B CELL RESPONSE TO SCHISTOSOME ANTIGENS IN ADULT MALES OCCUPATIONALLY EXPOSED TO *S. MANSONI* IN WESTERN KENYA

BY

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ABSTRACT

Increasing levels of membrane CD23 (CD23) on B cells and soluble CD23 (sCD23) in plasma are related to development of resistance to re-infection with *Schistosoma mansoni*. However, the mechanisms underlying CD23 expression on B cells remains less understood. In this study, *in vitro* B cell response to crude schistosome antigen stimulation as well as sCD23 and IgE levels in plasma and culture supernatants from *S. mansoni* exposed male adults were investigated. Blood was obtained from 34 adult males occupationally exposed as car washers or sand harvesters along the shores of Lake Victoria in Kisumu. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized fresh blood and B cells were obtained from the PBMCs by negative selection using magnetic bead separation. B cells were cultured at approximately $20x10^6$ cells/mL for 5 days in RPMI-1640 alone or with soluble egg antigen (SEA), soluble worm antigen preparation (SWAP), recombinant IL-4 (rIL-4) and anti-CD40 mAb (each alone, and, the first two in combination with one or both of the third and fourth). To determine whether B cells respond differently in pure culture or in PBMC, some 7 PBMC cultures were stimulated under similar conditions with pokeweed mitogen (PWM), anti-IgE mAbs, SWAP, SEA, or phytohaemagglutinin (PHA). B cell expression of membrane-bound CD23 was determined by flow cytometry using FACSCalibur (Becton Dickinson) while the quantitative release of sCD23, and IgE in cell-free culture supernatants and plasma were measured by direct ELISA. Flow cytometry data was analyzed on FlowJo (Treestar, 2008). Regression, correlation and ANOVA analyses were done using GraphPad Prism software v. 5. Both CD23 expression on B cells and sCD23 release into culture supernatants were significantly raised in the presence of SEA, rIL-4 and anti-CD40 mAb. The expression of CD23 on B cells was inversely proportional to sCD23 levels, directly related to IgE levels but unrelated to *S. mansoni* egg counts in stool, but these observations were not statistically significant ($p>0.05$). The factors involved in CD23 cleavage from cell surfaces and further breakdown remain unclear and warrants detailed studies that involve biochemical and molecular analyses.

1.0 GENERAL INTRODUCTION

1.1 Background

Schistosomiasis is an intravascular metazoan parasitic disease that affects at least 200 million people globally. One of the critical areas of schistosomiasis research under focus presently is whether substantial acquired immunity develops following initial infection. Different studies have generally shown a tendency to develop resistance to re-infection, but the patterns observed thereof differ depending largely on the populations under scrutiny (Karanja *et al.*, 2002). For example, preliminary data from parts of Kisumu in Western Kenya indicate that occupationally employed car washers working at a beach on Lake Victoria had reinfection intervals with *Schistosoma mansoni* different from those of commercial sand harvesters working at an adjacent beach. These patterns change over time, and part of the possible explanations for this difference could be based on different exposure patterns and duration of exposure since childhood (Colley D.G., 2007-personal communication).

Water exposure frequency, 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, there was no association between HIV-1 infection, CD4 T cell counts, or CD4 T cell counts of HIV-1 positive individuals with time to reinfection (Karanja *et al.*, 2002). 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 (Caldas *et al.*, 2000; Matupi, 2001).

The process by which immunity to reinfection by any given parasite is conferred is complex and may involve many cells and cellular products. In helminthic infections, the immunoglobulin IgE plays a central role in the destruction of the worm. Infection by *S. mansoni* is associated with a powerful 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 (Janeway *et al.*, 2001).

All antibodies are produced by mature and properly activated B cells, upon whose appropriate antigenic stimulation 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 (Chung *et al.*, 2002). The specific importance of IgE response in parasitic diseases remains unclear though in some cases IgE production has correlated with resistance to helminthic infection (Caldas *et al.*, 2000), cellular killing of parasites *in vitro* and the pathological processes associated with some clinical disorders (Callard *et al.*, 1988). In schistosomal infections, high titres of parasite-specific serum IgE are always achieved, but the exact role of this class of antibody is not well understood in these infections (Caldas *et al.*, 2000). 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 (Callard *et al.*, 1988; Paul, 1993: Karanja *et al.*, 2002). It is also known that IgE levels are significantly raised during parasitic infections (Karanja *et al.*, 2002).

IgE most likely exerts its functions through its receptors which include the high affinity receptor FcεRI and the low affinity receptor FcεRII (CD23). CD23 is a 45 kD type II membrane protein and is unique among Fc receptors in that it shares homology to C-type (calcium-dependent) lectins (Hibbert *et al.*, 2005). The expression of CD23 on B cells has been shown to be related to resistance to re-infection in a multiply treated cohort (Mwinzi *et al.*, 2009). The production and regulation of IgE may be regulated through this receptor. The membrane CD23 is cleaved by not yet understood proteases, under unclear circumstances, that generate the soluble form sCD23 (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, may regulate the synthesis of IgE (Aubry *et al.*, 1992).

Paradoxical activities have been confirmed to be produced by different fragments of CD23 following cleavage by endogenous proteases at different points. Studies have shown that soluble CD23 monomers (derCD23) inhibit while oligomers (the trimeric lzCD23) stimulate IgE synthesis (McCloskey *et al.*, 2007). Apart from IgE role, the exact 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. Again, it will be important to better understand the level of B cell proliferative response to schistosome antigen stimulation, and if this is related to CD23 expression and/or IgE production.

1.2 Problem Statement

The specific role of IgE in *S. mansoni* infections remains unclear, even as several field studies have shown relationship between high IgE levels and resistance to re-infection with schistosomes. CD23, both the cellular and soluble forms, are thought to be involved in the regulation of IgE production by mature, activated B cells, alongside crucial roles of different cytokines including IFN-γ, IL-4, IL-10, and IL-13, among others. Determining how B cells may alter their CD23 expression following encounter with schistosome antigens will give mechanistic insights into our understanding of B cell involvement in human schistosomiasis.

1.3 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 diseases now categorized as *previously neglected* by the World Health Organization, WHO (www.who.int/tdr/diseases/schisto/default.htm). While schistosomiasis is highly treatable, with good prognosis following praziquantel therapy among most patients, the high exposure indices among those living around water bodies continuously predispose the individuals to infection, watering down the essence of chemotherapeutic efficacy (Matupi, 2001; Karanja *et al.*, 2002). At present, there are heightened research efforts to understand the pathological implications of schistosomiasis, a keen focus being on the possibility of designing a potent vaccine. To this end, the immunological intricacies involved in the development of immunity, and the pathological aspects thereof, need to be understood. B cell behaviour is thus vital to investigate, especially in the process of IgE synthesis and regulation. This study was pivotal in creating an understanding of the role of CD23 in the regulation of IgE synthesis, and so the

defense against schistosomiasis re-infection. More specifically, the fundamental role of the cell membrane-bound CD23 and the soluble component cleaved enzymatically was investigated. This will begin to unravel our understanding of the involvement of B cells in the process of anti-schistosome immunity development.

1.4 General Objective

To determine B cell response on stimulation with schistosome antigens

1.4.1 Specific objectives

i To determine the proportions of $CD23^+$ B cells and CD23 intensity on B cells in blood and on post culture cells during *S. mansoni* infection.

ii To determine the relationship between B cell-surface CD23, sCD23 and IgE levels in plasma and culture supernatants.

iii To determine the association between IgE, CD23, sCD23 and intensity of *S. mansoni* infection

1.5 Hypothesis

Cell surface expression of CD23 is directly proportional to sCD23 and IgE secretion by schistosome-activated B cells

2.0 LITERATURE REVIEW

2.1 Introduction

Schistosomiasis, otherwise known as 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. The cercarie, which are released by freshwater snails, penetrate the skin of people in the water, often causing a transient dermatitis called *swimmer's itch* (Greenwood *et al.*, 2002). In the body, the larvae develop into adult schistosomes, which live in the blood vessels. The females release eggs, some of which are passed out of the body in the urine or faeces and others are trapped in body tissues, causing an immune reaction. 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. Control of schistosomiasis is based on drug treatment, snail control, improved sanitation and health education. Infection is widespread with relatively low mortality rate, but high morbidity rate, causing severe debilitating illness in millions of people (Karanja *et al.*, 2002). The disease is often associated with water resource development projects, like dams and irrigation schemes, where the snail intermediate hosts of the parasite breed (Greenwood *et al.*, 2002; www.who.int/tdr/diseases/schisto/default.htm *accessed September 1st 2008*).

2.2 Geographical Distribution

Schistosomiasis is found in tropical countries in Africa, Caribbean, eastern South America, east Asia and in the Middle East. *Schistosoma mansoni* is found in parts of South America and the Caribbean, Africa, and the Middle East; *S. haematobium* is prevalent in Africa

and the Middle East; and *S. japonicum* is widely distributed in the Far East. *S. mekongi* and *S. intercalatum* are found focally in Southeast Asia and central West Africa, respectively (Greenwood *et al.*, 2002).

An estimated 207 million people have schistosomiasis; 120 million symptomatic, and a few countries have eradicated the disease, with many more working towards it. The World Health Organization is promoting efforts towards this goal (Matupi, 2001). In some cases, urbanization, pollution, and/or consequent destruction of snail habitat has reduced exposure, with a subsequent decrease in new infections. The most common way of getting schistosomiasis in developing countries is by wading or swimming in lakes, ponds and other bodies of water which are infested with the snails (usually of the *Biomphalaria*, *Bulinus*, or *Oncomelania* genus) that are the natural reservoirs of the *Schistosoma* pathogen (Greenwood *et al.*, 2002; www.who.int/tdr/diseases/schisto/default.htm *accessed September 1st 2008*).

2.3 Immunity to Schistosomiasis

The immune response and immunopathologic manifestations in schistosomiasis are largely dependent on antigen-specific $CD4^+$ Th cells. In turn, the stimulatory/regulatory function of the Th cells is dependent on signals emanating from accessory cells. B cells are capable of functioning as accessory cells (Hernandez *et al.*, 1997). Infection by *S. mansoni* is associated with a powerful 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 (Janeway *et al.*, 2001; Mwinzi *et al.*, 2004).

The process by which immunity to reinfection by any given parasite is conferred is complex and may involve many cells and cellular products. In helminthic infections, the

immunoglobulin IgE plays a central role in the destruction of the worms (Nutman *et al.*, 1984; Janeway *et al.*, 2001). 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. 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. 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 (Nutman *et al.*, 1984). However, certain recent studies have pointed to tendency of individuals with high titres of IgE to resist re-infection with *S. mansoni* when exposure index is similar to those with lower parasite-specific serum IgE titres (Paul, 1993).

2.4 B cell Development

B cell development proceeds in an orderly and sequential manner, giving rise to mature cells capable of interacting with antigen through surface immunoglobulin (Ig). In the bone marrow, B- cell differentiation leads to generation of a broad repertoire of antibody-bearing cells in an antigen-independent manner (Janeway *et al.*, 2001; Blanco-Betancourt *et al.*, 2008). Immature B cells are the first B-cell subset to express the B-cell receptor, BCR, and the process yields a number of potentially self-reactive clones which can be eliminated by apoptosis (clonal deletion), modified by secondary rearrangements (receptor editing), or rendered hyporesponsive (anergy). In the mouse, for example, $2x10^7$ immature B cells are generated every day, about 90% of which undergo negative selection (Blanco-Betancourt *et al.*, 2008). These cells leave the bone marrow and develop further into mature cells. Of the about

$2x10^7$ IgM^+ B cells which develop in the murine bone marrow daily, only ~10% exit to the periphery. Of these émigrés, a larger proportion progresses to the immature B cell pool. In contrast to our knowledge of early events in the bone marrow, the features of later peripheral development are relatively obscure. Specifically, the factors that determine the selection of late immature B cells into the long-lived pool are not well understood despite their potential importance in immune repertoire formation (Janeway *et al.*, 2001; Chung *et al.*, 2002).

2.5 Transitional Immature B cells

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). Before migrating to the periphery, immature B cells develop into $IgM^{high}IgD^{low}CD21^-$ $CD23^-$ type 1 (T1) transitional B cells (Carsetti *et al.*, 1995), which differentiate in the spleen into $IgM^{high}IgD^+CD21^+CD23^+$ type 2 (T2) transitional B cells, and then into either $IgM^{high}IgD^-CD21^+CD23^-$ marginal zone or $IgML^{ow}IgD^{high}CD21^+CD23^+$ follicular mature B cells. The final developmental stage in the bone marrow before emigration is the late immature B cell that has high expression of surface IgM (sIgM) (Hernandez *et al.*, 1997; Blanco-Betancourt *et al.*, 2008).

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; Allman *et al.*, 1993). As with late stage bone marrow immature B cells, they are heat-stable antigen $(HSA)^{high}$ (CD24) 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 (Rolink *et al.*, 1998; Chung *et al.*, 2002).

Transitional B cells themselves represent a heterogeneous population of cells with phenotypic variability which can be further subdivided by the presence or absence of surface proteins such as CD21, CD23 and IgD (Kobayashi *et al.*, 2002). HSA^{high} transitional B cells can be divided into two subsets defined by their expression of CD23, with different functional characteristics although neither subset differs detectably in their intrinsic response to BCR cross-linking. However, functional differences exist between these two populations in their relative abilities to respond to T cell help. 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. While $CD23^-$ transitional B cells express low levels of IgD, $CD23^+$ transitional B cells and B cells from non-irradiated adult spleens express similarly higher levels (MacLennan, 1998). Lastly, HSA levels have been found to be comparable, although slightly higher, in the $CD23^-$ transitional B cells compared to $CD23^+$ transitional B cells (Melchers *et al.*, 1989).

$CD23^-$ and $CD23^+$ transitional B cells respond similarly to BCR cross-linking. Transitional B cells isolated from auto-reconstituted spleens undergo apoptosis in the presence of BCR cross-linking, whereas mature B cells respond to the same stimulus by proliferation

(Sater *et al.*, 1998). $CD23^-$ and $CD23^+$ transitional B cells share these critical functional characteristics. In contrast to mature B cells, $CD23^-$ and $CD23^+$ transitional B cells are both susceptible to apoptosis in response to BCR cross-linking. $CD23^-$ transitional B cells exhibit a higher spontaneous ligand-independent apoptosis rate as well as a slightly higher level of apoptosis for any given amount of BCR stimulation compared to the their $CD23^+$ counterparts (Chung *et al.*, 2002). In addition, neither $CD23^-$ nor $CD23^+$ immature B cells proliferate to BCR cross-linking, whereas both are induced to proliferate to lipopolysaccharide (LPS) (Sater *et al.*, 1998). The different degrees of proliferation to LPS in the two subsets may be related to survival differences, but the exact mechanism remains unclear (MacLennan, 1998; Sater *et al.*, 1998; Chung *et al.*, 2002).

$CD23^-$ and $CD23^+$ transitional B cells respond differently to T cell help signals. It has been shown that transitional B cells fated to undergo apoptosis could be rescued by the addition of IL-4 and/or activating anti-CD40 antibodies, mimicking both soluble and contact T cell activation signals; $CD23^+$ transitional B cells are rescued to a greater extent compared with $CD23^-$ transitional B cells from apoptosis. While CD40 stimulation has a greater impact than IL-4 to the survival of transitional B cells, the combination of the two signals appears to play a synergistic role in their rescue (Crow *et al.*., 1989; Petro *et al.*, 2002).

Neither $CD23^+$ nor $CD23^-$ transitional B cells have the ability to up-regulate B7-2 to BCR-mediated stimulation. CD80 (B7-1) and CD86 (B7-2) are highly homologous molecules which are up-regulated in B cells in response to various activating stimuli including BCR ligation (Carsetti *et al.*, 1995; Chung *et al.*, 2002). Their up-regulation thus transforms a resting B cell into a fully competent APC. The interaction of TCR with the MHC class II peptide and CD28 with B7 on mature B cells leads to the full activation of T cells with subsequent

production of IL-2 and the induction of Bcl-xL. Mature B cells increase their surface expression of B7-2 as early as 3h after BCR cross-linking and maintain its expression for >24h. However, while expressing low levels of B7-2 on their surface constitutively, neither $CD23^-$ nor $CD23^+$ transitional B cells can be induced to up-regulate B7-2 in response to BCR ligation (Chung *et al.*, 2002). Immature B cells have in some studies failed to up-regulate B7-2 even 72 h after anti-Ig stimulation or after anti-Ig and CD40 co-ligation, and, neither mature nor immature B cell subsets up-regulate B7-1 during the long incubation (Hathcock *et al.*, 1994; Fulcher and Basten, 1997; Chung *et al.*, 2002).

Both transitional B cell subsets appear to be short-lived (3 to 4 days) and non-dividing *in vivo*, although some studies have shown that T2's proliferate and up-regulate survival signals, whereas T1's die after *in vitro* BCR engagement (Su *et al.*, 2002). T1's express CD95 but not the anti-apoptotic molecule, bcl-2 (Carsetti *et al.*, 1995), further suggesting that T1 might be the target of the B cell negative selection occurring in the periphery. Such transitional B cell subsets have not yet been defined in humans, and current characterization of human peripheral blood B cells is based on the expression of CD27, which distinguishes unmutated $IgM^+IgD^+CD27^-$ naïve cells (about 60% in adults) from somatically hypermutated $CD27^+$ memory cells (40%, of which about 40% are IgM^+IgD^+) (Blanco-Betancourt *et al.*, 2008).

2.6 CD23 Expression and Function on Activated B cells

The low affinity receptor for IgE (FcεRII), which has been shown to be identical to the B-cell differentiation marker CD23 on B cells, and its proteolytic cleavage products, the IgE binding factors (IgE-BF, sCD23), are involved as regulatory molecules in immune functions. The existence of CD23 has been shown on B cells, T lymphocytes, monocytes, eosinophils,

platelets, and epidermal Langerhans' cells (Kicza *et al.*, 1989). Kikutani *et al.*, (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. $CD23^+$ cells and sCD23 act synergistically on the proliferation of CD23 B cell populations. Studies in cynomolgus monkeys (*Macaca fascicularis)* show that their peripheral blood B cells have a smaller fraction of $CD27^-$ (naive) cells (about 40%), as compared to human blood samples (about 70%). Similar to humans, though, an early activation marker, CD23, is expressed more on $CD27^-$ *M. fascicularis* naive B cells, as compared to $CD27^+$ B cells. The mean fraction of B cells exhibiting a memory phenotype is similar to that seen in human blood (Vugmeyster *et al.*, 2004).

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). CD23 is a 45 kD type II membrane protein and 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.*, 2005).

Furthermore, paradoxical activities have been confirmed to be produced by different fragments of CD23 following cleavage by endogenous proteases at different points. Studies have shown that 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). In mice, the shed FcεRII from B cells does not bind to IgE compared to sCD23 in humans. Since sCD23 is released from B

cells and monocytes, a regulatory circuit via sCD23 might be possible. It has been indeed 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).

2.7 B cell Activation and IgE Production

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 (including inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), usually generated by the engagement of the B cell receptor by antigen), protein kinase C activation, and an increase in intracellular free Ca^{2+}. In addition, regulatory proteins such as NF-κB are translocated to the nucleus to participate in elevated transcription of mRNA for immunoglobulin (Paul, 1993; 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. LPS, pokeweed mitogen, staphylococcus organisms), B cells convert into proliferating lymphoblasts. These are larger cells, which have much more cytoplasm, with clusters of ribosomes and a conspicuous Golgi apparatus. Depending on the nature of the stimulus, some of the replicating B cells will be destined to become memory cells (Paul, 1993).

Although helminth parasites induce the production of high levels of IgE antibodies, the immunoregulatory mechanisms determining this remains poorly understood. The regulatory

events controlling the synthesis of IgE have been extensively studied in rodent models using both *in vivo* and *in vitro* systems. These studies have shown the overall T cell-dependence of IgE antibody production and the homeostatic role of T helper and T suppressor cells (Allman *et al.*, 1993). Success with these systems have been documented both in patients with high serum IgE, in atopic patients, and normal donors. Some workers have succeeded in enhancing IgE production by pokeweed mitogen (PWM) in cells from normal donors (Kimata *et al.*, 1983; Saxon *et al.*, 1980).

The specific importance of IgE response in parasitic diseases remains unclear though in some cases IgE production correlated with resistance to helminth infection (Karanja *et al.*, 2002), cellular killing of parasites *in vitro* and the pathological processes associated with some clinical disorders (Hernandez *et al.*, 1997; Janeway *et al.*, 2001). 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 normal participants, while the mitogen enhances significant IgE production by PBMC of individuals with different helminth infections (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 (Kicza *et al.*, 1989; Corominas *et al.*, 1998). 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).

2.8 IL-10 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. IL-10 is a major regulatory cytokine involved in inflammatory responses. It is a general inhibitor of proliferation and cytokine responses in T cells. The functions of IL-10 in B cell IgE synthesis are debatable and in one study by Kobayashi *et al.*, (2002), IL-10 was found to facilitate IgE production by both human PBMC and highly purified B cells. It also enhanced B cell proliferation, and most importantly, promoted the generation of plasma cells. However, it did not enhance the expression of germline ε transcripts.

2.9 IFN-γ and CD23 Regulation

CD23 plays a role in IgE production, and cytokines involved in IgE synthesis also modulate CD23 expression on B cells (CD23 modulation on T cells remains entirely unclear), the best known of which are IL-4 and IFN-γ (Corominas *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).

However, in similar but unrelated studies among atopic donors (participants with generally higher IgE and eosinophils in circulation compared to normal counterparts), however, the use of IFN-γ as stimulus achieved little modulation of CD23 expression by PBMC-derived B cells (up to 1.2%) though IFN-γ led 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 (Matupi, 2001).

2.10 CD27 Expression on B cells

CD27, a disulfide-linked 120 kDa type I transmembrane glycoprotein belonging to the TNF receptor family, has been characterized as an antigen on majority of human peripheral T cells, medullary thymocytes (Kobata *et al.*, 1995), natural killer cells, plasma cells and memory B cells (Steiniger *et al.*, 2005). In human blood CD27 is present on B memory cells with hypermutated variable region genes, while naïve B cells are IgD^{+} $CD27^{-}$. Memory cells represent about 40% of blood B cells in humans but only 5% in mice. Human blood memory B cells form at least four subpopulations. Two large populations comprise 'switched' $CD27^{+}$ cells and $IgD^{+}IgM^{+}$ $CD27^{+}$ cells, respectively (Weill *et al.*, 2004). In addition, there is a smaller population of $IgD^{-}IgM^{+}CD27^{+}$ cells, called 'IgM only' B memory cells, which seems to depend on the presence of the spleen. Finally, a fourth $IgD^{+}IgM^{-}$ $CD27^{+}$ human memory B cell population, called 'IgD only' cells, occurs at very low frequency and exhibits high immunoglobulin mutation rates (Agematsu, 2000). In human and rodent lymphatic organs

recirculating naïve IgD^+ B cells are present in primary follicles or in the mantle zone surrounding germinal centres (GCs) of secondary follicles (Steiniger *et al.*, 2005).

CD27 binds to its ligand, CD70, a member of the TNF family, and induces T cell costimulation and B cell activation. CD27 is expressed on resting T and B cells, while CD70 is expressed on activated T and B cells. CD70 is a type II transmembrane glycoprotein belonging to the TNF family and is expressed by activated T and B cells (Kobata *et al.*, 1995).

The regulation of human CD27 expression is complicated and differs in human B and T lymphocytes. While CD27 is absent in naïve recirculating B cells, it is present in recirculating T cells. B memory and plasma cells express CD27 (Agematsu, 2000; Weill *et al.*, 2004). Certain T memory cells are $CD27^+$ in humans, but CD27 is down-regulated on $CD8^+$ T effector cells. The same may also be true for mice. The function of CD27 on B cells also differs among species. Thus, ligation of CD27 on activated mouse B cells promotes B memory cell development and inhibits plasma cell formation, while plasma cell differentiation is obviously promoted in humans (Agematsu, 2000). In addition, CD27 does not represent a memory B cell antigen in mice. The role of CD27 on human centroblasts, centrocytes and plasmablasts has not been studied in detail. Engagement of CD27 on human T cells appears to support activation and proliferation (Steiniger *et al.*, 2005; Agematsu *et al.*, 2000).

Klein *et al.* (1998) found that about 40% of human blood $CD27^+$ memory B cells carry IgG, IgE or IgA and are IgD^-IgM^-, 40% only express IgD and IgM, and 20% are IgD^-IgM^+. The latter population does not switch to other immunoglobulins and is called 'IgM-only' B memory cells. Recent results indicate that 'IgM only' B cells expressing hypermutated immunoglobulin may develop independent of T cells and thus do not represent memory cells at all, but special natural reactive B cells (Weill *et al.*, 2004).

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted at the Car Wash and Usoma beaches along the shores of Lake Victoria, on the outskirts of Kisumu city, western Kenya. Fishing is the key economic activity in the study area, while many men are also involved in sand harvesting for the construction industry. These young adult males stand in snail-infested lake water for up to six hours daily retrieving sand deposits from the lake or washing cars. This being the major (and only for most persons) source of income, the men mostly stay at the lakeshore, whether there is work or not, increasing their contact with the water (Karanja *et al.*, 2002).

3.2 Population and Sampling

The study recruited 34 adult males (age $\geq$ 18 yrs) working as sand harvesters or car washers along the shores of Lake Victoria. Participant selection was non-random such that all persons meeting the criteria were eligible to participate. Based on differences in mean CD23 expression between the resistant and susceptible groups in previous findings, power size calculation was used to arrive at the sample size, with a power of 0.95, and type I error margin of 0.05. This group was of choice since the participants herein stand for long hours in the lake water, thus at increased risk of schistosomal infection. Each prospective study participant was individually explained to the import of the proposed study; both benefits and potential dangers, and a written, informed consent obtained prior to sample collection (Appendix I).

3.3 Sample Collection and Processing

All prospective study participants were requested to submit stool samples on three consecutive days to allow diagnosis of schistosomiasis and other soil transmitted worms by microscopy (Kato Katz technique-Belo Harizonte, Brazil). Those found positive for

schistosomiasis or other worms were treated with standard doses of *praziquantel* and albendazole respectively. Anemic participants were provided with iron tablets while those positive for malaria were treated with first line antimalarial therapy, according to Ministry of Health guidelines.

About 30 mL of peripheral venous blood was obtained by a qualified, KEMRI phlebotomist from each participant; each sample was collected in three 15 mL tubes coated with heparin as anticoagulant (each containing about 10 mL of blood). Each tube was labeled with the date of collection and sample ID. Samples were placed in an ice box and sent to the lab within four hours of collection. Separately, about 2 mL of blood was collected from respective study participants in sterile tubes with EDTA (BD Vacutainer™ Systems) for preliminary surface phenotyping of whole blood prior to culture to allow objective comparison with post-culture characteristics. All samples were taken for hemoglobin level/anemia test, and CD4/CD8 quantification test (FACSCount™ Systems, USA).

Untouched B cells were isolated by negative selection (Miltenyi Biotech Inc., USA) from peripheral blood mononuclear cells (PBMC) isolated by the Ficoll Hypaque density gradient separation technique (Atlanta Biologicals, USA).

In 7 randomly selected samples, the isolated PBMCs were then cultured at $20x10^6$ cells/mL in complete medium (Appendix II (d)) at 37°C in a water-jacketed incubator with 5% CO_2 for 5 days, in 6 wells with 5 single-antigen-stimulated wells as follows: 20µL (14.81 µL/mL) of pokeweed mitogen, 2 µL (1.48 µL/mL) anti-IgE antibody (eBiosciences, San Diego-USA), 150 µL (111.11 µL/mL) of SEA, 150 µL (111.11 µL/mL) of SWAP and 150 µL (111.11 µL/mL) of PHA. The sixth was the unstimulated control well, having no antigen added

to culture medium (SEA and SWAP were prepared by Dr. Evan Secor). For some 5 randomly picked samples, CFSE staining was done before culture to illustrate proliferation.

For 34 samples, cultures were done on isolated B cells at $20x10^6$ cells/mL in RPMI-1640 alone or stimulated with SEA, SWAP, rIL-4 and anti-CD40 mAb, singly or in combination. Cell-free culture supernatant was harvested and frozen for sCD23 and IgE assays, while the cell pellet at the bottom of the wells were carefully harvested and transferred into 5mL flow cytometry tubes, stained with fluorochrome-labeled antibodies (CD4FITC, CD4PE, CD4PECy5, CD4APC, CD19PECy5, CD23PE & CD27APC) and analyzed by 4 colour flow cytometry on a FACSCalibur (BD Biosciences, USA) to enable the characterization of post-culture populations.

3.4 Data Analysis

Following sample acquisition on FACSCalibur, data was transferred from the Mac computer using *Transmac* software, for analysis by *FlowJo* Windows® version 7.2.4 (Treestar, 2008) on PC to identify and quantify different cell sub-populations, the main groups of interest being $CD23^+$ and $CD27^+$ B cells ($CD19^+$). Cells harvested from the culture wells were stained and analyzed by flow cytometry, using *FlowJo*.

The amount of IgE and sCD23 in the respective plasma and culture supernatants was determined by direct ELISA (Bender MedSystems; Vienna, Austria) and results analyzed together with FlowJo analysis using *GraphPad Prism*® Version 4 (2008) to establish the relationship between the various parameters previously cited (mainly CD23 expression, sCD23 release, IgE production and worm burden). The expression of CD23 on whole blood and post-culture B cells was measured by flow cytometry by first measuring the side scatter [SSC]

against forward scatter [FSC] in order to gate the parent lymphocyte population, from which $CD19^+$ cells were quantified, followed by $CD19^+/CD23^+$, and $CD19^+/CD23^+/CD27^+$, according to the staining procedure described previously, and analyzed using *FlowJo,* and *GraphPad Prism* version 5.

3.5 Ethical Considerations

Ethical approval for the study was obtained from Ethical Review Committee of the Kenya Medical Research Institute. This being part of an on-going KEMRI study, authority to obtain samples had been sought from the Provincial Administration, Local Authority, the Ministry of Health, and the Kenya Medical Research Institute. Further approval was requested from Maseno University's School of Public Health and Community Development to allow the study to proceed, being the basis for an MSc thesis, whose research proposal was submitted and all clearance obtained in time for the study to start.

3.5.1 Written informed consent

Informed consent for study participation was obtained from the adult participants by signing the consent forms (see Appendix I). All prospective participants were accorded complete explanation of the study design and goals, and their participation requested in the native language (predominantly Dholuo), English or Swahili, depending on individual choice. Each prospective participant was explained to in detail both the expected benefits and potential dangers of the proposed study, and a written, informed consent obtained prior to sample collection. They were allowed to ask questions as appropriate, regarding the study, and had the freedom to refuse to or provide written consent before enrolment; participation was voluntary.

They were also informed of the freedom to quit at will, without the fear of any reprimand or discrimination in enjoying study benefits.

3.5.2 Risks and benefits

The study required about 30 mL of venous blood from each participant; a safe volume of blood to draw. Bleeding was done by trained and KEMRI-authorized personnel. All participant samples were sent to the lab within 4 hours of sample collection. All found positive for schistosomiasis (and willing) were treated with standard doses of praziquantel while all willing participants were treated with albendazole for all the other intestinal worms. Anemic participants were diagnosed for malaria and all positive and willing given standard antimalarial therapy, depending on clinical presentation.

3.5.3 Respect and confidentiality

All the samples collected from the study participants were allocated unique participant identification codes to obviate complications of confidentiality of participant test results. Each personnel involved were expected to uphold utmost respect for the participants before, during and after sample collection, processing and in reporting of results and relevant participant information (where applicable). Particularly, field assistants and officers followed the existing code of practice in handling both participants and samples, both in the field and *en route* to the laboratory. All laboratory processes, including procedures involving blood were conducted in accordance with KEMRI and international guidelines on Good Laboratory Practice (GLP).

4.0 RESULTS

Table 1 shows a summary of the lab findings for 34 participants, of whom 7 had no *S. mansoni* eggs in their stool. There were 5 samples with hookworm and 2 with *Trichuris trichiura* (whipworm) eggs; no other soil-transmitted worms were diagnosed among the 34 individuals. Anemia was not a complication among the 34 adult males given the normal physiologic range is 9–17 µg/mL of blood.

Table 1. Summary of preliminary lab findings on blood and stool tests

	Mean	Std. Deviation	Std. Error	Range
***S. mansoni* eggs/gram of stool**	**101.4**	**233.0**	**48.58**	**0 - 1049**
PBMC ($x10^6$)	**35.73**	**16.75**	**3.493**	**13.75 - 71.25**
B Cells ($x10^6$)	**3.64**	**1.494**	**0.3115**	**1.5 - 6.07**
CD4 (cells/mL of blood)	**717.83**	**281.6**	**58.73**	**272 - 1405**
CD8 (cells/mL of blood)	**473.70**	**222.6**	**46.42**	**123 - 907**
CD4/CD8 ratio	**1.52**	**0.6991**	**0.1458**	**1.0 - 3.18**
Hb (µg/ml)	**13.99**	**1.913**	**0.4079**	**9.9 - 17**

4.1 Intensity of Schistosomal Infection, CD23 Expression and sCD23 Release

Figure 1 represents typical flow cytometry acquisition plots confirming the isolation process. Dot plot **A** shows PBMC as separated from whole blood, in which B cells ($CD19^+$) constitute about 12% of total lymphocytes, while dot plot **B** shows B cells following magnetic bead separation; B cells attain a purity of about 94%.

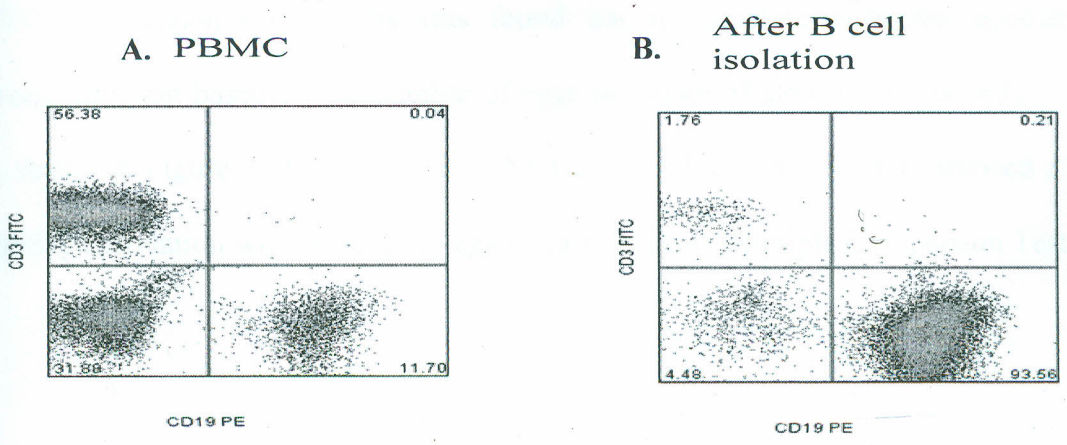

Figure 1. B cell separation (purity) confirmation from PBMC

As illustrated by Figure 2, FlowJo was used to systematically quantify the cell populations of interest, either in whole blood or culture supernatant samples. CD19 positive cells (B cells) were gated following gating on the lymphocyte pool.

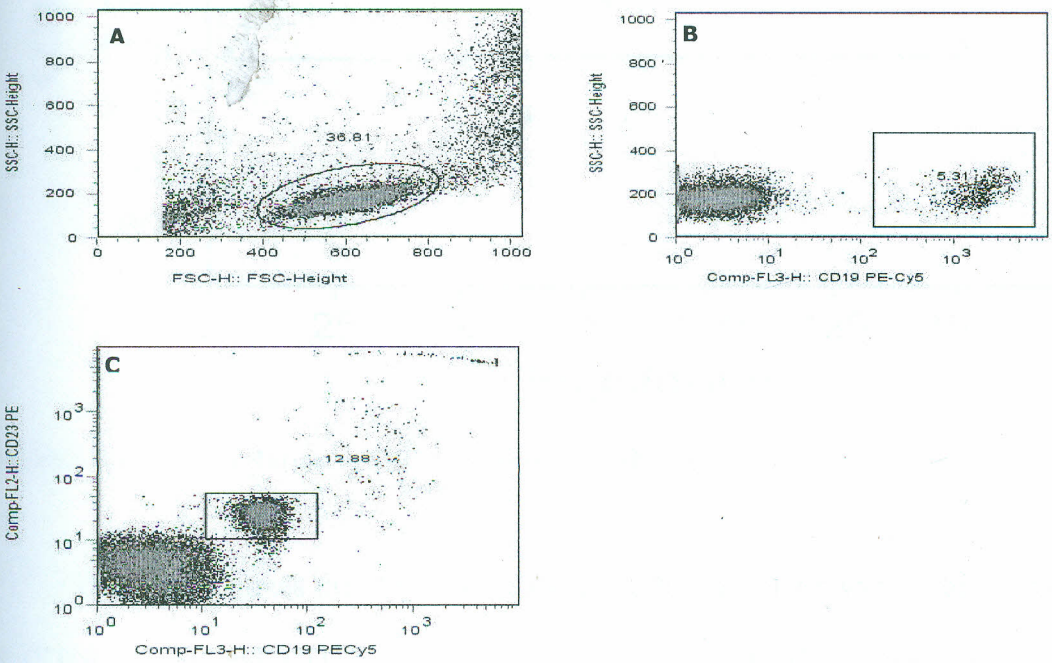

Figure 2. FlowJo analysis of FACS data: A is gate on the lymphocyte population; B is a gate on the $CD19^+$ subpopulation, and C is a gate on $CD19^+/CD23^+$ lymphocytes.

CD23 expression on B cells was found not to correlate with the intensity of schistosomal infection based on the number of eggs per gram of stool (r^2 = 0.00002135; α = 0.05), as shown in Figure 3. The expression of CD27 on $CD23^+$ B cells only showed a very weak negative correlation with *S. mansoni* egg counts (Tukey's Multiple Comparison Test; p = 0.9865).

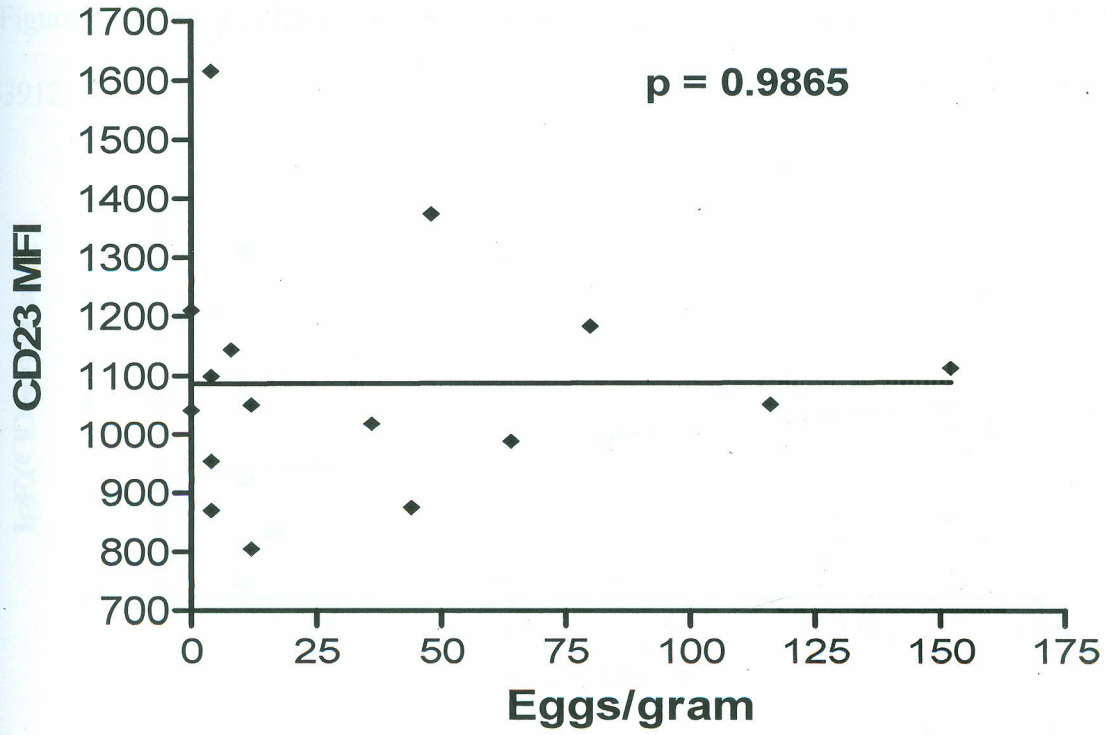

Figure 3. Linear correlation of CD23 expression with egg counts

Table 2 illustrates the slope, correlation coefficients and p values for lines of regression of CD19, CD23 and CD27 expression on egg count. CD27 expression, judging by the correlation coefficients (r^2) and p values, though not significantly related to egg counts, seemed the strongest of the three, CD23 looking almost totally unrelated to egg counts.

Table 2. Regression analysis of CD19, CD23 and CD27 expression on egg count.

	CD19MFI	CD19/CD23MFI	CD19/CD23/CD27 MFI
Slope	0.6253 ± 1.230	0.02016 ± 1.166	-0.02274 ± 0.02454
r^2	0.02107	0.00002135	0.05781
p value	0.6205	0.9865	0.3697

IgE levels in culture were increased with raised egg counts per gram of stool, as shown in Figure 4. There was however not a significant relationship, with a *p* value of 0.2621; r^2 = 0.03912.

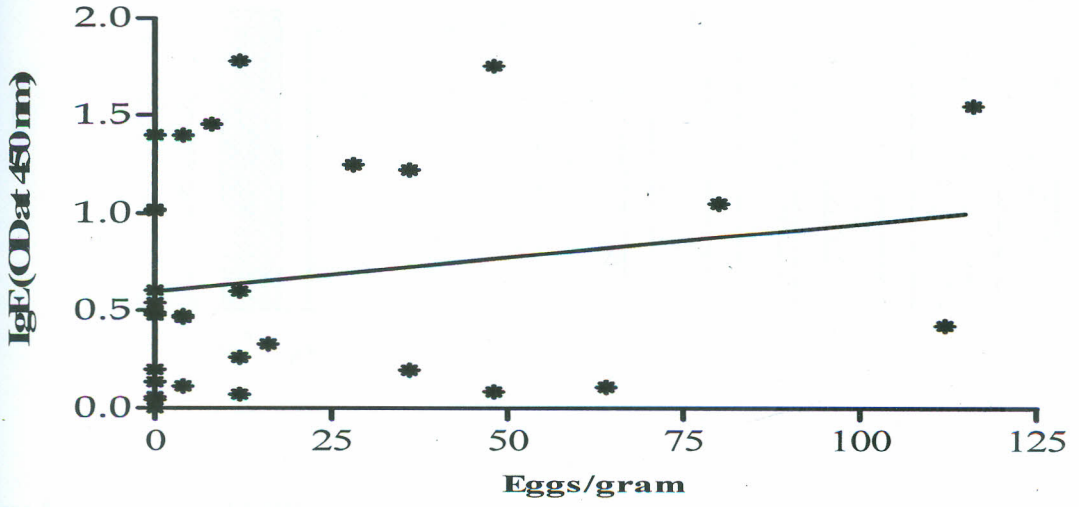

Figure 4. Linear regression of culture supernatant IgE with egg counts per gram of stool

4.2 B cell Yield, CD23 Expression and sCD23 Release from Culture

The 5-day cultures yielded adequate quantities of lymphocytes (~36 million cells), from which mean fluorescence intensity (MFI) of CD19 on B cells (~3.6 million cells) was analyzed. There were no significant differences in MFI of CD19 between the various culture stimuli used, as shown in Figure 5.

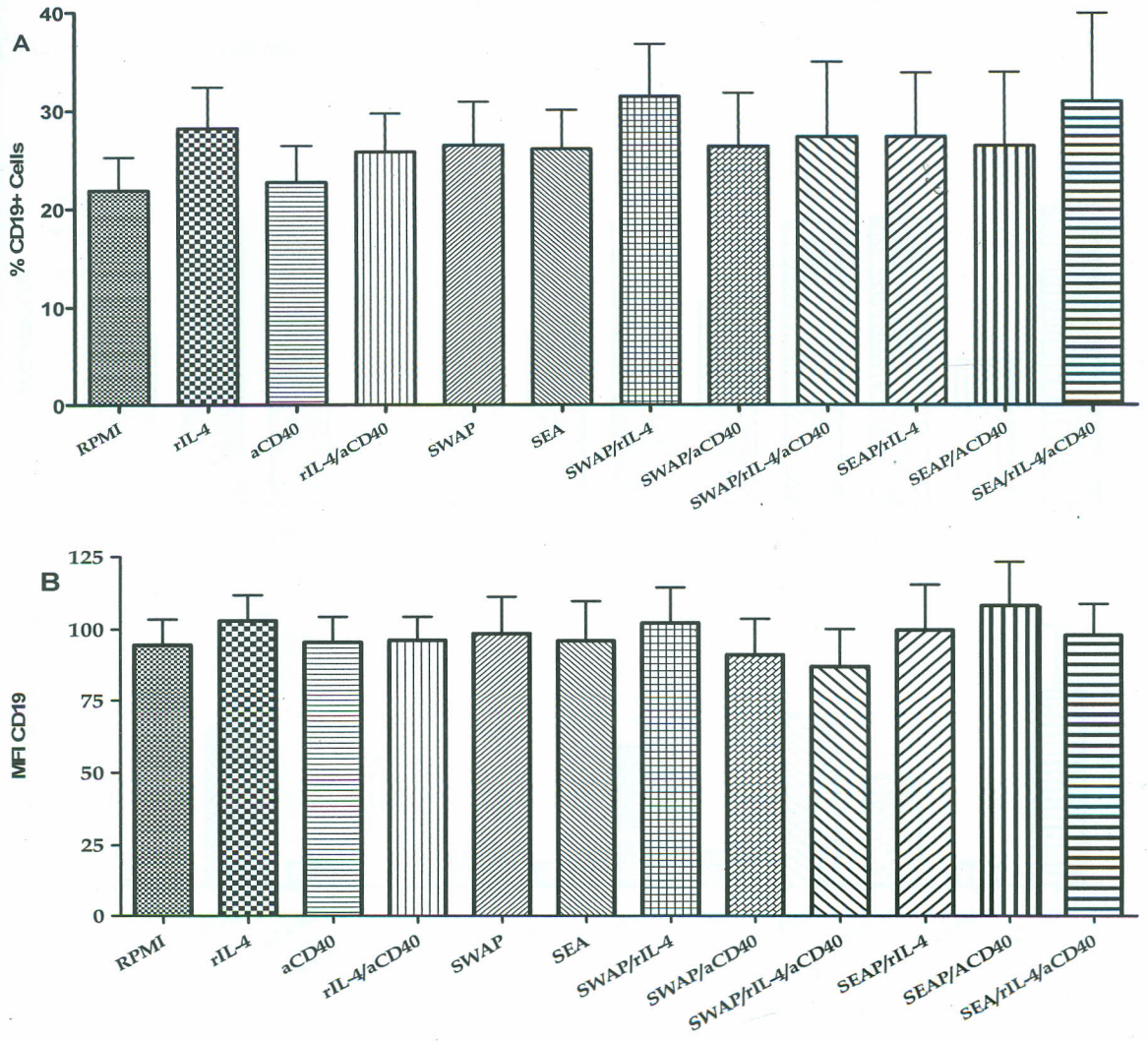

Figure 5. Expression of CD19 on cultured peripheral B cells (n = 34). Bar graph A shows the number of B cells ($CD19^+$ cells; as % of lymphocytes), while B represents the geometric mean of CD19 fluorescence (mean fluorescence intensity; MFI) on the cultured B cell isolates, as determined by flow cytometry.

On the other hand, significant differences were seen in B-cell CD23 expression from different stimulations, with great differences in the CD23 MFIs between the different stimulations (Figure 6). The results were statistically significant by Tukey's Multiple Comparison Test; $p<0.05$. Of interest, aCD40, SWAP, SEA, SWAP/aCD40 and SEA/aCD40 did not stimulate significantly different CD23 surface expression relative to observations on RPMI-1640 cultures. rIL-4 was useful in enhancing CD23 expression on B cells, alone or in

combination with the other antigens, but the most significant elevation was recorded in rIL-4/aCD40/SEA stimulations.

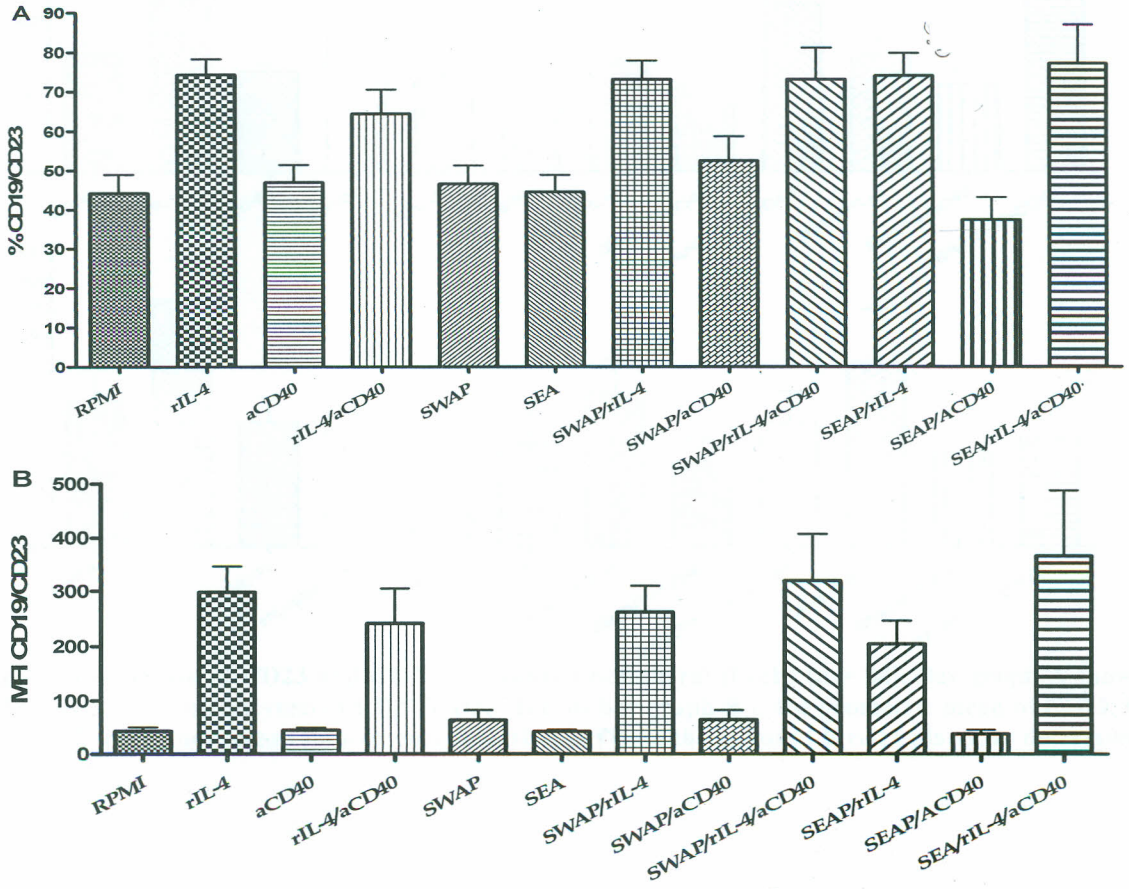

Figure 6. Expression of CD23 on cultured peripheral B cells (n = 34). Bar graph A (above) shows the number of $CD23^+$ B cells (as % of lymphocytes) while B shows the geometric mean of CD19 and CD23 fluorescence (mean fluorescence intensity; MFI) on the cultured B cell isolates, as determined by flow cytometry.

Recombinant IL-4 addition to B cell cultures led to a significant increase in CD27 expression on CD23+ B cells (Figure 7), followed by rIL-4/SEA while cultures stimulated with SWAP/rIL-4, SWAP, SEA, aCD40, aCD40/SWAP, aCD40/SEA, and rIL-4/aCD40/SEA were not different from unstimulated cultures in RPMI-1640 (Tukey's Multiple Comparison Test; $p<0.05$).

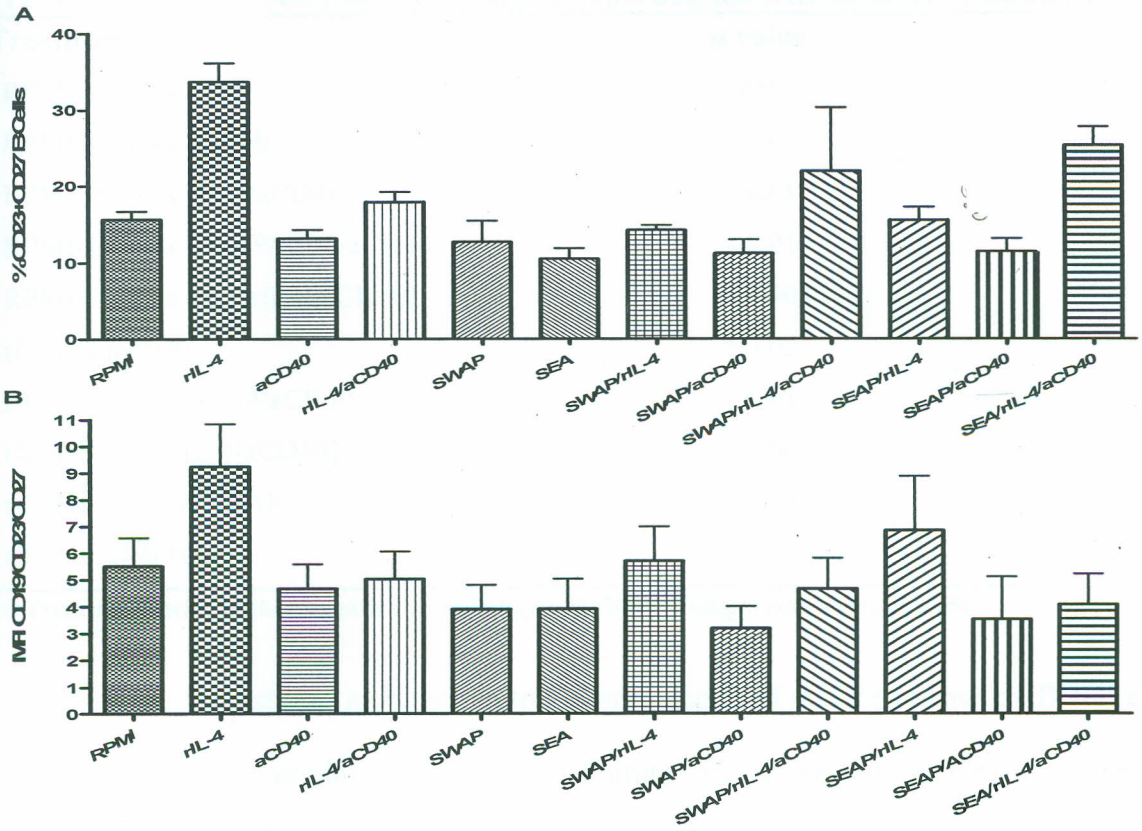

Figure 7. Expression of CD23 and CD27 on cultured peripheral B cells (n = 34). Bar graph A shows the number (as % of lymphocytes) of $CD27^{+}CD23^{+}$ B cells bar graph B is the geometric mean of CD19, CD23 and CD27 fluorescence (mean fluorescence intensity; MFI) on the cultured B cell isolates, as determined by flow cytometry.

As depicted in Table 3, the amount sCD23 in culture supernatants varied greatly between different culture stimulants, the only exceptions being seen in environments where rIL-4 or anti-CD40 mAbs were used as single stimuli, or used in combination with each other or other antigens but compared against other wells stimulated with either or both of the two. Highest levels of sCD23 were seen in culture supernatants from cells that had been stimulated with rIL-4 and anti-CD40 mAbs together with SEA (p <0.001; Tukey's Multiple Comparison Test).

Table 3. sCD23 in cell-free B cell culture supernatants with different stimuli (n = 34)

Treatment	p value
RPMI-1640 vs rIL-4	> 0.05
RPMI-1640 vs aCD40	> 0.05
RPMI-1640 vs rIL-4/aCD40	**< 0.01**
RPMI-1640 vs SWAP/rIL-4/aCD40	**< 0.01**
RPMI-1640 vs SEA/rIL-4/aCD40‡	**< 0.001**
rIL-4 vs aCD40	> 0.05
rIL-4 vs SWAP/rIL-4/aCD40	**< 0.01**
rIL-4 vs SEA/rIL-4/aCD40‡	**< 0.001**
rIL-4/aCD40 vs SWAP	**< 0.01**
rIL-4/aCD40 vs SEA	**< 0.01**

‡ Treatments with higher significances (Tukey's Multiple Comparison Test; $p<0.05$)

Figure 8 illustrates a column graph of stimulation of sCD23 release by B cell cultured for 5 days, compared to plasma levels before culture. All culture settings yielded higher sCD23 titres relative to the plasma levels, although degrees of significance varied from insignificant to highly significant ($p<0.01$). Compared alone, culture sCD23 levels were significantly elevated only in wells that had both rIL-4 and anti-CD40 mAbs, alone or together with either SWAP or SEA. However, marked differences were reported in wells stimulated with rIL-4, anti-CD40 mAbs, and SEA ($p<0.05$; Tukey's Multiple Comparison Test). Much higher sCD23 concentrations were detectable in cultures stimulated with SEA/rIL-4/aCD40 compared to any of the single antigens (SEA, SWAP, rIL-4 & aCD40), and a combination of SEA/rIL-4.

The combinations rIL-4/aCD40 ($p<0.01$), SWAP/rIL-4/aCD40 ($p<0.01$), and SEA/rIL-4/aCD40 ($p<0.001$) produced significantly higher sCD23 levels compared to unstimulated RPMI-1640 cultures, the first two not being significantly different from each other, while the third was different from both of the first two, and the other settings (Tukey's Multiple Comparison Test; $p<0.05$).

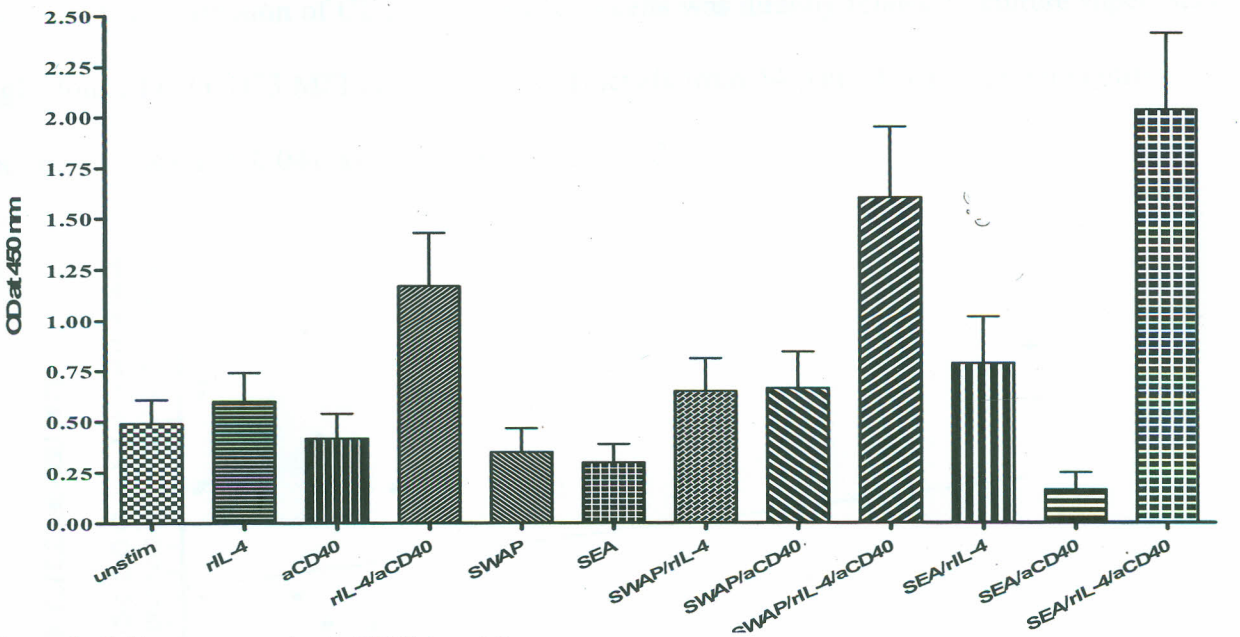

Figure 8. Culture supernatant sCD23 (n = 16)

The amount of sCD23 detectable in culture supernatants was inversely proportional to the level of CD23 expression on B cells (two sCD23 and three MFI CD19/CD23 outliers removed), but this correlation was not significant among 19 participant samples, as indicated in Figure 9.

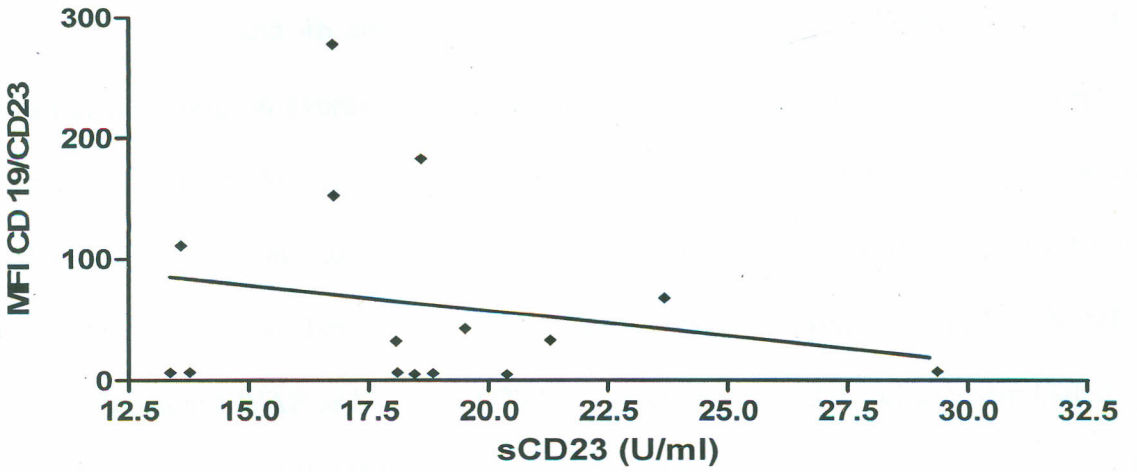

Figure 9. Regression of sCD23 MFI on CD23 on cultured B cells (n = 19)

The expression of CD23 on cultured B cells was directly related to culture supernatant IgE (four CD19/CD23 MFI outliers removed) levels from 34 patients (though not significantly so: $p = 0.3683$, $r^2 = 0.04$), as illustrated in Figure 10.

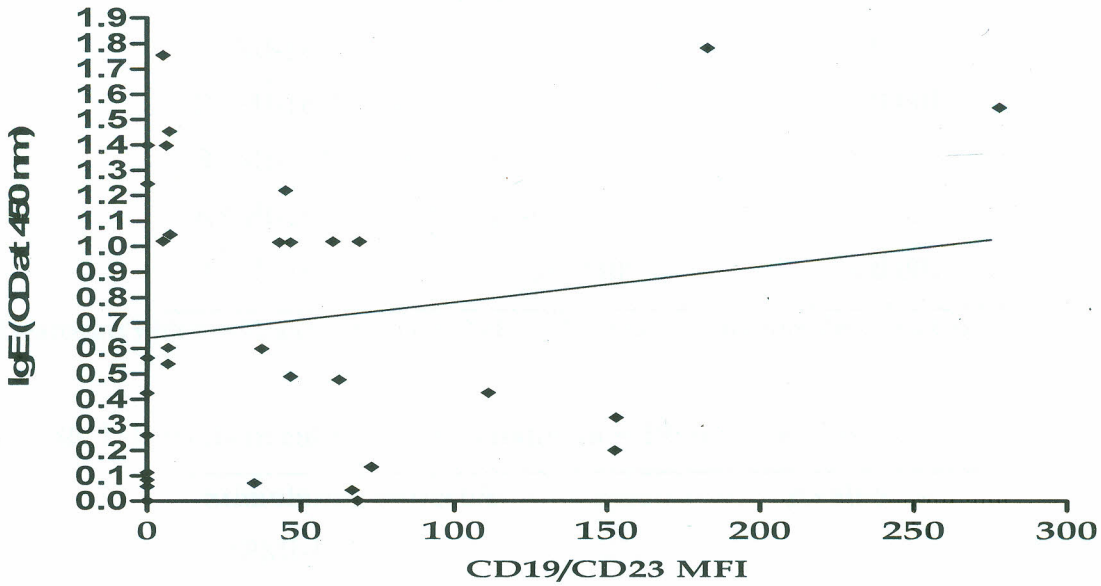

Figure 10. Linear regression of culture IgE levels with CD23 expression on B cells

4.3 sCD23 and IgE in Patient Plasma and Culture Supernatants

Tables 4a and 4b show that the yields of IgE and sCD23, respectively, in culture supernatants were on average not significantly different from plasma levels, except in media stimulated with SWAP/rIL-4/aCD40 or SEA/rIL-4/aCD40 (Tukey's Multiple Comparison Test; $p<0.001$). sCD23, and not IgE (Table 4b), was in addition significantly elevated (Tukey's Multiple Comparison Test; $p<0.05$) 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-1640 (Tukey's Multiple Comparison Test; $p>0.05$).

Table 4a. Comparison of culture sCD23 levels (n = 19; matched cases)

Stimulating antigen/s	*p* value
RPMI-1640 alone	
RPMI-1640 + rIL-4/aCD40‡	**< 0.001**
RPMI-1640 + SWAP/rIL-4	> 0.05
RPMI-1640 + SWAP/aCD40	> 0.05
RPMI-1640 + SWAP/rIL-4/aCD40‡	**< 0.001**
RPMI-1640 + SEA/rIL-4	> 0.05
RPMI-1640 + SEA/aCD40	> 0.05
RPMI-1640 + SEA/rIL-4/aCD40‡	**< 0.001**

‡ Treatments with significant differences (Tukey's Multiple Comparison Test; $p<0.05$)

Table 4b. IgE levels in culture supernatants (n = 19; matched cases)

Stimulating antigen/s	*p* value
RPMI-1640 alone	
PMI + rIL-4/aCD40‡	**< 0.01**
RPMI-1640 + SWAP/rIL-4	> 0.05
RPMI-1640 + SWAP/aCD40	> 0.05
RPMI-1640 + SWAP/rIL-4/aCD40‡	**< 0.01**
RPMI-1640 + SEA/rIL-4	> 0.05
RPMI-1640 + SEA/aCD40	> 0.05
RPMI-1640 + SEA/rIL-4/aCD40‡	**< 0.001**

‡ Treatments with higher significant differences (Tukey's Multiple Comparison Test; $p<0.05$)

There was an inverse relationship between levels of culture sCD23 and IgE in 16 matched cases as shown in Figure 11, from where it is evident that high sCD23 titres in plasma corresponded with low IgE titres in the respective patients (one sCD23 and one IgE outlier values removed).

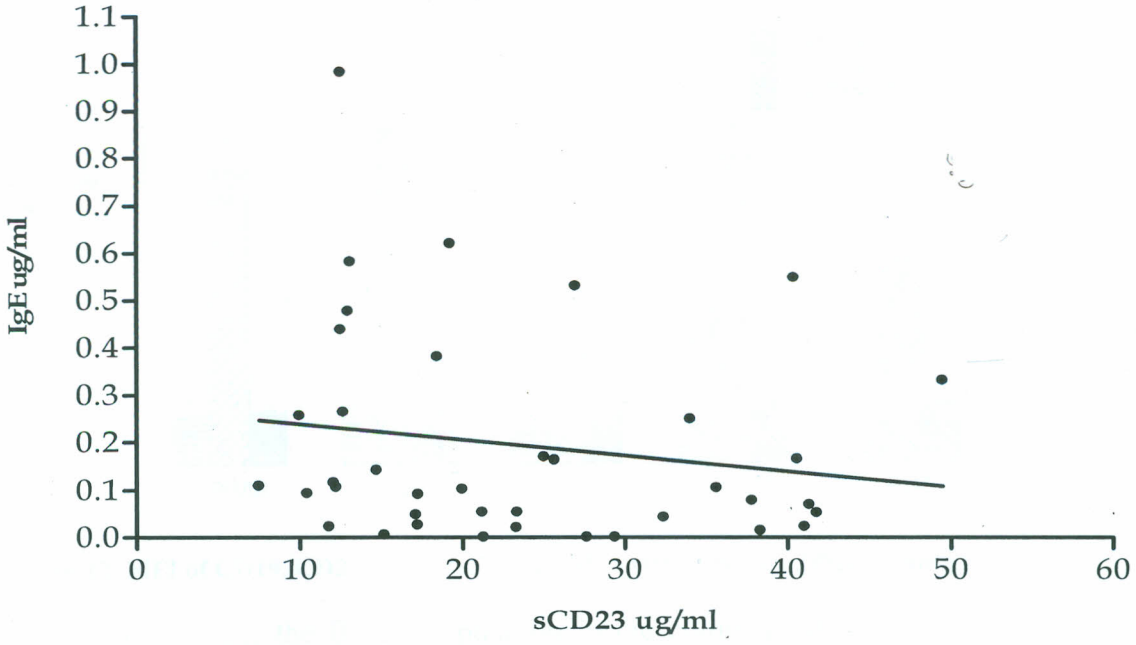

Figure 11. Linear regression of culture IgE and sCD23 levels for 41 participants

4.4 PBMC Stimulation by Different Antigens

Separately, an additional seven PBMC samples were used to evaluate the differences in the level of stimulation of B cell activation, given the same conditions of temperature, humidity, pressure, for 5 days. While the primary goal was to assess the performance of pokeweed mitogen, it was evident that used in complete media as single antigens, pokeweed mitogen, anti-IgE monoclonal antibodies, and SWAP resulted in much elevated CD23 expression on B cells compared to PHA and SEA (Figure 12). All stimulant antigens yielded good ratios of CD19/CD23 cells, with low proportions of $CD19^{+}$ cells.

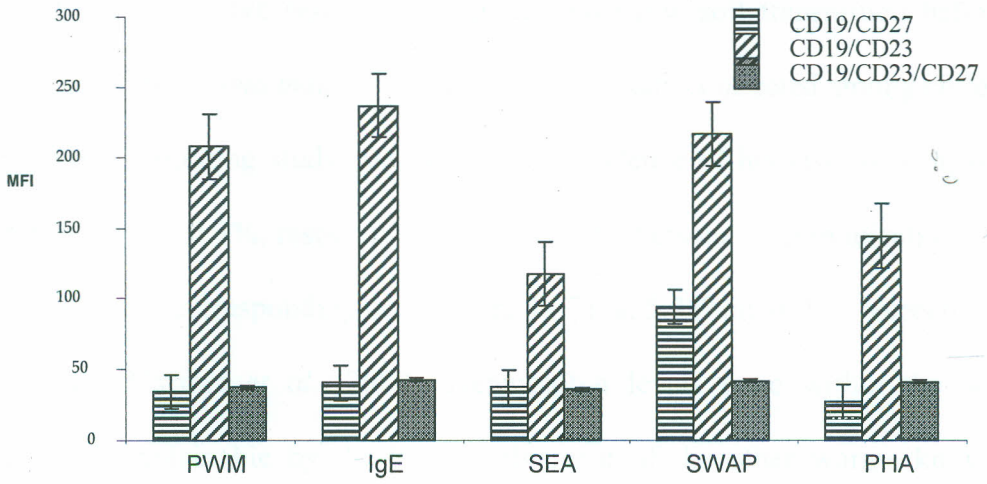

Figure 12. MFI of CD19, CD23, and CD27 on PBMCs stimulated by different antigens

On average, the B cell population in the cultured PBMCs (regardless of stimulating antigen) constituted just about 5% of the entire pool, out of which approximately 48% was CD23+, and, of this, about 45% co-expressed CD27.

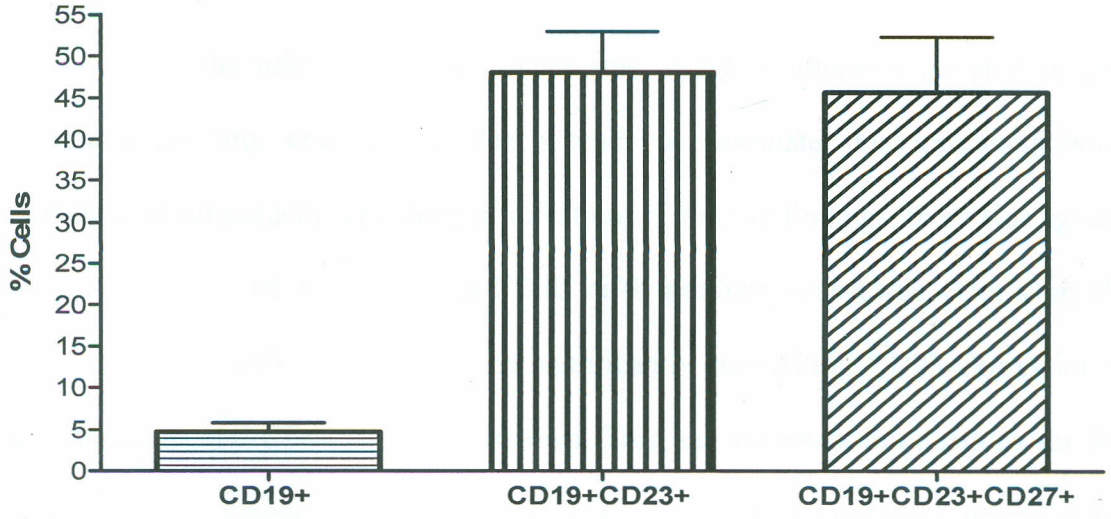

Figure 13. Average cell populations from cultured PBMCs. Only a small fraction (5%) of the resultant population consists of B cells

5.0 DISCUSSION

From the above results, adult males have few soil transmitted helminthes problems (other than *Schistosoma mansoni* infections). In a study conducted among children aged 9 to 14 years in a neighboring study site, the point prevalence of hookworm, whipworm and ascaris were 15%, 9% and 3%, respectively (Verani 2008, personal communication). In the this study community, the corresponding levels were 14.71%, 5.88% and 0%, respectively, for the same helminthes (Mwinzi *et al.,* 2009). Hemoglobin levels were within the normal range, an observation explainable by the low involvement of the other worms known to suck large volumes of blood. Malaria was not prioritized in this study and could have contributed to the cases of low hemoglobin levels (though not necessarily anemic). The B cell proportions of lymphocyte populations also fell within normal ranges (study average was 10.19%; normal range is 10-20%). CD4/CD8 ratios were also averagely within the normal range, with study average being 1.76 (in a range of 1.0 - 13.18, while the normal healthy range is 0.6-5.0 (95% confidence).

While both the role of and the mechanisms of IgE synthesis regulation in general remain almost obscure, emerging evidence tends to associate this immunoglobulin to protection against subsequent schistosomal infections. From the findings, there was agreement with existing reports that IL-4 and anti-CD40 mAb produce synergistic effects on B cell expression of CD23, with rIL-4 used alone significantly stimulating CD23 expression on B cells as opposed to anti-CD40 mAbs on its own. This has important implications for studies and/or interventions designed to target CD23 expression. Part of important directions would include interventions seeking to raise IL-4 levels, thereby enhancing CD23 B cell expression, and consequently resistance development. In agreement with previous works (Chung *et al.,* 2002; Petro *et al.,* 2002), anti-CD40 mAbs without rIL-4 did not produce B-cell activation, a

feature attributable to the absence of T cells that are necessary for activating resting B cells through CD40L engagement with CD40 molecules on B cells (Janeway *et al.*, 2001).

Significant differences were also seen in $CD23^+$ B-cell CD27 expression from different stimulations but with variations not as much as in CD23 expression. It was observed that rIL-4 used alone in RPMI-1640 produced the greatest stimulation of CD27 expression on B cells. This is understandable given the role of IL-4, like IL-6, in stimulating cell growth, and IgE production (Janeway *et al.*, 2001). rIL-4/SEA, and rIL-4/SWAP used in RPMI-1640 also produced good stimulation of CD27 expression on $CD23^+$ B cells, the other combinations being either lower or not significantly different from RPMI-1640 alone.

The synergistic effect was also observed on sCD23 levels in culture supernatant, but rIL-4 did not produce significantly higher titres than RPMI-1640 alone, although levels were more significantly raised compared to plasma sCD23 levels. Both CD23 expression on cultured B cells and sCD23 culture titres were elevated when rIL-4 and aCD40 mAbs were used together with SEA or SWAP, and the greatest levels were reached in rIL-4/aCD40/SEA-stimulated wells. It has been indeed 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).

B-cell expression of CD23 was not related to the schistosomal infection intensity as determined by egg counts per gram of stool sample processed. The expression of CD27 was very weakly associated with egg counts ($r^2 = 0.05781$; $p = 0.3697$). This considered the co-expression of CD27 and CD23, the latter of which is documented to be higher in CD27 negative B cells, CD23 being an early activation marker (Vugmeyster *et al.*, 2004). Therefore, enhancing CD23 expression may as such play an important role in regulation B cell transition

to memory cells, an important feature in secondary immune reactions or responses to chronic infections (Chung *et al.*, 2002).

sCD23 levels were inversely related to CD23 expression, and the soluble egg antigen (in synergy with anti-CD40 mAbs and recombinant IL-4) elicited the greatest CD23 expression on B cells as well as highest release of sCD23 in culture supernatants. Culture cellular CD23 expression was directly associated with IgE tires in culture supernatants. Plasma sCD23 levels were positively related to plasma IgE levels (although not significantly; $p > 0.05$) in the 34 participants. Culture sCD23 levels were however inversely associated with culture IgE titres. This difference in observations could have been the result of differences in the preferential release of monomeric or oligomeric isoforms of sCD23 by proteolytic cleavage (McCloskey *et al.*, 2007). Previous findings indicate that sCD23 is involved in regulation of IgE synthesis, and that high sCD23 levels are associated with increased IgE synthesis during human *S. mansoni* infections (Kicza *et al.*, 1989; Matupi, 2001; McCloskey *et al.*, 2007) but the mechanisms underlying the relationship and/or regulation remain unclear. Studies have shown that soluble CD23 monomers inhibit while oligomers stimulate IgE synthesis (McCloskey *et al.*, 2007), but the little understanding of the circumstances surrounding the monomeric/oligomeric sCD23 balance within the human hinders comprehensive elucidation of the observed relationships.

Plasma IgE levels only differed significantly from culture IgE levels in wells stimulated with a combination of either rIL-4/aCD40/SWAP or rIL-4/aCD40/SEA. Unlike with sCD23, IgE titres in culture were not significantly raised by rIL-4/aCD40 used in RPMI-1640 without either SEA or SWAP. IgE levels in culture were increased (though not significantly) with

raised schistosome egg counts in stool (p = 0.3697; r^2 = 0.05781), an observation consistent with previous findings (Caldas *et al.*, 2000).

It was also noted that the differences in IgE titres were not as great as those observed with sCD23. IgE synthesis was perhaps not as much affected by the different cascades involved in cultured cells, being well regulated by a number of unknown mechanisms, while sCD23 is believed to be cleaved by yet unknown enzymes whose regulatory mechanisms and the factors involved also remain unknown (Matupi, 2001; Kobayashi, 2002).

On comparison of other antigens singly used to stimulate PBMCs, IgE, SWAP and PWM (decreasing order) showed the highest stimulation of CD23 expression on B cells, with PHA and SEA trailing without significant differences between the two. Only subtle differences were observed in the expression of CD27 on $CD23^+$ B cells following stimulation with each of the stimuli, although IgE, SWAP and PHA each seemed to have higher stimulation than PWM or SEA. The B cell yield from PBMC culture was relatively low, but consistent with many works done (Nutman *et al.*, 1984; Saxon *et al.*, 1980).

6.0 CONCLUSION

B cell surface CD23 expression significantly varies for different Antigenic stimuli, with IL-4 producing the highest effect on CD23 expression and sCD23 release. The presence of schistosome-derived antigens (SWAP and SEA) played a synergistic effect on CD23 expression. This is consistent with other studies, and interventions targeting CD23 must take into account the contribution of IL-4 in CD23 and perhaps in resistance development.

IgE titres in culture supernatants were raised above plasma levels, and were directly proportional to CD23 expression and inversely to sCD23 levels (not significant). There could have been interplay of factors, more so regarding cleavage of CD23 from the surface.

Schistosoma mansoni egg counts in stool was not significantly associated with B cell surface CD23 expression, sCD23 release or IgE production. This in part could be attributable to the fact that egg release by infected persons depends on many factors other than the mere presence of a mature female worm.

7.0 Suggestions for Further research

The role of CD23 in immunity to schistosomiasis, especially molecular aspects of B cell CD23 expression, and the mechanisms of IgE involvement, require detailed and specific attention. This would include investigating factors involved in CD23 mRNA isoform gene transcription, translation, and possible role of gene polymorphisms.

Furthermore, factors involved in CD23 cleavage from cell surfaces and further breakdown warrant detailed studies. This will help give a holistic view of the dynamics of CD23 expression, shedding light on the balance between cell-bound and soluble CD23 forms.

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