specific role in the regulation of IgE synthesis remain less understood (Kicza *et al.*, 1989; McCloskey *et al.*, 2007). Particularly in helminthic diseases, the preferential expression of the CD23 isoforms (CD23a and CD23b) is less characterized, and so are mechanisms involved in its cleavage from cell surfaces.

This study has established a predominant CD23b expression, in both *S. mansoni*-infected and non-infected persons. This is in contrast with evidence among non-endemic populations, in which CD23a is more predominant (Yokota A *et al.*, 1988). One possible explanation here could be that CD23b being inducible, is triggered by a host of factors, including highly prevalent helminthic infections that induce IL-4 production. CD23a is constitutively expressed by B cells (Yokota *et al.*, 1988). Studies on the drivers of CD23 isoform expression have shown that whereas the CD23a promoter is activated only by IL-4, the CD23b promoter is also stimulated by IL-4, anti-mu, and anti-CD40 (Kolb *et al.*, 1994; Onguru *et al.*, 2011a). The activation of CD23 transcription has been reported to depend on two putative STAT6 binding sites, the first of whose deletion poses an abrogative effect on IL-4 driven activation of CD23a transcription, while the deletion of both is requisite for the expression of CD23b (Ewart *et al.*, 2002). The dominance of CD23b expression in B cells may thus be attributable to yet to be established helminth-triggered factors that result in editing both STAT6 binding sites, or that enhance IL-4 production.

Because of the multiplicity of possible causes of raised IL-4, both schistosome-infected and non-infected individuals tend to be predisposed to higher levels of CD23b. The difference could also possibly be from a lack of IgE bound to the cells which have been found to downregulate CD23b (Griffith *et al.*, 2011). While this study finds CD23b as more prominently expressed by B cells, it does not provide the specific details on the extent to which the observation is attributable to *S. mansoni* infection.

5.3 B cell surface CD23, sCD23 and soluble IgE

The expression of CD23 on B cells was directly associated with plasma IgE tires, consistent with previous findings (McCloskey *et al.*, 2007). This further confirms the pivotal role CD23 plays as a receptor for IgE-mediated antihelmintic responses. The amount of sCD23 detectable in plasma was positively correlated with the mean expression (MFI) of CD23 on B cells (Hibbert *et al.*, 2005b), comparable to findings in among Ugandan fishermen, which revealed similar trends in IgE reduction following chemotherapy with praziquantel (Fitzsimmons *et al.*, 2004). This explains the plausible role of IgE in anti-schistosome responses, which are known to be mediated by CD23 through cross-linking with antigens and other receptors (Kolb *et al.*, 1994).

Following different antigenic treatments of cultured B cells, the yields of IgE and sCD23 in culture supernatants were generally not significantly different from plasma levels, except in media stimulated with SWAP/rIL-4/aCD40 or SEA/rIL-4/aCD40. sCD23, and not IgE, was in addition significantly elevated in supernatants from cultures with either rIL-4 or aCD40 (without SWAP or SEA). sCD23 levels were not raised in cultures stimulated with rIL-4, aCD40, SEA or SWAP as single antigens compared to RPMI. This confirms the previously reported pivotal role

of IL4 in the stimulation of CD23 expression on B cells (Yokota *et al.*, 1988; Ewart *et al.*, 2002; Hibbert *et al.*, 2005b).

5.4 B cell surface CD23 and surface-bound IgE

There was considerable amount of IgE on the surface of B cells of fresh whole blood, bound to CD23. This finding is in tandem with other studies, and further supports the possible involvement of CD23 in the regulation of IgE synthesis and function (Aubry *et al.*, 1992; Hibbert *et al.*, 2005b; Griffith *et al.*, 2011). Being one of the receptors for IgE, and carrying the isotype on the surface of B cells enables the molecule to induce signaling mechanisms by the B cells that are crucial in the development of adaptive immune responses, especialy given CD23 also binds several other receptors, including CD21, CD11b and CD11c (White *et al.*, 1997). The binding of IgE to CD23 on B cell surface is important as it facilitates cross-linking of antigens, and possibly other receptors, triggering specific signaling pathways needed to propel antigen-specific immune responses, as previuously described (Kolb *et al.*, 1994).

5.5 CD23 expression and B cell activation

B cell interaction with other cells, notably T cells, is improved by the expression of accessory molecules like CD40 (Liebig *et al.*, 2009). From the analysis, there was a statistical correlation between CD40 and CD23 expression by B cells, perhaps bacuase CD40 expression is stimulated by several factors that activate B cells, and CD23 is key in stimulation by *S. mansoni* antigens. While the number of B cells expressing CD40 remained generally low, the mean exression was relatively high. This is consistent with other findings exerting the role of CD40 expression as necessary for optimal primary B cell responses (Lee *et al.*, 2003), and given its role in the

activation of dendritic cells and other APCs (Caux *et al.*, 1994a; Caux *et al.*, 1994b; Banchereau *et al.*, 1995).

5.6 CD23 and TLR4 expression by B cells

This current study found no significant correlation of fresh whole blood B cell surface expression of CD23, CD69 and TLR4 with worm intensity measured in eggs per gram (EPG) of stool. This is consistent with previous observations, where egg counts have not been correlated with other cell surface markers in several analyses (Karanja *et al.*, 2002a; Black *et al.*, 2010).

The percentage of TLR4-bearing B cells positively correlated with percentage B cell CD69 and CD23 expression. CD69 is an early marker of activation and the association of TLR4 levels with its rise may indicate active involvement of TLR4 in the recognition of schistosome antigens. Previous studies have demonstrated an association between human schistosomiasis and TLR4 expression by B cells (Takeda and Akira, 2003; Takeda *et al.*, 2003; Onguru *et al.*, 2011b), and a correlation between B cell CD23 expression and resistance to reinfection with *S. mansoni* (Mwinzi *et al.*, 2009). The failure to see a correlation between CD23 and CD69 expression by B cells may well be explained by the fact that CD23 is always expressed on B cells, especially the IL-4-induced CD23b isoform which has been shown to predominate in the study population (Onguru *et al.*, 2011a).

The proportion of B cells in whole blood samples did not differ significantly between the those who had been previously treated severally using praziquantel and those who had no history of treatment using the same drug. This could be attributable to the fact that several factors may

influence cellular balance and dynamics. The higher mean fluorescence intensity of CD19 and greater heterogeneity in praziquantel-naive than in praziquantel-reated participants need further investigations as this study cannot at this point provide a valid explanation. A possible proposition could be that the untreated group could have more aggressively involved B cells hence the expression of more CD19, a receptor with B cell regulatory functions (Sato *et al.*, 1997; Watanabe *et al.*, 2007).

The expression of TLR4 by B cells was not different between Untreated and Treated (p=0.6083), although TLR4 MFI on CD19 was lower on Treated than Untreated persons (p=0.0392). The former observation could be because upon challenge, these innate receptors might be activated regardless of prior exposure and/or treatment, resulting in similar expression profiles between the two groups. The extent of expression by individual cells within a particular exposure/treatment group is however expected to be different, as drug treatment alters the immune profiles of responding individuals (Black *et al.*, 2010) as a consequence of exposure of worm antigens from dying worms, as previously reported (Mutapi, 2001; Fitzsimmons *et al.*, 2004). B cell expression of CD69 was not different between Treated and Untreated groups, as CD69 indicates activation, and each groups showed activation, reflecting the involvement of TLR4, which has also been shown to be crucial in poke weed mitogen-induced proliferation of B cells (Bekeredjian-Ding *et al.*, 2012).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The mRNA for isoform CD23b was found to be predominantly expressed over that of CD23a by B cells of adults exposed to *S. mansoni*. The IL-4 driven immune responses characteristic of schistosome infections also favour CD23b expression, and this may be as a result of so many other factors.

This study concludes that B cells use CD23-bound IgE for their responses to *S. mansoni* antigens, and that there is the involvement of both the cell surface-bound and cleaved forms of CD23 in IgE-mediated responses to *S. mansoni* infection.

It is evident that B cell CD23 expression increases with increase in the expression of the markers of B cell activation and function, especially during the early stages (CD69) and late stages (CD40) of activation. B cells thus use CD23 at all stages of their responses to *S. mansoni* infection.

This study reports that B cell CD23 expression increases with increasing TLR4 expression during immune responses to *S. mansoni*. It is therefore therefore highly possible that CD23 is involved in the innate recognition of *S. mansoni* antigens, and may play a role in TLR4-mediated B cell activation following *S. mansoni* infection.

6.2 Recommendations

1. This study recommends more detailed studies on the determinants of CD23 isoform expression by B cells, due to the fact that many factors may drive preferential CD23 expression. Such studies should cover a larger population, invoving both exposed and non-exposed individuals, considering the potential for other infectious diseases to interfere with the immune profile.

2. There is need to study the factors that influence CD23 expression and cleavage from B cell surface, IgE production and binding to both cell surface and soluble CD23 molecules in *S. mansoni* infected individuals.

3. This study suggests that mechanistic studies be conducted to investigate the specific roles and dynamics of CD23 (surface and soluble forms) during B cell activation following *S. mansoni* infections. These studies should involve studying the various B cell activation pathways, and particularly describe the specific relationships between CD23 and the markers of B cell activation, CD40 and CD69.

4. Finally, this study recommends that studies be conducted on the mechanisms involved in TLR4-mediated B cell activation following *S. mansoni* infections, with the specific objective of defining the role of CD23 during the activation process. These studies should involve both B cell-surface and soluble CD23 levels, and isoform expression, studied in the context of TLR4-mediated activation.

6.3 Suggestions for further research

Given CD23b is predominantly expressed by B cells of individuals infected by *S. mansoni*, coupled to other reports linking CD23-IgE to other immune responses like allergy, studies on the role of CD23 gene polymorphisms in B cell role in anti-schistosome responses are hereby suggecsted. This study suggests that the proposed studies should be conducted on highly exposed populations, which have no exposure to any chemotherapy for schistosomiasis. The enrollment of participants should consider the history and frequency of exposure, coinfections and other immunologic disorders like allergy and atopy, which influence IgE production, and therefore CD23 expression.

This study further suggests that the proposed studies should include detailed investigations on CD23b transcription and surface expression, especially on other cells such as T cells, monocytes, eosinophils. Further, the factors influencing CD23 cleavage from the cell surface also need to be investigated, as this will provide better understanding of CD23 role in *S. mansoni* infection. Such studies should also include the dynamics IgE binding on CD23 on the various cells relative to plasma IgE levels, and compared to the levels of related receptors, including CD21, CD40, CD69 and TLR4. In addition, these studies should include gene transcription analyses, to provide a deeper picture of CD23 role in *S. mansoni* infection.

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APPENDIX I: INFORMED CONSENT FORM

TITLE OF STUDY:
Immunoepidemiology of CD23 Expression by schistosome-activated B Cells

Principal Investigator:
Daniel Onguru[1,2];

Co-Investigators
Ayub V. O. Ofulla, PhD.[1]
Pauline Mwinzi, PhD.[2]
Lisa Ganley-Leal, PhD.[3]
Diana M. S. Karanja, PhD.[2]

Participating Institutions:
[1]Biomedical Sciences and Technology Department; School of Public Health and Community Development, Maseno University. P.O. Box Private Bag, Maseno-Kenya.

[2]Kenya Medical Research Institute, Centre for Global Health Research (KEMRI-CGHR), P.O. Box 1578, Kisumu, Kenya

[3]Boston University School of Medicine, Section of Infectious Diseases, Department of Microbiology 650 Albany Street, Boston, MA.

Participation Information:

You are being asked to take part in a medical research study being performed by the Kenya Medical Research Institute (KEMRI) in collaboration with University of Georgia, USA and the Boston School of Medicine. It is very important that you understand the following general principles that apply to all participants in our studies:

1) Your participation is entirely voluntary

2) You may withdraw from participation in this study or any part of this study at any time with no penalty

3) Please ask any questions that will allow you to understand the study more clearly.

Introduction:

Schistosomiasis, also known as Bilharzia, or snail fever, is a disease caused by worm parasites transmitted by snails. The snails live in different types of water including ponds, rivers and lakeshores. Persons whose activities cause them to come into contact with infested water where there may be transmission going on are likely to suffer from the disease. Schistosomiasis worm parasites penetrate your skin when you are in the water of a lake, river, stream, or pond. Schistosomiasis can sometimes be serious or even fatal if not diagnosed and treated properly.

Purpose of the Study

The purpose of this research study is to learn the reasons that some people get infected with worms again after they have been treated, while other people do not, even though they each get exposed by being in the water, and why some people seem to get infected less often after many treatments. We hope that learning these things will lead to better ways to control this disease. Any questions you may have about this study will be answered prior to you being in this study. The benefits to you will be the diagnosis and treatment of the parasites we find that you have. You can quit the study at any time without penalty. If you choose to quit the study, you will still be offered treatment for schistosomiasis, malaria, and/or intestinal worms that we have shown that you have at that time.

Who Can Participate in the Study?

You must be over 18 years old to participate

Risks, Hazards and Discomforts of Participation:

The risks or hazards to you if you take part in this study are minimal. Drawing of blood is almost always a harmless procedure with only slight discomfort when the needle is put in. Sometimes a slight bruise will appear for one or two days after you give blood. Someone trained to draw blood will draw the blood. It will be done using sterile methods, so that you will not be harmed by the process.

Benefits:

There are NO direct benefits to be expected from the study, except the diagnoses and treatment offered to the study participants and upon request from time to time.

Procedures to be Followed:

For this study, you will be asked to give between 25 ml and 40 ml of blood two times per year, over 3 years. About 40 ml of blood will be drawn when you first enter the study, and every six months thereafter for three years. The drawing of your blood is for research. You cannot be forced to give blood and if you decide that you do not want to have blood drawn, you will still be treated for whatever parasites that you have. Stool samples will also be collected from you every 2 months, and they will be looked at to see if you have worms. If you do have worms, you will be offered treatment for them by the study nurse. Your blood will be looked at to see if you have malaria. If you do have malaria, you will be offered treatment for it by the study nurse. Every two years, you will also be examined by feeling your stomach and using a sound wave machine to determine if your worms have caused any swelling of your spleen or liver. If they have, you

will be referred to Nyanza Provincial Hospital for more tests, follow-up, and treatment, if needed.

1. Day 0. You will be informed about the study. If you're interested in joining the study, you will be enrolled in the study and your informed consent will be obtained. A study number will be assigned to the study subject and all specimens will be labeled with this number upon collection.

I. 25-40 ml of venous blood will be drawn.

II. Stool cups will be provided.

III. The blood will be processed into B cells and serum and immunologic assays done on these specimens.

2. Day 0 or 1 (and subsequent 3 days as needed to obtain stool specimens)

I. Stool specimens will be requested and used to diagnosis bilharzia infection.

3. From Day 2-3

II. You will be offered treatment for malaria, soil-transmitted helminths, or schistosomiasis by the project nurse, should any of those infections be diagnosed.

4. After 2 months

I. Stool cups will be provided to those who were treated for schistosomiasis on Day 2 or 3.

II. This will be repeated every two months and you will be offered treatment whenever found positive for worms or malaria

5. Every 25 weeks (6 months), you will be asked to donate 40 ml of blood for immunologic studies. This process will be repeated every 6 months for as long as you wish to remain in the study.

6. Within first 30 days and after 2 years, patients will be offered ultrasonography by the project ultrasound team, to determine if they have developed hepatic morbidity due to schistosomiasis. This will be scheduled during one of their twice a year blood drawing appointments described in #5. Any subjects (the number will be small) who have ultrasonographic evidence of hepatic morbidity (periportal fibrosis) due to schistosomiasis will be offered follow-up physician appointments at Nyanza Provincial Hospital.

7. At any time point that parasitologic diagnostics indicate infection with soil-transmitted helminths, malaria or schistosomiasis, the patient will be offered appropriate treatment for that condition by the project nurse.

Estimated duration of enrollment:

Enrollment of study participants into this study will be stopped in the second year of study; you will be followed for parasite evaluation, treatments and blood evaluation for a total duration of not more than three years.

Storage and exportation of samples:

Some of your blood will be frozen for further studies of how your body can respond to worms. In addition, part of your sample may be shipped to the United States of America (USA), for futher research on how our bodies may respond to worms, using equipment that we do not yet have in our country. If any sample will be taken to the United States, Dr. Mwinzi will accompany the sample in person and will be the one to carry out the research in USA. This further research may help us find out what is important for the body's defense against worms and may help someone

to make a vaccine against this disease. This is done so we can learn more about the disease, it does not affect your treatment and these results will not be told to you. Your blood will be stored as long as the study goes on. This is so we can study it when we have new tests that we can run to learn more about this disease. If you do not want to have your blood stored for future testing, you may say this now or at any time in the future. If at a later date you decide that you do not wish your samples to be stored, please contact Dr. Mwinzi and your samples will be destroyed. Also, some of your blood cells will be frozen so that we can do tests to see whether your cells are capable of making some parts of the immune response, or not. These tests on your cells will be done to help us learn why some people do not get infected again and why other people keep getting infected with this disease. These results will also not affect your treatment but we hope they will lead to knowing more about how people resist infections with worms.

Participation is voluntary:

Your being in this study is by your own choice and you are free to leave the study at any time without penalty. This includes if you decide that you do not want your blood drawn after the first time or if you do not want your sera or cells tested at some time in the future. If you decide not to be in the study, this will not have any impact on the care you get. You will be treated for free for parasites if you need it, but you will not be paid for being in this study. If you agree to be in this study, please sign the attached consent form. Before you sign the consent form, please ask questions about things that you do not understand. If you have questions during the course of the study, you may call Dr. P Mwinzi at CGHR, KEMRI. The telephone number is: 020-2022929. Further questions regarding the study may be directed to The Secretary, KEMRI/National Ethics Review Committee, Tel. 2722541, or 0722202901, or 0733400003; Nairobi.

CONSENT AGREEMENT FORM

I agree to take part in a research study titled "**Immunoepidemiology of CD23 Expression by schistosome-activated B Cells"** which is being conducted by Mr. Daniel Onguru, Dr. Pauline Mwinzi, Dr. D Karanja, all of the Center for Global Health Research the Kenya Medical Research Institute, in Kisian, Kenya, tel 057-2022923, Prof. Ayub Ofulla of the School of Public Health Health and Community Development-Maseno University, and Dr. Lisa Marie Ganley-Leal of Boston School of Medicine. I do not have to take part in this study and I can stop taking part at any time without giving any reason, and without penalty. I can ask to have information related to me returned to me, removed from the research records, or destroyed.

Name_____Age_____Sex_____

Location_____

Signature_____Date_____

Name of Witness_____Date_____

Signature_____

For patients who cannot read, this form was read to them in a way that they understood:

Name of Reader_____

Signature_____Date_____

Mark of Volunteer_____

Name of Witness_____Date_____Signature_____

CONSENT AGREEMENT FOR SAMPLE STORAGE AND SHIPMENT

Serum and cells from your blood will be frozen and stored for future testing of their ability to respond to the parasite and for genes that affect your responses, and some will be transported to the United States for futher research. Do you also agree to have blood stored and/or exported for future use? If you do agree, sign below.

Signature_____Date_____

Name of Witness_____Date_____Signature_____

APPENDIX II: REAGENT RECIPES

a. FACS Wash Buffer (B Cell Phenotyping)

1X PBS + 0.1% sodium azide + 1% FBS or BSA

b. FACs Lysing Solution

BD FACs lysing solution diluted 1:10 in distilled water

c. Fixative (for Flow Cytometry)

BD Cytofix buffer

d. Complete Media (For B Cell Culture)

2 ml L-Glutamine + 2 ml Pen-Strep + 10 ml Heat-inact

ivated NHS + 146 ml RPMI

e. Antigen Preparation concentration

SWAP 10 µl/ml SEA 5 µl/ml

f. ELISA Blocking Buffer

25g non-fat milk + 1.5 ml Tween 20 + 1X PBS (bring volume to 500 ml)

g. Cytokine ELISA Wash Buffer

3L 1X PBS + 1.5 ml Tween 20; mix well

h. sCD23 ELISA Reagent

i. Wash buffer: 50 ml wash buffer concentrate + 950 ml dH_2O

ii. Assay buffer (1-12 strips): 5 ml Assay buffer concentrate + 95 ml dH_2O

iii. Biotin-conjugate (1-12 strips; 1:100 dilution): 0.06 ml BC + 5.94 ml Assay buffer

iv. Standard: Add volume of dH_2O as stated on specific lyophilized sCD23 Standard

v. Streptavidin-HRP (1-12 strips): 0.12 ml Streptavidin-HRP + 12 ml Assay buffer

vi. TMB Substrate solution (1-12 strips): 6 ml Solution I + 6 ml Solution II

i. CFSE Buffer (PBS/5% FBS)

95 ml 1X PBS + 5 ml FBS; syringe filter

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KEMRI/RES/7/3/1 **November 6, 2009,**

TO: **DANIEL O. ONGURU AND DR. PAULINE MWINZI, (PRINCIPAL INVESTIGATORS)**

THROUGH: **DR. JOHN VULULE, THE DIRECTOR, CGHR KISUMU.**

RE: **SSC PROTOCOL NO. 1611 (*INITIAL SUBMISSION*): IMMUNO-EPIDEMIOLOGY OF CD23 EXPRESSION BY SCHISTOSOME-ACTIVATED B-CELLS.**

This is to inform you that during the 172nd meeting of KEMRI/National Ethics Review Committee held on Tuesday 3rd November 2009, the above study was reviewed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **15th day of October 2009,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **Thursday, 14th October 2010.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **Thursday, 2nd September 2010**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Kindly include the contact of the KEMRI/NERC in the Informed Consent Document (ICD), it is The Secretary, Tel. 2722541, 0722 205901, 0733 400003 and remit a revised copy to the secretariat for our records.

Yours sincerely,

R.C. Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

APPENDIX IV: MAP OF STUDY AREA