DEVELOPMENT OF T CELL IMMUNITY TO *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN-1 IN A POPULATION EXPOSED TO HOLOENDEMIC MALARIA

BY

Kiprotich Chelimo

A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of a Degree in Doctor of Philosophy in Biomedical Science and Technology (Immunology Option)

School of Public Health and Community Development

MASENO UNIVERSITY

ABSTRACT

Plasmodium falciparum malaria is a major cause of mortality and morbidity in children under the age of five years in malaria endemic areas of sub-Saharan Africa. Clinical immunity to malaria develops in holoendemic areas by the age of 5 years and is characterized by diminished disease severity and less frequent infections. Protective immunity against *P. falciparum* malaria is mediated by both antibodies and cytokines. IFN-γ is considered a key cytokine that mediates protection, however, the development of immunologic memory may involve differentiating functional aspects of T cell subsets. Naïve and memory T cells can be categorized into subsets based on surface marker expression and ability to secrete cytokines. This study aimed at investigating the development and regulation of *P. falciparum* merozoite surface protein-1 (MSP-1) 42kDa 3D7 strain specific memory T cell subsets in a population exposed to holoendemic malaria. To do this, a cross-sectional study was designed to evaluate children of different age groups and adults from Kanyawegi, a malaria holoendemic region in western Kenya. Isolated peripheral blood mononuclear cells were either freshly stained for *ex vivo* T cell surface molecules or stimulated with *P. falciparum* MSP-1 for IFN-γ ELISPOT and intracellular cytokine staining enumeration of T cell subsets by flow cytometry. Culture supernatants were tested for the presence of Th1 and Th2 cytokines. Results showed that MSP-1 stimulated both $CD8^+$ and $CD4^+$ T cells to produce IFN-γ, however, the phenotype of these antigen-specific T cells differed by age. The frequency of IFN-γ producing T cells to MSP-1 increased with age and was associated with overall T cell maturation from a naïve (T_N: CD62L+/CD45RA+) to differentiated effector (T_{EM}: CD62L-/CD45RA-) or central memory (T_{CM}: CD62L+/CD45RA-) T cell phenotypes.

CD4 T *ex vivo* mature T_{CM} and T_{EM} significantly increased while T_N decreased with age ($p = 0.0001$, 0.0002 and 0.0003, respectively). Within CD8 subsets, only T_N significantly decreased with age ($p = 0.0046$). There was a strong negative correlation between *ex vivo* CD4 and CD8 T_N and both T_{CM} and T_{EM}. The frequencies and magnitude of IFN-γ responses to MSP-1 measured by ELISPOT increased significantly with age ($p = 0.023$ and 0.0011, respectively). The prevalence of MSP-1 specific IFN-γ producing CD4 and CD8 T_{CM} and T_{EM} subsets were similar across the age groups but only CD4 but not CD8 T_N significantly decreased with age ($p = 0.0119$ and 0.246, respectively). Correlation of all IFN-γ producing CD4 and CD8 cell subsets and IFN-γ ELISPOT responses across the age groups were not significant. No significant differences were observed in the surface expression of T cell costimulatory molecules: CD28, CD152 and CD154 across the age groups (all $p > 0.05$) but the ratio of CD154/CD152 CD4 T cell subset were significantly lower in infants and adults compared to children between the age of 1 and 5 years. The frequencies and levels of Th1 and Th2 cytokines tested revealed a similar non-significant difference across the age groups. However, apart from IL-4 levels, all other cytokines positively correlated with more than two other cytokines from either the Th1 or Th2 group (all $p < 0.05$). These findings suggest that frequent exposure to endemic *P. falciparum* induces antigen specific memory T cell subsets and cytokines that may be essential for the development of immunologic memory to malaria. Understanding the natural correlates of protection and evolution of immunologic memory to *P. falciparum* MSP-1 will assist in vaccine design and targeting populations at risk.

CHAPTER ONE

1.0. INTRODUCTION

Malaria, a disease caused by microscopic *Plasmodium* parasites and transmitted by *Anopheles* female mosquitoe, is currently estimated to threaten 40% of the global human population. Four human *Plasmodium* species; *vivax, ovale, malariae* and *falciparum* have species-specific distribution in endemic tropical regions of Africa, Asia and America. In sub-Saharan Africa, *P. falciparum* is responsible for the high morbidity and mortality associated with malaria infections particularly in young children and pregnant women (Uneke., 2007). Globally, over 90% of the 300-500 million reported cases and 1.5-2.7 million mortalities due to malaria are found in sub-Saharan Africa (W.H.O., 1990). Based on mathematical modeling, it is estimated that everyday in Kenya alone, 72 and 400 children less than 5 years of age die or develop clinical malaria, respectively (Snow *et al.*, 1998).

Integrated malaria control measures targeting *Anopheles* vectors, *Plasmodium* parasite and human hosts have been employed yet it remains a global challenge to eradicate malaria in the tropics. Moreover use of insecticides that helped eradicate malaria vectors in temperate countries in 1960s was restricted in 1970s when it was discovered that most vectors where developing resistance to them and also had some other environmental implications (Mendis *et al.*, 2009). Management of clinical malaria using inexpensive drugs such as chloroquine and sulphur based compounds has also resulted into a complex scenario due to parasite drug resistance (Lusingu and Von Seidlein., 1998). Since vector and parasite resistance have limited tools for malaria

control there is an ever increasing need to develop more sophisticated methods to manage malaria (Good *et al.*, 2005).

As integrated control measures are put in place to prevent and control malaria transmission, design and development of anti-malarial vaccines remains the long term goal for elimination and eradication of malaria. Currently three types of malaria vaccines are being developed based on the complex life cycle within the mosquitoe and human hosts. Transmission blocking vaccines are designed to interfere with development and transmission of sporozoite within the mosquito to human (Arakatawa *et al.*, 2009). Pre-erythrocytic vaccines are developed to inhibit the invasion and growth of sporozoites in hepatocytes while erythrocytic vaccines aim at the merozoite invasion and growth within the erythrocytes (Speake and Duffy, 2009). Erythrocytic stage of malaria parasites is associated with most clinical and pathologic symptoms of malaria infection hence the focus of developing a vaccine based on this parasite stage would eventually reduce high morbidity and mortality cases due to merozoite invasion and destruction of erythrocytes.

The basis of designing and developing an effective malaria vaccine lies in understanding the *Nature* of immune correlates of protection which include immunogenic surface proteins expressed by *Plasmodium* parasites and the constituents of the human immune system that recognize them. Natural exposure to high and stable perennial malaria transmission is characterized by gradual decrease in the clinical malaria cases observed during the first five years of life (Bloland *et al.*, 1999). These changes likely involve both humoral and cellular compartments of the immune system. During *P. falciparum* blood stage infection, antibodies are believed to be the major immune mediator as depicted by higher frequencies and levels in immune compared to non-

immune individuals (Okech *et al.*, 2006; Kumar *et al.*, 1995). Transfer experiments have shown that IgG is the main effector antibody isotype that suppress blood stage infection (Sabchareon *et al.*, 1991) as they bind to the surface of infected erythrocytes preventing invasion of erythrocytes and allow for antibody dependent parasite growth inhibition (Woehlbier *et al.*, 2006).

The role of T cells during blood stage infection has been more challenging to demonstrate although it is well established that T helper cells assist B cells switch their antibody isotypes to cytophilic IgG1 and IgG3 subclasses and that these subclasses are specific for *P. falciparum* merozoite antigens (Su *et al.*, 2002). The expansion of CD4 T lymphocyte population during *P. falciparum* infection (Farouk *et al.*, 2006) further suggest a significant role for CD4 T cells for fighting malaria infections. Naïve T cells are immature and nonspecific but once they recognize antigens they undergo a process of maturation and differentiation to form a population of memory T cells, which is characterized by clonal expansion and ability to respond faster to antigen-specific stimuli upon re-exposure (Esser *et al.*, 2003). In fact CD4 and CD8 expressing memory receptor (CD45RO) inhibit parasite growth via production of IFN-γ, TNF-α (Fell *et al.*, 1994) and activation of antibody secretion (Kabilan, 1997; Troye-Blomberg *et al.*, 1990). Age-related development of clinical immunity to malaria may be associated with evolution of antigen-specific memory T cells in *P. falciparum* exposed individuals. Understanding the dynamics of T cell development within the context of the *P. falciparum* exposure in infants and children may help in targeting antigen specific T cells relevant to malaria immunity that will assist in anti-malarial vaccine design and evaluation strategies.

1.1. Hypotheses

1.1.1. General hypothesis

The general hypothesis was that age-related development of immunity to *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) is mediated by IFN-γ producing central memory T cells (IFN-γ^+, CD45RO$^+$, CD62L$^+$) expressing high levels of CD28 and CD154 and have high proliferative potential upon re-exposure to malaria antigens.

1.1.2. Specific hypotheses

The specific hypotheses were:

a) The proportion and magnitude of *P. falciparum* MSP-1 specific IFN-γ responses increase with age from infants to adults.

b) There is gradual increase in the number of mature IFN-γ secreting memory T cell subsets specific to MSP-1 with an increase in age.

c) There are age-related changes in the frequency of T cell co-stimulatory molecules (CD28, CD152, and CD154) with an increase in age.

d) There are age-related changes in the frequency and levels of cytokines responses specific to *P. falciparum* MSP-1.

1.2. Main Objective

The main objective of the study was to evaluate age-dependent differences in T cell memory subsets to *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) in children and adults residing in a malaria holoendemic area.

1.3. Specific Objectives

a). Determine the proportion of *P. falciparum* MSP-1 specific IFN-γ responses in adults and children exposed to holoendemic malaria transmission.

b). Phenotypically characterize and determine the prevalence of MSP-1 specific memory CD8 and CD4 T cell populations (CD62L, CD45RA, IFN-γ) in adults and children exposed to holoendemic malaria transmission.

c). Phenotypically characterize and determine the prevalence of CD8 and CD4 T cell co-stimulatory molecules (CD154, CD152, and CD28) in adults and children exposed to holoendemic malaria transmission.

d). Determine MSP-1 antigen specific T cell cytokine (Th1 and Th2) responses in adults and children exposed to holoendemic malaria transmission.

1.4. Justification

In regions of chronic and persistent *P. falciparum* infection, children present with clinical symptoms and immunopathology due to invasion and destruction of infected erythrocytes by merozoite stages of malaria parasites. However, children in areas under perennial intense malaria transmission acquire clinical immunity to malaria by the age of 5 years (Bloland *et al.*, 1999). The mechanism underlying clinical immunity to malaria are not clearly understood but is thought to involve both antibody and T cell responses (Stephens and Langhorne, 2006). The role of B cells during *P. falciparum* blood stage infection relate to high frequencies and levels of IgG antibodies that bind to infected erythrocytes, released merozoites and parasite toxins (Okech *et al.*, 2006; Kumar *et al.*, 1995). B cells however, require CD4 T cells for activation, isotype switching and effective secretion and maintenance of antibodies (Stephens and Langhorne, 2006). Nevertheless, the mechanism and phenotype of CD4 helper T cells involved in providing assistance to B cells has not been well characterized during *P. falciparum* blood stage infection.

Cellular communication between CD4 T cells and B cells may occur through cell surface co-stimulatory molecules and/or secretion of cytokines that activate B cells to undergo maturation and differentiation to assist in parasite clearance. Chronic *P. falciparum* infection may actively suppress the development of immunologic memory through the down regulation of essential costimulatory molecules or cytokines required for effective activation and maintenance of memory T cells. An insight into the changes in cell surface expression of these molecules may help in defining malaria specific immunity and future design of vaccines. This study was therefore designed to investigate the phenotypic (global and antigen specific T cell subsets) and functional (cytokines and

costimulatory molecules) *Nature* of T cells in a population naturally exposed to high and stable *P. falciparum* transmission. Understanding the development of immunologic T cell memory under natural *P. falciparum* transmission may be fundamentally critical in determining clinical endpoints for vaccine trials in African infants and children living in malaria endemic regions.

In Rome, malaria devastated the invaders of the ancient Roman Empire and was associated with marshes and swamps. People blamed the rot and decay that brought a bad smell to the air hence arose the Italian name *mal' aria* meaning bad air. The cause of malaria was discovered in 1880 by Charles Laveran and 18 years later, Sir Ronald Ross attributed malaria transmission to *Anopheles* mosquitoe (Beltran, 1981; Guillemin, 2002).

By the mid-20th century, malaria related mortality decreased, mainly because of the spontaneous decline in contact between human and vector populations due to improved living conditions as well as by the vector control measures. By the early 1950s, endemic malaria disappeared from North America and Europe. However, in the malaria endemic tropics, prevention measures have faced continued challenges due to insufficient vector control programs and increased migration of the human hosts who are carried on the boats, trawlers, ships, jets and surface transport (Kaneko, 1998).

2.2. *Plasmodium* Life Cycle and Malaria Transmission

Malaria is caused by a single-celled microscopic parasite of genus *Plasmodium*. There are over 100 different species of *Plasmodium* of which four species *P. vivax*, *P. ovale*, *P. malarie* and *P. falciparum* infect humans. *P. falciparum* is the most common and lethal species that causes the highest cases of malaria morbidity and mortality in sub-Saharan Africa (Magill, 2006).

Female *Anopheles* mosquitoes are the main vectors and intermediate hosts for *Plasmodia* parasites (Coluzzi, 1993). Malaria transmission occurs when an infected female *Anopheles* mosquitoe injects infective sporozoites to a human host during blood meal. Sporozoites travel to the liver and establish themselves in the hepatocytes where they undergo schizogony into thousands of schizonts that are released into the blood

stream where they invade circulating erythrocytes. While in the erythrocytes, they undergo further asexual multiplication to produce merozoites, which are released to the blood stream thereby infecting new erythrocytes and amplifying the peripheral parasite density. A synchronous burst of erythrocytes and release of hemozoin and other toxins leads to characteristic periodicity in fevers. After several cycles of erythrocytes re-infection, merozoites can transform into sexual stage gametocytes, which are infective when ingested by an *Anopheles* mosquitoe during blood meal. Male and female gametocytes sexually unite in the mosquitoe stomach forming zygotes, which develop into ookinates and eventually transforming to sporozoites in the salivary glands hence completing the malaria parasite life cycle (Bynum, 1999; Good *et al.*, 2004). Other modes of malaria transmission include the transfusion of parasite contaminated blood or transplacental infection of the fetus.

At different developmental stages, the *Plasmodium* parasite expresses stage specific proteins that can be processed and presented by the human immune system (Reviewed in Todryk and Walther, 2005). Some of these malaria proteins have been evaluated as potential anti-malarial vaccine candidates (Bojang *et al.*, 2005; Druilhe *et al.*, 2005; Audran *et al.*, 2005; Flanagan *et al.*, 2006).

Malaria transmission periodicity and intensity varies between different localities due to several biological and environmental factors. In areas of high and stable malaria transmission (holoendemic), the resident population is exposed to repeated infections that may gradually lead to build up of some protective immunity. Many of them carry infections at any given time hence acting as potential reservoirs for transmission by mosquitoes. In areas where the rate of infection is low (epidemic), people may not

develop immunity because of infrequent exposure to the parasites and therefore might suffer higher morbidity and mortality during an outbreak (Snow *et al.*, 1994).

Parasite and mosquitoe behaviors are modulated by climate changes. Mosquitoe populations are normally high in humid conditions and expand during rainy seasons (Gil *et al.*, 2007). Malaria parasites require 10-14 days to complete the sexual cycle within the mosquitoe from gametocyte to sporozoite. Vectors live long enough to allow parasites to complete their development hence environmental factors that affect mosquitoe survival, such as indoor residual spraying decreases malaria incidence (Cot *et al.*, 2001). Reduction of ambient temperatures slows the parasite development and this explains why parasites do not thrive well in temperate areas.

2.3. Malaria Incidence, Morbidity and Mortality

Malaria is the most important, widespread vector-borne disease that is estimated to threaten 40% of the global human population (W.H.O., 1990). It is endemic in 91 countries within the tropics of Africa, Asia and Latin America extending into intertropical regions (Carme and Venturin, 1999; Wernsdorfer and Wernsdorfer, 2003; Ezhov *et al.*, 2004). Because of free movement of people from one continent to another, malaria has once again become a concern in temperate countries where it had been eradicated (Ezhov *et al.*, 2004; Chalumeau *et al.*, 2006).

In early 90's, Sub-Saharan Africa contributed to over 90% of 300-500 million malaria cases reported globally (W.H.O., 1990). However, global efforts to eradicate malaria have led to elimination of malaria in low transmission areas with a long term goal of elimination in high transmission areas (Mendis *et al.*, 2009). It is estimated that between 1.5 - 2.7 million people die of malaria related illness yearly, most of whom are

children below 5 years of age (Reviewed in Greenwood and Mutabingwa, 2002). Based on mathematical modeling, it is estimated that everyday in Kenya 72 and 400 children less than 5 years of age die or develop clinical malaria, respectively (Snow *et al.*, 1998) illustrating that malaria is one of the main causes of child mortality in this region. Malaria burden impairs social and economic development in endemic countries (Guinovart *et al.*, 2006) and there is need for integrated control measures to reduce malaria related mortality.

2.4. Malaria Immunopathology

Infection by *P. falciparum* leads to clinical immunity in some individuals and immunopathology in others. What underlies differences in susceptibility to clinical malaria lies within the host immune response. Immunopathology may result from direct destruction of erythrocytes by the parasites as well as uncontrolled activation of immune cells by parasite toxins during blood stage malaria. The multiplication of *P. falciparum* parasites in erythrocytes and synchronous burst of erythrocytes causes reduction of erythrocyte numbers and activates cytokine cascades leading to malaria symptoms such as nausea, headache and complications such as malaria related anemia (Good *et al.*, 2004). By yet undefined mechanisms, parasites suppress the generation of new erythrocytes leading to thrombocytopenia, which may contribute further to malaria-induced anemia (Abdalla and Wickramasinghe, 1988). Cerebral malaria is another complication resulting from sequestration of *P. falciparum* parasites in the brain tissues and localized cytokine secretion (Good *et al.*, 2004). Persistent *P. falciparum* infection may also result into multi-organ failure that is fatal in most cases (Krishnan and Karnad, 2003).

Destruction of erythrocytes and release of parasite toxins and parasites antigens recognized by the human immune cells leads to the activation of the immune system (Stevenson and Riley, 2004). Pro-inflammatory cytokines such as tumor necrosis factor - alpha (TNF-α) and interferon gamma (IFN-γ) are secreted early during infection by macrophages and natural killer cells in an attempt to suppress the parasites (Krishnegowda *et al.*, 2005; Miller *et al.*, 1989). Optimum levels of these cytokines have been shown to be protective against the parasites but over production or lack of negative feedback regulatory mechanisms induce malaria fevers and exacerbate related complication such as cerebral malaria (Clark *et al.*, 1991). Counter regulatory anti-inflammatory cytokines (IL-10, TGF-α) are normally secreted during persistent infections and are thought to quench the effect of pro-inflammatory cytokines though the evidence is controversial (Brown *et al.*, 1987; Day *et al.*, 1999). Although anti-inflammatory cytokines are required to counteract type 1 cytokines, it has been shown that they are the essence of chronic malaria infection and act to modulate the host immune response (Plebanski *et al.*, 1999).

There is also a general deficiency in mounting an appropriate immune response during *P. falciparum* infection (Williamson and Greenwood, 1978). The mechanism underlying this phenomenon is not well understood but may involve inappropriate apoptosis of immune cells (Kemp *et al.*, 2002) and/or blockage of dendritic cell function by infected erythrocytes via CD36 (Urban *et al.*, 2001).

In general, the erythrocytic stage of *P. falciparum* is responsible for the pathology associated with malaria. It is hypothesized that protective immunity against malaria prevents the establishment of an erythrocytic stage infection either at the level of the liver

stage infection (which is asymptomatic) or merozoite invasion into new erythrocytes. Malaria vaccine strategies likewise target these stages of the parasite life cycle.

2.5. Malaria Vaccines

The drive to develop a malaria vaccine stems from the unique challenges involved in the implementation of an integrated malaria eradication program in malaria endemic, resource constrained countries where mosquitoes are resistant to insecticides such as DDT and the parasites are resistant to inexpensive chemotherapeutic agents, such as chloroquine – two powerful tools that were instrumental in the eradication of malaria in North America in the 1960s (Good *et al.*, 2005).

Vaccines have been effective tools in mass control of bacterial and viral diseases such as small pox and polio yet there is no licensed vaccine for any parasitic infections including malaria. The ultimate goal of developing malaria vaccines has been complicated by the complex *Nature* of the *Plasmodium* parasite, *Nature* of the cells infected and the complex interaction between human cells and the parasites (Kitua, 1997). Different developmental stages in both mosquitoe and human hosts present additional challenges with stage-specific protein expression patterns and functions, some of which are yet to be defined (Stevenson and Zavala, 2006). Recent completion of the mosquitoe, *P. falciparum* and human genomes will enhance our understanding of the interaction between the parasites proteins and human immune responses, yet these studies are arduous and have just begun (Gardner *et al.*, 2002; Reviewed in Sims and Hyde, 2006).

In 1970s, protective immune responses elicited in mice by use of irradiated sporozoites indicated that anti-malarial vaccine was possible (Nussenzweig *et al.*, 1967).

However, use of irradiated sporozoites for mass immunization strategies is not necessarily practical since it is expensive in terms of production and maintenance. Although this method is being pursued (Hoffman and Chattopadhyay, 2008), other less labor-intensive techniques have been developed using specific proteins expressed by the parasites. The leading sporozoite vaccine candidate, circumsporozoite surface protein (CSP), is the dominant protein expressed on the surface of sporozoites that elicits both antibody and T cell immune responses (Nardin *et al.*, 1999). Recently, a study in The Gambia demonstrated that CSP-specific CD4 T cells protect against malaria infection and disease through secretion of IFN-γ (Reece *et al.*, 2004). At the liver stage, the liver stage antigens (LSA) 1-7 have been identified and are being evaluated as vaccine candidates (Krzych *et al.*, 1995; Morrot and Zavala, 2004).

The cellular mechanism of protection from sporozoite and liver stage infection is thought to be mediated by cytotoxic T lymphocyte (CTL) activity and secretion of IFN-γ by CD8 cells, which recognize antigens presented by the liver cells in the context of MHC Class I (Krzych *et al.*, 1995; Nardin *et al.*, 1999). IgG antibodies to these proteins have also been detected in children and adults residing in malaria endemic areas (Wang *et al.*, 2003; Chelimo *et al.*, 2005). Both sporozoite and liver stage infections are not associated with any malaria symptoms and vaccine candidates targeting these stages are being designed to block blood stage infection which causes symptoms and immunopathology (Labaied *et al.*, 2007).

During blood stage infection, merozoite surface antigens are expressed on the surface of infected erythrocytes that are also being examined as vaccine candidates (Holder, 1994). Merozoite surface protein-1 (MSP-1) is one such antigen and is the

leading blood stage vaccine candidate which is currently undergoing field trials in malaria endemic areas (Genton *et al.*, 2002). Antibodies have been demonstrated to play a major role in blood stage immunity by binding to the surface antigens on merozoites leading to agglutination, opsonization and phagocytosis, preventing cytoadherence, and neutralizing toxins hence preventing invasion of new erythrocytes (Riley *et al.*, 1990; O'Donnell *et al.*, 2001; Perraut *et al.*, 2005).

Studies in experimental mice have shown that depletion of T lymphocytes reduces or even ablates the effect of these antibodies to merozoites suggesting that T cell help is required for efficient B cells activation and antibody production (Langhorne *et al.*, 1990; Vanham and Bisalinkumi, 1995; Xu *et al.*, 2000). Since erythrocytes do not express MHC molecules, T cells will not directly recognize infected erythrocytes. In mice malaria models, macrophages and dendritic cells engulf the infected erythrocytes and toxic products and present them in the context of MHC-II to CD4 T lymphocytes leading to their activation, maturation and secretion of cytokines (Langhorne, 1994; Ing *et al.*, 2006). Cytokines may directly kill the parasite, activate cellular innate immunity, and activate B cells and antibody production against merozoites and their related toxins (Hisaeda *et al.*, 2005; Stephens and Langhorne, 2006).

2.6. *Plasmodium falciparum* Merozoite Surface Protein-1 Vaccines

During *P. falciparum* blood stage infection, several proteins are expressed by the merozoites one of which is merozoite surface protein -1 (MSP-1). MSP-1 is one of the leading blood stage candidate vaccine against *P. falciparum* malaria undergoing different phases of clinical trials in different malaria transmission settings (D'Alessandro *et al.*, 1995; Genton *et al.*, 2000; Genton *et al.*, 2003). It is a 200 kDa protein expressed on the

surface of intact merozoites and anchored to erythrocyte cell membrane by glycosylphosphatidylinositol (GPI) protein. It is primarily proteolytically cleaved into N-terminal and central regions of p83, p30, p38 and C-terminal region of p42 fragments (Woehlbier *et al.*, 2006). Secondary processing of the 42kDa fragments to a final protein of approximately 33kDa and 19kDa occurs before the merozoites enter the erythrocytes (Blackman and Holder, 1992). The 33kDa is shed off while MSP-1_{19} fragment remains on the surface and carried into parasitized erythrocytes during invasion (Wipasa *et al.*, 2002). MSP-1_{19} contains two epidermal growth factor-like domains that are involved in interaction with erythrocytes and are essential for merozoite invasion of erythrocytes (Blackman and Holder, 1992; Nikodem and Davidson, 2000).

Recombinant MSP-1_{42} and MSP-1_{19} vaccine candidates derived from two established laboratory *P. falciparum* lines/strains (3D7 and FVO) have been evaluated in animal models (Singh *et al.*, 2006) and clinical trials in humans (Huaman *et al.*, 2008). Both vaccines elicited very high and broad antibody responses that was associated with protection in experimental models (Darko *et al.*, 2005) but not in humans (Ogutu *et al.*, 2009). Further analysis on protective IgG responses elicited by MSP-1_{42} vaccine in mice and monkeys were shown to be due to both MSP-1_{19} and not the MSP-1_{33} subunit. The mechanism of protection is thought to involve IgG antibodies that block cleavage and processing of MSP-1_{42} into its smaller fragments hence limiting merozoite invasion (Guevara et *al.*, 1997). Functional invasion and inhibitory antibody assays show that IgG antibodies to MSP-1_{42} and MSP-1_{19} suppress parasite growth and inhibit erythrocyte invasion (Chang *et al.*, 1996; John *et al.*, 2004). It is postulated that B and T cell epitopes are contained within MSP-1_{19} and MSP-1_{33}, respectively, hence MSP-1_{33} is thought to be

a target of T cell based vaccines (Wipasa *et al.*, 2002). However, trials on MSP-1_{33} did not correlate with any clinical protection even though it evoked high antibody titers (Ahlborg *et al.*, 2002).

Analysis of MSP-1 protein from different strains of *P. falciparum* has shown that it contains some conserved as well as dimorphic regions especially within the MSP-1_{19} region (Miller *et al.*, 1993). There are four common allelic variants due to single amino acid substitutions observed in laboratory strains and additional polymorphism in the field isolates leading to a further classification into K and MAD20 alleles (Miller *et al.*, 1993; Woehlbier *et al.*, 2006; Takala *et al.*, 2007). The presence of multiple polymorphisms in laboratory and field isolates posses a great challenge to development of blood stage vaccine. Since MSP-1_{42} from 3D7 and FVO strains contain sequence that cover antigenic diversity in MSP-1_{33} and MSP-1_{19}, using the 42kDa in vaccine trials addresses the problem of polymorphisms.

2.7. Immunity to Blood Stage *P. falciparum*

Development of immunity to malaria infection depends on malaria transmission dynamics. In areas of unstable malaria transmission, immunity to malaria may not be acquired due to lack of frequent exposure to the parasites hence prevalence of clinical malaria during outbreak is high and similar across different age groups (Hogh, 1996; Rogier, 2000). In contrast, adults residing in areas of perennial high malaria transmission intensity acquire immunity against high parasitemia and clinical malaria leaving only children aged below five years at risk (Hviid, 1998; Bloland *et al.*, 1999; Rogier, 2000). Age-related reduction in parasitemia and clinical malaria in immune populations is associated with humoral and cellular immunity to blood stage parasites (Artavanis-

Tsakonas *et al.*, 2003; John *et al.*, 2004). Therefore, there is need to understand the development and stability of immunity in populations naturally exposed to *Plasmodium* infections in order to inform malaria vaccine design strategies.

2.7.1. Innate immunity

Innate immunity is the first line of defense against invading foreign pathogens and signals cells to acquire immunologic memory (Reviewed in Stevenson and Riley, 2004). Innate immune cells are not antigen specific but recognize pathogen associated molecular pattern molecules that are common to microbial pathogens. Natural killer cells, natural killer T cells, neutrophils, macrophages/monocytes and dendritic cells comprise innate immune cells and upon seeing pathogens secrete pro-inflammatory cytokines as a first line of defense (Urban *et al.*, 2005). In the case of malaria infection, these cytokines directly act on merozoites or may activate other cells of the immune system enabling effective control of the infection (Krishnegowda *et al.*, 2005). Recently, it was demonstrated that different subsets of NK cells exposed to *P. falciparum* infected erythrocytes respond with different regulatory molecules through secretion of IFN-γ, up-regulation of perforin, granzyme A secretion and lyses of infected erythrocytes (Korbel *et al.*, 2005). Macrophages bind to *P. falciparum* infected erythrocytes via CD36 molecules leading to phagocytosis, production of pro-inflammatory cytokines, and release of nitric oxide (Serghides *et al.*, 2003; Good *et al.*, 2005; Krishnegowda *et al.*, 2005). Macrophages and dendritic cells also act as antigen presenting cells (APCs) therefore becoming a strong link between the innate and adaptive immune systems.

2.7.2. Acquired immunity

2.7.2.1. Acquired humoral immunity to blood stage P. falciparum

Immature B cells secrete IgD and IgM but in the event of pathogenic/immunologic insult to the immune system, B cells are activated by T helper cells to switch their antibody isotypes to cytophilic IgG1 and IgG3 subclasses that are more effective against *P. falciparum* merozoites (Su *et al.*, 2002). Antibody levels to malaria blood stage antigens are higher and protect against clinical malaria in people residing in malaria endemic areas (Ferreira-da-Cruz *et al.*, 1995; Roy *et al.*, 1998). Antibodies are the major effectors of blood stage immunity in animal models and human *Plasmodium* infection (Kumar *et al.*, 1995; Okech *et al.*, 2006). Transfer experiments have shown that IgG is the main effector antibody isotype that suppress blood stage infection (Sabchareon *et al.*, 1991). Secreted IgG binds to the surface of infected erythrocytes preventing invasion of erythrocytes and also allowing for antibody dependent parasite growth inhibition (Woehlbier *et al.*, 2006). Moreover, antibodies prevent cytoadherence of parasitized erythrocytes on endothelial surfaces by blocking CD36 (Gruarin *et al.*, 2001). Chronic *P. falciparum* may lead to highly specific antibodies to malaria antigens because of affinity maturation in B cells.

2.7.2.2. Acquired cellular immunity to blood stage P. falciparum

Lymphocytes recognize *P. falciparum* blood stage antigens presented by antigen presenting cells (APC) in the context of MHC-II molecules (Esser *et al.*, 2003). Upon engagement of the MHC class II-peptide complex with T cells receptor, T lymphocytes undergo a maturational process that involves the up-regulation of co-stimulatory

molecules (Banchereau and Steinman, 1998). Naïve T lymphocytes become activated and differentiate into effector cells that secrete sig*Nature* cytokines that stimulate B cells, isotype switching and antibody production (Snapper *et al.*, 1992).

The role of CD4 T lymphocytes during blood stage *Plasmodium* infection is controversial and is yet to be resolved. During *P. falciparum* infection, there is expansion of CD4 T lymphocyte population (Farouk *et al.*, 2006) suggesting that they play a role during malaria infection. Depletion of CD4 T lymphocytes has been shown to abrogate immune response to *Plasmodium* infection (Langhorne *et al.*, 1990; Hirunpetcharat *et al.*, 1999) but seem to have no effect in other studies (Hirunpetcharat *et al.*, 1997). CD4 and CD8 expressing memory receptor (CD45RO) inhibit parasite growth via production of IFN-γ, TNF-α (Fell *et al.*, 1994) and direct antibody secretion through cytokine secretion (Reviewed in Kabilan, 1997; Troye-Blomberg *et al.*, 1990).

2.7.3. Global T cell phenotypic changes in *P. falciparum* malaria endemic regions

The gradual decline of naïve T cell production in the thymus due to normal aging leads to reduction in the replacement of peripheral T cell repertoire (Reviewed in Chidgey *et al.*, 2007). Naïve cells have specific T cell receptors that recognize only a single epitope expressed by multiple microbes found in the environment leading to clonal expansion of antigen specific T cells. This suggest that, although there is continuous production of naïve T cells, exposure to polymicrobial infections may lead to physiological changes in the proportion of T cell subsets with age. Different T cell subsets have been characterized based on expression of cell surface markers as well as intracellular molecules. Naïve T cells express CD45RA and CD62 selectin, central memory cells lack CD45RA but express CD62L while effector memory lack the expression of both molecules but have

immediate effector functions (cytokine and perforin release). The terminally differentiated T cells re-express CD45 but lack CD62L and are prone to apoptosis (Sallusto *et al.*, 1999b).

Phenotypic changes accompanying normal growth and development may contribute to variation on the level of immunity witnessed in different age groups exposed to the same environmental factors. In case of exposure to persistent chronic *P. falciparum* infection, older children and adults are more protected from infections and clinical malaria compared to younger children below the age of five years (Baird, 1998). Hence, this study was set to evaluate the age related physiological changes in the composition of T cell subsets that protect upon exposure to *P. falciparum.*

2.7.4. T cell costimulatory molecules for cellular interaction

CD28 is a cell adhesion molecule (CAM) that functions as a ligand for CD80 and CD86 antigens that are expressed by antigen presenting cells. Interaction of CD28 and its ligands co-stimulates CD2 and CD3 T cell receptor (TCR) dependent activation pathway (Park *et al.*, 1997). CD28 is predominantly expressed in CD4 (95%), some CD8 (50%) T lymphocytes and at low levels in immature thymocytes. Upon T cell activation, they are found to increase on the surface of mature CD3, CD4 and CD8 T lymphocytes. Differential expression of CD28 by CD4 and CD8 T cells affects the functional aspects of these cells (Truneh *et al.*, 1996).

Since CD28 is an important molecule for T cell activation, its expression profile on the surface of T cells may influence the clinical presentation of some disease conditions. In fact, expression of CD28 has been associated with development of some

diseases in both human and animal (Arreaza *et al.*, 1997; Zhu *et al.*, 2001; Zuberek *et al.*, 2003). Reduced expression of CD28 is also associated with reduction in IFN-γ and IL-2 secreting cells in autoimmune neuritis (Zhu *et al.*, 2001; Norton *et al.*, 1992). Cytotoxic T cell lymphocyte antigen (CTLA-4)/CD152 is a negative regulator of T cell activation and competes with CD28 for CD80/CD86 ligands (Lenschow *et al.*, 1993). CTLA-4 has high affinity for these ligands than CD28 hence can competitively inhibit or interfere with CD28 signaling (Engelhardt *et al.*, 2006).

CD154/CD40L is a member of TNF family of glycoproteins that is expressed on the surface of activated CD4 T cells and a small subset of activated CD8 T cells. Upregulation of this molecule on T cells and binding to its ligand CD40, leads to T cell proliferation and cytokine secretion (Fanslow *et al.*, 1994). Binding of CD154 to its receptor leads to B cell proliferation, immunoglobulin switching and prevents apoptosis of germinal center B cells (Yang *et al.*, 2000). The mechanism of T cell activation via CD154 involves CD40 binding that eventually up regulates the expression of CD80 and CD86 molecules on APCs (Young *et al.*, 2006). Studies on the role of CD40-CD40L in maintenance and proliferation of memory T cells has shown that memory cells can proliferate independently of this molecular interaction but cannot differentiate to cytokine producing effector cells (MacLeod *et al.*, 2006). Studies in mouse infected with *Salmonella* demonstrated that CD40-CD154 cooperation induces IFN-γ, IL-12 and NOS during acute phase of the infection, antibody production and generation of memory cells that protect against subsequent infections (al-Ramadi *et al.*, 2006). Furthermore, individuals with sezary syndrome, an advanced form of cutaneous T-cell lymphoma, have

defective IL-12 and TNF-α secretion due to impaired CD40L expression (French *et al.*, 2005).

The expression profiles of costimulatory molecules on the surface of T cells have been shown to affect cytokines (IFN-γ, IL-2, IL-12 and TNF-α) that are important in suppression of malaria infection and have been used in defining memory T cell subsets (Sallusto *et al.*, 1999b; Taylor-Robinson and Smith, 1999) suggesting that modulation of different costimulatory molecules may influence development of memory T cell subsets during malaria infection. Therefore, better understanding of immunologic T cell memory in a population exposed to holoendemic malaria requires investigation of expression profiles of these molecules.

2.7.5. Cytokine responses to asexual *P. falciparum* infection

Measuring a single or a few parameters may not give a clear picture of the complexity of the normal physiological processes. Until a few years ago, evaluation of multiple proteins at the same time was not possible until the advent of bioplex/multiplex technology which enables simultaneous analyses of multiple proteins using very limited sample volumes (Khan *et al.*, 2009). This technology has been applied in assaying for multiple cytokines in plasma or culture supernatants. Cytokines are immune regulatory proteins secreted by different cells of the immune system for cellular communication when they are activated. During *P. falciparum* blood stage infection, both pro-inflammatory and anti-inflammatory cytokines are involved in protective and pathologic aspects of infection (Wroczynska *et al.*, 2005; Yazdani *et al.*, 2006). Pro-inflammatory cytokines which include IL-1, IL-2, IL-6, IL-12, IL-18, IFN-γ and TNF-α are secreted by both innate and adaptive immune cells during the early days of infection and are involved in the initial

phases of parasite clearance (Wroczynska *et al.*, 2005). IL-1 in synergy with TNF-α stimulate acute phase response which triggers increase in serum concentration of mannose binding proteins and C reactive proteins that aid in parasite recognition leading to activation of the complement system and IgM antibody production against the parasites (Pied *et al.*, 1989).

Severe malaria in non-immune adults has been associated with increased levels of IFN-γ, IL-18 and reduced levels of IL-12 (Wroczynska *et al.*, 2005). IL-12 secreted by dendritic cells and macrophages is essential for directing the cytokine profiles of the activated T lymphocytes leading to production of IFN-γ (O'Garra *et al.*, 1995; Su and Stevenson, 2002) and activation of NK cells (Su and Stevenson, 2002). IFN-γ secreted by macrophages and NK cells upregulate the secretion of TNF-α from monocytes leading to increased production of nitric oxide synthetase (NOS) that mediate anti-parasitic effects in *P. chabaudi* infected mice (Jacobs *et al.*, 1996). In the presence of IL-12 and IL-18, NK cells are activated by *P. falciparum* infected erythrocytes to secrete IFN-γ (Artavanis-Tsakonas *et al.*, 2003). Further secretion of IFN-γ by activated T lymphocytes modulates the phagocytic and parasicidal activity of neutraphils and macrophages leading to parasite clearance (Hisaeda *et al.*, 2005; Su *et al.*, 2002). Optimum production of pro-inflammatory cytokines is protective against malaria related morbidity but over production may lead to immunopathologies (Good *et al.*, 2005).

Anti-inflammatory cyokines such as IL-2, IL-4, IL-5, IL-10, IL-13 and TGF-α among other anti-inflammatory cytokines are released later during the course of *P. falciparum* infection and counteract the effect of pro-inflamatory cytokines hence regarded as immunosuppressive or regulatory cytokines (Brown *et al.*, 1987; Day *et al.*,

1999). In pregnancy malaria, the levels of anti-inflamatory cytokines remained very low while levels of pro-inflammatory cytokines increased during infection (Fievet *et al.*, 2001). In some studies, the levels of Th2 cytokines have been shown to be higher in malaria patients compared to uninfected naïve controls (Wroczynska *et al.*, 2005) and regulate the levels of IL-12 production by DC and macrophages hence indirectly suppressing IFN-γ by NK and T cells (D'Andrea *et al.*, 1993). All these point to the fact that immunity or pathology due to malaria is a factor of relative balance between inflammatory and anti-inflammatory cytokines.

2.7.6. Interferon gamma responses to *P. falciparum* infection

Both antibodies and cellular components of innate and adaptive immunity play a critical role in reducing the episodes of clinical malaria in *P. falciparum* endemic areas (Doolan *et al.*, 2006). Cytokines form the major subset of functional molecules secreted by activated cells and play a central role in protection against *P. falciparum* pre-erythrocytic and erythrocytic stages. Interferon gamma, a type 1 cytokine secreted mainly by T cells and macrophages, plays a crucial role in activating macrophages, B cells and T cells hence acting as an important molecule in suppression of malaria parasites as well as immunopathology. Infection by *P. falciparum* leads to increased levels of this cytokine especially during erythrocytic stages (Su and Stevenson, 2002) suggesting an immunoregulatory role during malaria infection. Development of T cell based malaria vaccines have focused on up-regulation and detection of this cytokine (Luty *et al.*, 1999). Understanding the developmental dynamics of interferon gamma cytokine in a population naturally exposed to high and stable malaria transmission would be a step further into the in-sights of protective cellular mechanisms.

2.7.7. Development and regulation of acquired immunity to *P. falciparum* infection.

Maternal antibodies protect children born and residing in malaria endemic areas during the first 4-6 months of their lives (Wagner *et al.*, 1998). When maternal antibodies wane, children become susceptible to *P. falciparum* infection and present with highest cases of clinical malaria at the age of 6 to 24 months (Bloland *et al.*, 1999; Branch *et al.*, 2000). It is thought that clinical immunity is progressively acquired and reaches immune adult levels by the age of 5 years (Taylor-Robinson, 2002). Acquired immunity to *P. falciparum* is never complete as demonstrated by immune people who present with clinical malaria when they revisit the endemic areas after being away for some time (Yazdani *et al.*, 2006). Acquisition of natural immunity to malaria may involve interaction of innate and adaptive immune cells but the mechanism is not fully understood.

The initial contact of *P. falciparum* parasites with infant immune system may determine whether the parasite is cleared or persists to form a chronic infection. Central to initiation of immunity in naïve infants are dendritic cells since they are the only APC that can present antigens to naïve T cells (Banchereau and Steinman, 1998). The location of antigen presentation to naïve T cells during blood stage *P. falciparum* is complex since blood stage antigens are not localized but are circulated in infected erythrocytes. It is possible that some antigen presentation occurs in the T and B cell germinal centers but this may also occur in the peripheral blood circulatory system. Therefore, dendritic cells are required during the initial blood stage infection if an appropriate adaptive immune

response is to be evoked. However, other types of APCs may be involved during repeated infections and in development of memory T cell immunity to malaria.

Immunity to *P. falciparum* infection is only attained after repeated bites from infective mosquitoes (Yazdani *et al.*, 2006). Clinically, immune adults have been shown to posses memory T and B lymphocytes and high antibody titers to MSP-1 (Deloron and Chougnet, 1992). Memory T cells secrete IFN-γ which is effective against *P. falciparum* infection but demonstration of memory CD4 T cells secreting IFN-γ during blood stage infection has been difficult to show directly due to the low frequencies and poor methods of detecting these memory cells. For rational design of an effective MSP-1 vaccine, memory CD4 T lymphocytes would need to be targeted for activation and expansion to boost cytokines and antibodies production.

It has been suggested that clinical immunity to *P. falciparum* is short lived and is lost within a short time in the absence of repeat exposure (Deloron and Chougnet, 1992). The lack of immunological memory to malaria may be due to the presence of T regulatory cells that suppress immunity by direct contact with immune cells or by secreting immunosuppressive cytokines (Schluns and Lefrancois, 2003; Achtman *et al.*, 2005; Stephens and Langhorne, 2006). CD4 and CD8 T regulatory cells are characterized by expression of a transcription factor *FoxP3* and secretion of high levels of IL-10 and TGF-β, which are known to suppress Th1 cytokines (Fantini *et al.*, 2004; Chen *et al.*, 2003). The availability of high levels of TGF-β may also enhance the development and expansion of T regulatory cells (Chen *et al.*, 2003) leading to further suppression of proinflammatory immune responses. T regulatory cells with characteristic *FoxP3* expression are induced during blood stage infection and are associated with

increased production of TGF-β, decreased proinflammatory cytokines and suppression of antigen specific immune response (Walther *et al.*, 2005). The presence of T regulatory cells and high levels of secretory TGF-β coincided with resistance of parasite clearance from the blood suggesting that malaria infection could induce these immune modulating populations of regulatory T cells (Walther *et al.*, 2005). However, the development of T regulatory cells and the cytokine profile during evolution of immunity in malaria endemic areas is yet to be well described in human populations.

2.7.8. Immunologic T cell memory to *P. falciparum* infection

T helper cells are a heterogeneous population of naïve and memory cells that have a crucial role in the development and maintenance of immunological memory. They can be distinguished based on cell surface receptors, migratory and effector function (Bergmann *et al.*, 1997). During normal development of CD4 T cells, naïve cells with diverse repertoire of antigen receptors are generated and circulate freely in the body searching for the presence of any antigen that has complementary structure to the receptors (Good *et al.*, 2004). Naïve T cells are immature and nonspecific but once they recognize antigens they undergo a process of maturation and differentiation to form a population of memory T cells, which are characterized by clonal expansion and ability to respond faster to specific stimuli upon re-exposure [Reviewed in Esser *et al.*, 2003)). Activation of T cells involves up regulation of co-stimulatory and chemokine molecules some of which will enable extravasation of the selected naïve cells to specific tissues that act as the source of the antigens (Campbell and HayGlass, 2000; Colantonio *et al.*, 2002; Sallusto *et al.*, 1999). The ability of memory cells to recall an antigen after a prolonged period suggests that memory cells do not need antigen exposure to be maintained. However, the

mechanisms responsible for their generation and maintenance have not been well characterized in human *Immunology* (Gray and Matzinger 1991; Mullbacher, 1994).

Memory CD4 T cells are subdivided into effector ($CD4^+CD45RA^-CD62L^-$) and central memory ($CD4^+CD45RA^-CD62L^+$) T cells characterized by immediate and delayed recall immune responses respectively (Sallusto *et al.*, 1999a; Esser *et al.*, 2003). Upon encounter with a recall antigen, CD4 memory T cells express activation signals leading to release of cytokines that will activate both innate (macrophages and neutrophils) and specific immune cells (cytotoxic T cells and B cells) at least in animal models (Von der Weid and Langhorne, 1993). The two populations of memory CD4 T cells secrete distinct cytokine profiles and chemokines. Effector memory T (T_{EM}) cells secrete higher levels of IFN-γ or IL-4 and down regulate CCR7 expression while central memory T (T_{CM}) cells secrete mainly IL-2 and IL-10 and upregulate CCR7 expression upon activation (Reinhardt *et al.*, 2001; Sallusto *et al.*, 1999b). IFN-γ suppresses *P. falciparum* infection through direct killing of parasites and via activation of innate immune cells such as neutrophils and macrophages that degranulate and engulf the parasites, respectively (Yoshida *et al.*, 2003). IL-4 is T helper type 2 cytokine also secreted by memory T cells that is known to activate memory B cells, promote isotype switching and antibody secretion during *P. falciparum* infection (Esser and Radbruch, 1990; Tsuji *et al.*, 1994).

People residing in areas with high perennial, stable malaria transmission acquire natural immunity to *Plasmodium* infection and clinical malaria with most cases of high parasitemia, malaria morbidity and mortality concentrated in children below the age of 5 years (Bloland *et al.*, 1999; Taylor-Robinson, 2002). Clinical malaria has been mainly

associated with blood stage *Plasmodium* infection that may present with tissue damage, destruction of infected and uninfected erythrocytes and release of parasite toxins that activate secretion of pro-inflammatory cytokines (Krishnan and Karnad, 2003; Helbok *et al.*, 2005; Nebl *et al.*, 2005). During *P. falciparum* blood stage infection, infected erythrocytes burst releasing merozoites that invade new erythrocytes. Since mature erythrocytes lack MHC molecules, T lymphocytes may not play a direct role but antibodies are able to directly block merozoite invasion of new erythrocytes (Schofield *et al.*, 1986; Yazdani *et al.*, 2006). The protective role of CD4 T cells during *P. falciparum* blood stage infection is not yet clear but it has been suggested that they recognize antigens presented by APCs and secrete cytokines that will activate B cells to produce antibodies specific to parasite antigens (Meding and Langhorne, 1991; Su *et al.*, 2002; Su and Stevenson, 2002; Ing *et al.*, 2006).

In human and murine malaria models, IFN-γ and IL-4 are two cytokines secreted by CD4 T lymphocytes during blood stage *Plasmodium* infection (Pouniotis *et al.*, 2004; Malhotra *et al.*, 2005). Expansion of memory CD4 T cells secreting IL-4 in cultures from malaria exposed populations has been demonstrated to correlate with protection when challenged with attenuated *P. falciparum* sporozoites (Bergmann *et al.*, 1997). However, the initiation and maintenance of T cell immunity for B cell help during blood stage infection has yet to be well characterized. In fact, the progressive development of immunity to malaria may depend on the factors controlling the initial interaction between parasites and host immune cells so that if the parasite is not cleared during its acute phase it enters the chronic phase and will only be limited by the memory T and B cells. Investigations of the initial and subsequent cellular and molecular immune responses in

P. falciparum infections areas may provide insight into the development of protective natural immunity. Hence, this study aimed at evaluating the presence of malaria-specific T cells subsets in infants, children and adults naturally exposed to holoendemic *P. falciparum* malaria.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study Area

The study participants were recruited from Kanyawegi sub-location, Maseno Division, Kisumu District in western Kenya, a region with high, intense and perennial (holoendemic) malaria transmission.

3.2. Study Population and Design

This was a cross sectional study involving enrollment of infants, children and adults from a malaria holoendemic area. A total of 88 participants from the following five age groups were enrolled (6 months {n=17}, 12 months {n=16}, 24 months {n=19}, 5 years {n=18} and adults {n=18}). Available literature suggest that children residing in malaria holoendemic areas gradually acquire immunity to malaria and develop clinical immunity by the age of five years (Taylor-Robinson, 2002). Therefore this age stratification was applied to capture the role of T cell susbsets in progressive development of clinical immunity to *P. falciparum* malaria. Due to the variation in experimental design and the high number of cells required to meet the different objectives proposed, different number of individuals were used to test each objective. All individuals recruited were first screened for *ex vivo* T cell subsets out of which 49 samples had enough cells to test for IFN-γ production to $MSP1_{42}$ (3D7 strain). Only subsets of positive responders by IFN-γ production to $MSP1_{42}$ (n = 26) were further recruited for intracellular cytokine staining, costimulatory molecules and Th1/Th2 cytokine assays.

3.2.1. Sample size determination

A total of 88 samples were recruited randomly in the field from the indivuduals who met the inclusion criteria. Based on G*power calculation (Faul *et al.,* 2007), the sample size of 88 had a critical power of 0.84 at $\alpha = 0.05$ to detect difference between groups (as shown by the thick line in figure 1). For immunophenotyping, at least 20,000 events (T cells) were acquired in a FACScaliber per sample hence giving more power for a smaller n value (n = 26 and 49) to adequately detect statistical differences during data analysis and interpretation.

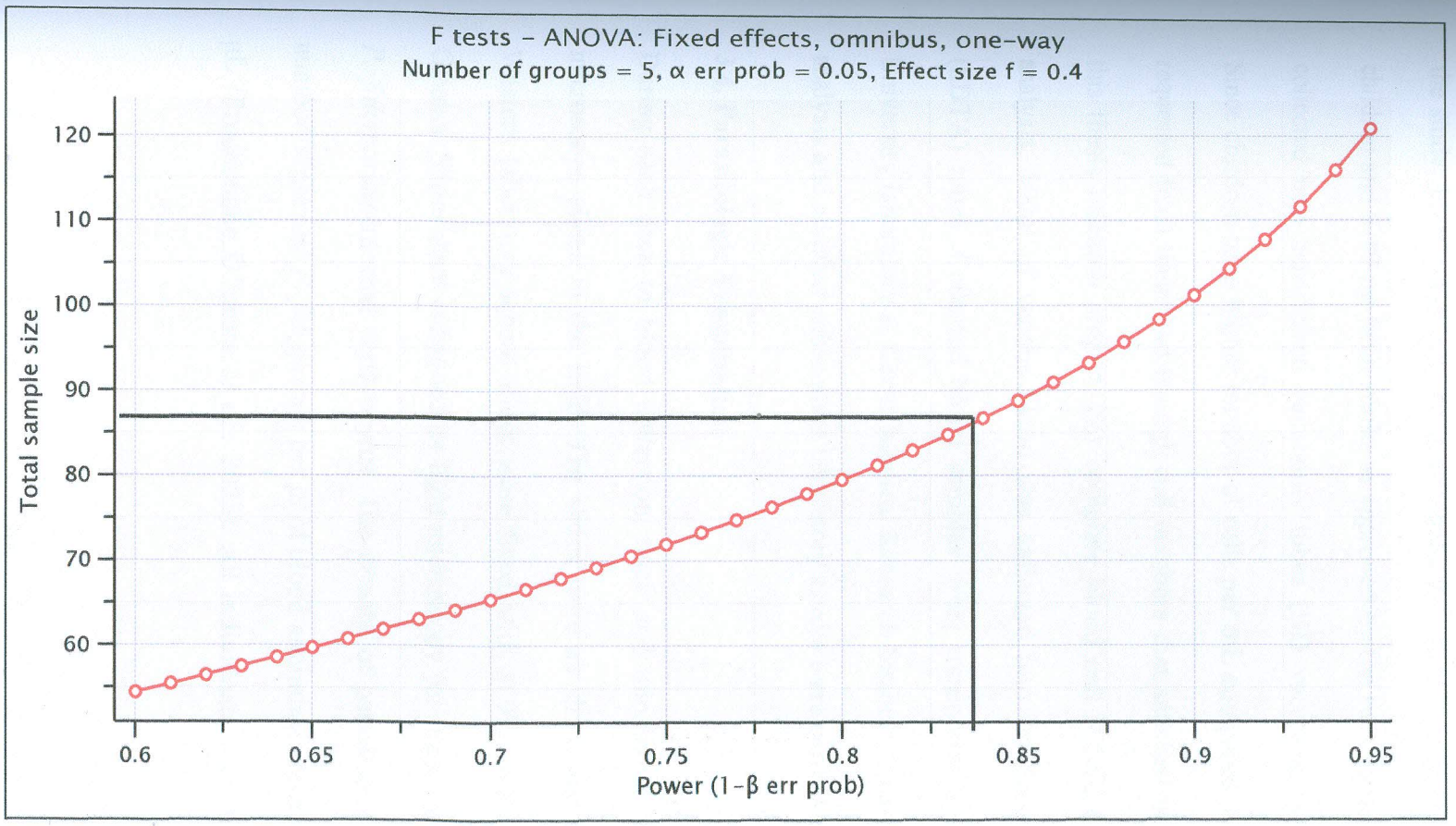


Figure 1. Power graph showing how the sample size was determined with a given critical power to detect statistical difference.

3.3. Physical Examination, Blood Collection and Full hemogram Analysis

After consenting and before enrollment, each study participant was physically examined by the project physician to ascertain if each of them was physically fit for enrollment into the study. From physically fit individuals, 2-5 ml of venous blood was requested from children and 8-10 ml from adults as approved by the two institutional review boards and collected into heparinised vacutainer tubes (BD vacutainer systems, Plymouth, UK). Since children have higher number of cells per mL compared to adults, 2-5 mL of blood requested from them had enough cell numbers that satisfied the objectives proposed in this thesis research project. For complete blood cell (CBC) counts or full hemogram analysis, 500ul of venous blood was collected into Ethylenediamine tetraacetic acid (EDTA) tubes. Collected blood samples were transported to Case Western Reserve University laboratory at Kenya Medical Research Institute, Kisumu where full hemogram analysis and all other subsequent laboratory assays were carried out.

3.4. Parasitologic Examination

During each time of blood collection, thick and thin blood smears was prepared on microscope slides in the field. The slides were air dried and transported to the Case Western laboratory where slides were fixed with 100% ethanol and stained with 5% Giemsa. Slides were examined by light microscopy for the presence and enumeration of *P. falciparum*-infected erythrocytes. The density of parasitaemia was expressed as the number of asexual *P. falciparum* per µl of blood assuming a leukocyte count of 8,000 per µL (Prudhomme O'meara *et al.*, 2005). All individuals with detected parasites in the

smear were treated for uncomplicated malaria with antimalarial (Coartem) as recommended by the Ministry of Health in Kenya (Appendix 1).

3.5. Peripheral Blood Mononuclear Cells (PBMCs) Isolation

The blood samples collected in heparin tubes were layered carefully on top of 5ml Ficoll-hypaque in sterile 15ml tubes. The caps were tightened and transferred to a centrifuge and spun at 350g for 30 minutes to allow separation of whole blood into three main components: plasma on top, PBMC in the middle and erythrocytes at the bottom of the tube. Plasma was aspirated and aliquoted into sterile Sarsdedt (Sarsdedt^{TM}, Newton, Germany) tubes and frozen at -80^{0}C for later use in antibody detection. The PBMC layer was transferred carefully into a new sterile 15mL tube and sterile 1×PBS added to a total of 12mL in each tube. This was mixed by gently inverting the tube 2-3 times then centrifuged at 250g for 15 minutes. The supernatant was aspirated carefully to avoid sucking the pellet. The pellet was flicked gently to break and resuspended in another 12mL of sterile 1×PBS followed by centrifugation at 250g for 10 minutes. At the end of spinning, supernatants was aspirated leaving a clean pellet of PBMC which was broken and resuspended in 1mL of culture media (RPMI 1640; GIBCO, Invitrogen, Paisley, Scotland, UK) supplemented with L-glutamine, human serum type AB, HEPES and gentamycin (cRPMI 1640). Suspended PBMC was mixed in 1:1 ratio with Turk's solution and 10μL of the mixture charged into a haemocytometer for PMBC enumeration.

3.6. Antigens for Evaluation of T cell Specific Responses

PBMC were stimulated with the following three different test conditions: PBS, *Staphylococcus* enterotoxin B (SEB), and MSP-1_{42}3D7. PBS is normal saline solution made of sodium and potassium salts with a Ph of 7.2. It does not contain any protein hence was used as a negative control for each sample. SEB is a bacterial toxin super antigen that stimulates about 5% of the total population of total T lymphocytes and was used as a positive control. MSP-1_{42}3D7 is *P. falciparum* antigen expressed during the blood stage infection and was used as test recombinant protein expressed in *Escherichia coli* for evaluation of T cell specific responses.

3.7. Cell Surface Staining for T cell Costimulatory Molecules

Freshly isolated PBMC or 36 hours cultured PBMC were used to assay for *ex vivo* T cell subset and costimulatory molecules, respectively. An aliquote of 100 µL containing 5×10^5 cells was sampled into 5mL polystyrene tubes and stained with fluorochrome conjugated antibodies (BD Pharmingen™, BD Biosciences, Pharmingen) against cell surface molecules (anti-CD3, CD4, CD8, CD45RA, CD62L, CD28, CD152, and CD154) at room temperature for 30 minutes. 2mL of wash buffer (0.5% BSA in 1×PBS) was added into each tube and centrifuged at 430g for 8 minutes to pellet the cells. Wash buffer was decanted carefully and tubes bloated on a paper towel. The pellet was vortexed and fixed with 500µL of 4% paraformaldehyde for 15 minutes at 4^0C in the dark after which a total of 10,000 cells were acquired using a FACscaliber™ (Becton Dickinson, USA).

3.8. Cytokine Enzyme Linked Immunospot (ELISPOT) Assay

Multiscreen ELISPOT plates (Millipore Corporation, Bedford, USA) were coated with capture anti-human IFN-γ antibody (Endogen M-700A, Worcester, MA) at a final concentration of 5µg/mL overnight at 4^0C. On the next day, the plates were washed x3 with 1xPBS and blocked with 100µL of 10% fetal calf serum for 2 hours at room temperature. Plates were washed ×3 with PBS then cells diluted to 5×10^6 per mL. 5×10^5 cells were seeded per well and each sample stimulated with the following three conditions: PBS, SEB and $MSP1_{42}$3D7 for 2.5 days in a humidified incubator with 5% CO_2 and temperature regulated at 37^0C. At the end of incubation period, plates were washed ×3 with 1×PBS followed by ×3 with 1×PBS containing 0.05% Tween (PBS-T) and secondary anti-human IFN-γ antibody (Endogen M-700B) added at a final concentration of 0.75µg/mL. After incubation for 1hour and 30 minutes at 37^0C, the plates were washed ×3 with PBS-T and horse reddish peroxidase (HRP) conjugated streptavidin (DAKO PO397) added at 1:2000 dilution and incubated for two hours at room temperature. Finally, plates were washed ×3 with 1×PBS and HRP substrate (1% 3-amino-9-ethyl-carbazole substrate in 0.1 M acetate buffer) added to develop the spots. This took between 20 to 40 minutes before stopping the reaction with distilled water.

3.9. Intracellular Cytokine Staining For Flow Cytometry

After counting, the resuspended PBMC was diluted to 5×10^6 cells/mL and cells seeded in 96 well culture plate at a cell concentration of 2.5×10^5 cells per 200 µL of cRPMI into two parallel wells. These cells were stimulated with either of the three test conditions (PBS, SEB or $MSP1_{42}$3D7) for 7 days in a humidified incubator with 5% CO_2 and temperature regulated at 37^0C. After 60 hours of stimulation, supernatants were removed

and 200μL of fresh cRPMI containing 20 units of IL-2 was added. During the last 6 hours of culture duration, intracellular cytokines was blocked with brefeldin A after which the supernatants from the cultured cells was removed leaving only a small volume with the pellet. The remaining pellet was suspended in 200μL of 20mM EDTA in PBS and cells transferred into 5mL polystyrene tubes, washed with 0.5% BSA and stained for cell surface molecules (anti-CD3, CD4, CD8, CD45RA and CD62L antibodies) at room temperature for 30 minutes. Fixing was done with 4% paraformaldehyde for 15 minutes at 4^0C in the dark. Cells were washed and cell membranes permeabilized for 30 minutes at 4^0C in the dark using ice-cold permeabilising solution (HEPES + 0.1% saponin). Cells were washed ×2 with wash buffer and incubated with anti-human IFN-γ antibodies for 30 minutes. Cells were fixed with 300μL of 4% paraformaldehyde for 15 minutes at 4^0C and washed. Cells were then suspended in wash buffer and at least 20,000 gated events were acquired per condition using a four colour flow cytometer (FACscalibur™ Becton Dickinson, USA). Since samples were acquired without compensation, FlowJo programme (Tree Star Inc, USA), which allows for post acquisition compensation was used for data processing and analysis. Appendix 2 shows the schematic representation of the gating strategy used to analyze T cell subsets

3.10. Bioplex Cytokine Assay

Bio-plex™ (Bio-Rad laboratories Inc, USA) cytokine commercial kit was used for detection of multiple Th1 and Th2 cytokines from the supernatants after PBMC stimulation with MSP-1_{42} for 60 hours. A sterile immunolon 96 well filter plate was pre-wetted using 100 μL/well of Bio-Plex assay buffer and aspirated by vacuum manifold.

The bottom of the filter plate was blotted with a clean paper towel. The multiplex bead working solution was vortexed for 15-20 seconds at medium speed and 50µL was aliquoted into the wells of the filter plate and removed by vacuum filtration. Each well was washed ×2 with 100µL of Bio-Plex wash buffer and the bottom of the plate was then blotted with a clean paper towel. Filter plate was put on a plate holder and 50µL of serially diluted cytokine standards and samples were put in each well, covered with a plate sealer and aluminum foil then placed on a microplate vortex-gene 2 shaker (Scientific Industries, Inc.) for incubation at room temperature. The speed of the vortex-gene 2 shaker was slowly increased to 1,100rpm for the first 30 seconds then reduced to 300rpm for 30min. At the end of 30min incubation, the buffer was aspirated and the plate washed ×3 using 100µL of Bio-Plex wash buffer then blotted on a paper towel. The Bio-Plex detection antibody working solution was gently vortexed and 25µL was added into each well, covered with a sealing tape and aluminum foil then placed on a microplate shaker for incubation at room temperature. The speed of the shaker was slowly increased to 1,100rpm during the first 30 seconds then reduced to 300rpm for 30min. After 30 minutes of incubation, the buffer was aspirated and plate was washed ×3 using 100µL/well of Bio-Plex wash buffer. Streptavidin-PE was vigorously vortexed and 50µL was added into each well, covered with a plate sealer and aluminum foil then placed on a microplate shaker for incubation at RT. The speed of the shaker was slowly increased to 1,100rpm for the first 30 seconds then reduced to 300rpm for 10 min. After 10 minutes of incubation, the buffer was aspirated and plate was washed ×3 using 100µL/well of Bio-Plex wash buffer. The beads in each well were resuspended in 125µL of Bio-Plex assay buffer, covered with a plate sealer tape and shaken at 1,100rpm for 30seconds at room

temperature. The sealing tape was removed and the plate was immediately read on the Bio-Plex™ (Bio-Rad laboratories Inc, USA) system.

3.11. Ethical Considerations

This study was part of the bigger study between KEMRI and CWRU to investigate development of human immunity to *P. falciparum* malaria in Western Kenya. As such this study was approved by the two institutional review boards with the following ethical considerations.

The minimal risk associated with participation was the discomfort of venipuncture and finger prick blood sample collection. There was a remote chance of secondary infection that is minimized by sterile precautions. Infection due to venipuncture has never occurred in the past studies contacted by this investigators. Drawing the proposed amount of blood from infants would not in any way endanger the child. Hemoglobin measurements were done prior to all venous blood sample collections to ensure each study participant was not anemic.

Direct benefits to study participants included clinical monitoring and treatment of malaria. When possible, those who experience severe illness were transported to the local hospital. This included those with hemoglobin concentrations <7 g/dL for children and <10 g/dL for adult women and men. The risks of venipuncture or finger prick were minimal relative to the immediate health benefit available to the community. Transport cost incurred by the study participants was re-imbursed. Any medical injuries as a result of participation in this study were covered.

The purpose of this study was explained to the study participants or the parents or guardian of all infant participants by the field assistants or hospital personnel in the local language. Those willing to enroll in the study were required to sign a written consent form in order to participate (Appendix 1). Recruitment was strictly voluntary. It was made clear that refusal to participate or withdrawal during the course of the study would not influence care or transport to local health facilities. It was also indicated that no monetary or other gains would be offered in exchange for participation. If the parent or guardian was literate, they were given a written document in Luo, Swahili or English. They were be asked to sign this document with a witness from KEMRI. Illiterate individuals were accompanied by literate village residents and the consent form was read to them. The consent form was co-signed by the village representative and a witness from KEMRI.

Each individual was assigned a unique identification number (UNID) which was used to label all samples collected for laboratory analysis. The UNID was linked to the personal identifiers in a data base that was key coded and only accessible to the investigators. All other information was stored in the data base by UNID. Original data collection forms, such as consent forms and blood collection forms were stored in a locked filing cabinet at the CWRU-KEMRI office at CGHR Kisumu. For malaria treatment purposes, blood smear data was made available to the clinical officer assigned to this project. No other information which contains personal identifiers was circulated. This database was stored on back-up files after analysis in case project records need to be reviewed.

No test was done for other potential microbial (parasitic, bacterial, fungal and viral) pathogens since ethical approval was only sought to investigate immunity to malaria. Informed consent was obtained from each adult and mothers/guardians of infants after explaining to them the purpose/objectives of the study. This study was approved by the Ethical Review Committee of the Kenya Medical Research Institute and the Institutional Review Board of Case Western Reserve University (Appendix 1).

3.12. Data Analysis

The frequency of IFN-γ cytokine responses measured by ELISPOT was compared between adults and children of different age groups and analyzed using multiple Chi-square tests. The magnitude of spot forming units (SFUs) per individual as measured by ELISPOT was compared between adults and children using Kruskal Wallis test. For surface staining and intracellular cytokine secretion, FlowJo software programme (Tree Star Inc., USA) was used to analyze the acquired events. The proportion of CD4 and CD8 T cells secreting IFN-γ cytokine and expressing cell surface molecules (CD28, CD152 and CD154) was compared between adults and children of different age groups (6, 12, 24, and 36 months, 5 years) using Chi-square. Cytokine levels across the different age groups were compared using Kruskal Wallis test. Dunn's post hoc test was applied to statistically significant groups to further confirm data from which reference age group differed from any other group. The relationship between multiple cytokine secretion and expression of cell surface molecules was tested using Spearmann's correlation analysis. For all statistical tests, Graphpad programme (Graphpad Prism™ La Jolla, USA) was used for analysis and any probability value less than 0.05 ($p < 0.05$) was taken as significant.

CHAPTER FOUR

4.0. RESULTS

4.1. Parasitemic and Full Hemogram Characteristics of the Study Population

Complete blood cell (CBC) counts and parasitemia were determined for all participants enrolled in the study (n = 88). The median levels with lowest and highest confidence intervals are shown in Table 1. The median parasitemia ($p = 0.0473$), white Blood Cells ($p = 0.0013$), lymphocytes ($p = 0.0009$), and monocytes ($p = 0.0435$) varied significantly between the age groups.

Table 1. Parasitemic and full hemogram characteristics of the study participants (n = 88).

Characteristic	**0.5 years** n = 17	**1.0 years** n = 16	**2.0 years** n = 19	**5.0 years** n = 18	**> 18 years** n = 18	***p* values**
Parasitemia	0 (0-3)	2 (0-91)	4 (0-1040)	2 (0-437)	0 (0-0)	**0.0413**
Hemoglobin	9.9 (6.8-12.1)	9.8 (5.8-12.1)	9.3 (4.4-10.9)	10.7 (7.7-12.9)	12.2 (6.1-14.7)	0.0898
WBC ($\times 10^3/\mu L$)	12.6 (6.2-32.5)	13.2 (6.1-20.8)	11.1 (7.0-33.3)	8.9 (4.5-12.2)	5.5 (3.5-7.9)	**0.0013**
LY# ($\times 10^3/\mu L$)	7.7 (4.4-11.9)	8.4 (4.7-13.5)	7.5 (4.5-23.4)	4.9 (2.7-7.5)	2.7 (1.5-6.2)	**0.0009**
MO# ($\times 10^3/\mu L$)	1 (0.2-1.8)	1.1 (0.5-2.3)	0.8 (0.3-1.8)	0.7 (0.2-1.1)	0.4 (0.2-0.8)	**0.0435**
GR# ($\times 10^3/\mu L$)	3.4 (0.4-8.6)	4.1 (2-7.2)	2.9 (2-8.1)	3.3 (1.7-5.8)	2.5 (1.4-4.0)	0.4076
RBC ($\times 10^3/\mu L$)	4.46 (2.33-5.28)	4.73 (3.17-5.09)	4.14 (3.98-5.46)	4.23 (3.44-5.46)	4.4 (2.47-5.46)	0.7543
Plt ($\times 10^3/\mu L$)	388 (135-562)	344 (148-515)	323 (131-732)	264 (135-553)	223 (110-346)	0.3040

Data for each age category were pooled then median levels and lower and upper confident intervals (in brackets) were calculated. Median values between groups were compared by Kruskal Wallis. Median levels of parasitemia, white Blood Cells (WBC), lymphocytes (LY#), and monocytes (Mo #) varied significantly across the age groups as shown on the table.

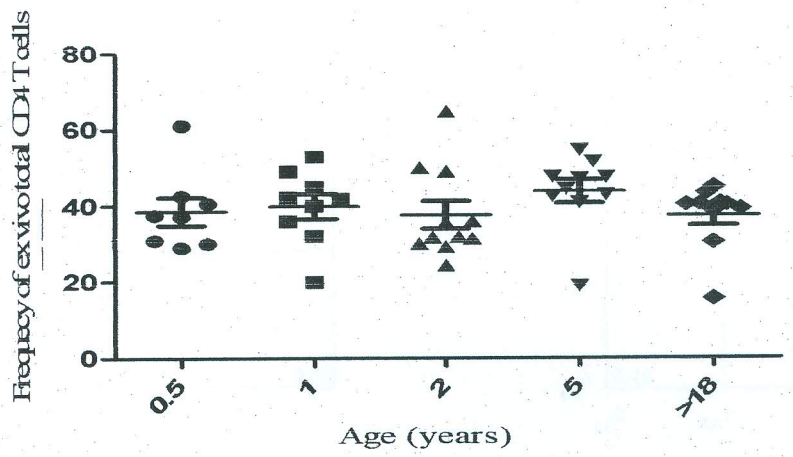
4.2. Characteristics of *Ex vivo* CD4 T Cell Subsets

The frequencies of total CD4 were measured for 88 participants and shown to be similar at approximately 40% in all age groups (Figure 2 {i}). However, adults displayed more of memory CD4 T cell phenotype (T_{CM} and T_{EM}) while infants had more naïve T cells (T_N) as illustrated in Figure 4. The frequency of CD4 T_{CM} and T_{EM} significantly increased with age (p =0.0001 and p = 0.0002, respectively) while T_N progressively decreased with age with significantly lower proportion observed in adults (p = 0.0003). The proportion of terminally differentiated memory (T_{EMRAs}) CD4 T cells were very low (less than 1% of total CD4) and similar across the age groups.

4.2. Characteristics of *Ex vivo* CD8 T Cell Subsets

Similarly, the frequencies of total CD8 T cells were measured for 88 participants and were approximately 18% and comparable between all age groups (Figure 2 {(ii}). The distribution patterns of CD8 T cell subsets were similar to those of CD4 subsets but the levels of significance were lower than those observed in CD4 compartment. As shown in Figure 5, CD8 T_{CM} and T_{EM} although seemingly higher in adults, did not reach any level of significance. But CD8 T_N frequencies reduced with age and was significantly lower in adults compared to children (p = 0.0046). However, CD8 T cells had more T_{EMRAs} as compared to almost negligible proportion of CD4 T_{EMRAs} that showed a trend of gradual increase with age though not at a significant rate (p = 0.0735). Figure 3 shows representative histograms of distribution of *ex vivo* CD4 and CD8 T cell subsets from an infant and adult.

(i)



(ii)

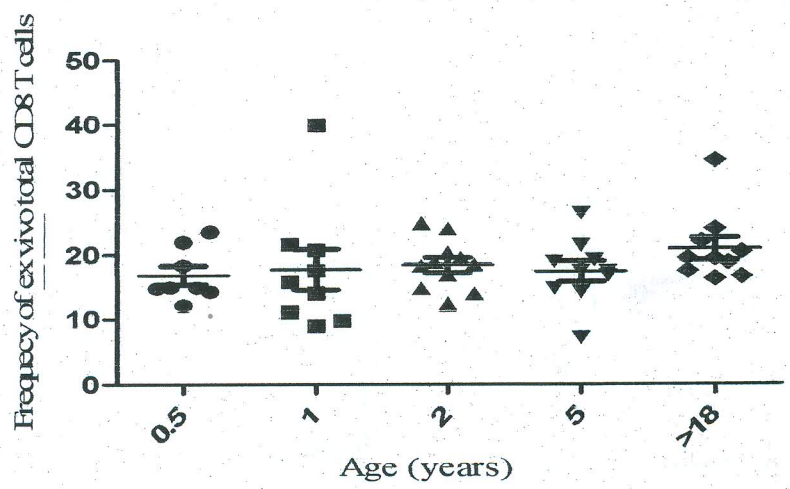


Figure 2. Proportion of total *ex vivo* (i) CD4 and (ii) CD8 T cell subsets in different age groups (n = 88). X- axis is age in years and Y-axis is the frequency of total CD4 or CD8 subsets. The median frequencies of CD4 and CD8 T cells were 40% and 18% respectively in all age groups.

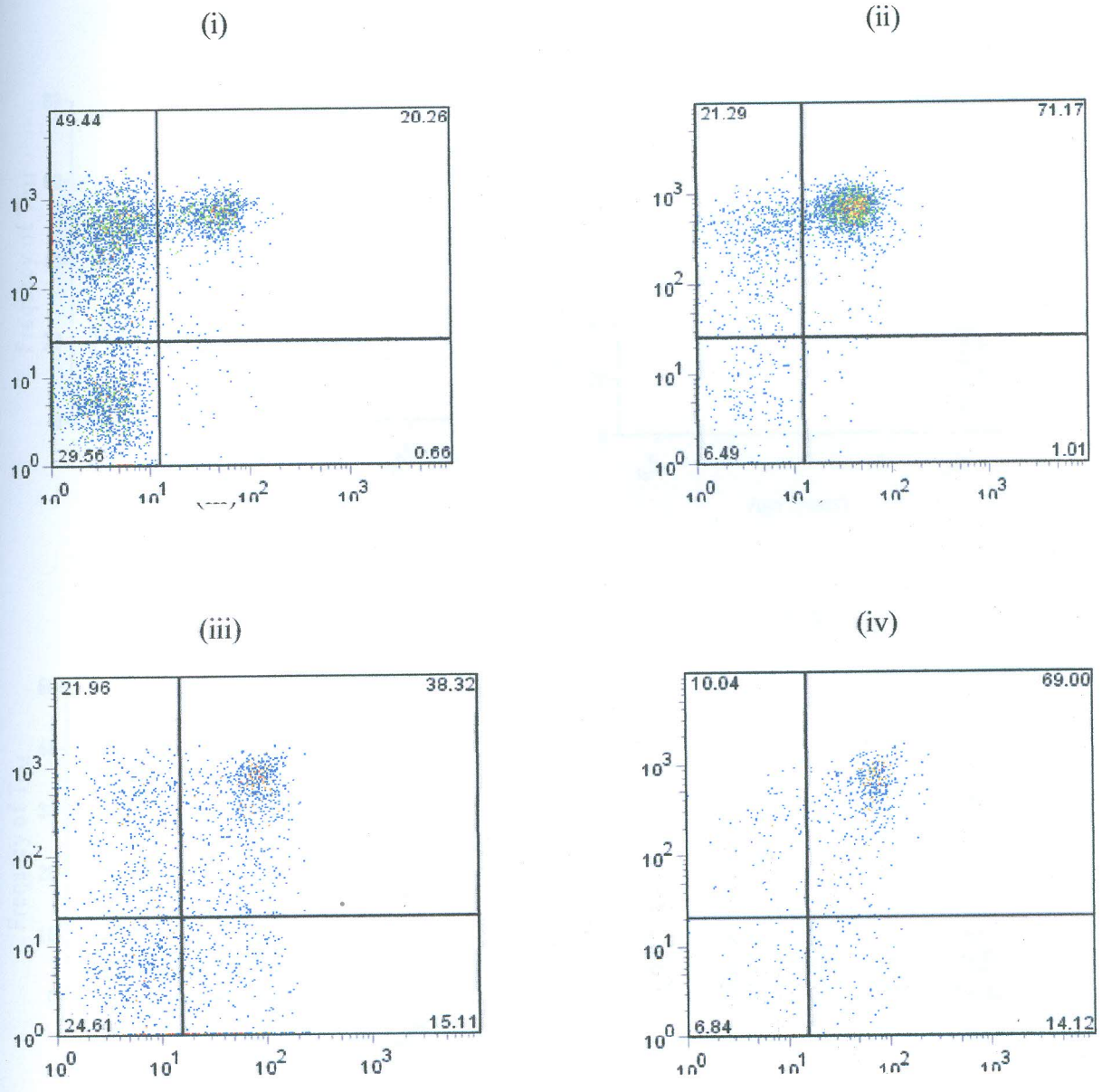


Figure 3. Representative histograms showing the proportions of CD4 and CD8 T cells subsets in adults and children. Histogram is (i)-Adult CD4, (ii)-Child CD4, (iii)-Adult CD8 and (iv)-Child CD8. CD45RAFITC is a naïve T cell receptor bound to a green fluorochrome while CD62LPE is a selectin molecule bound to a red fluorochrome.

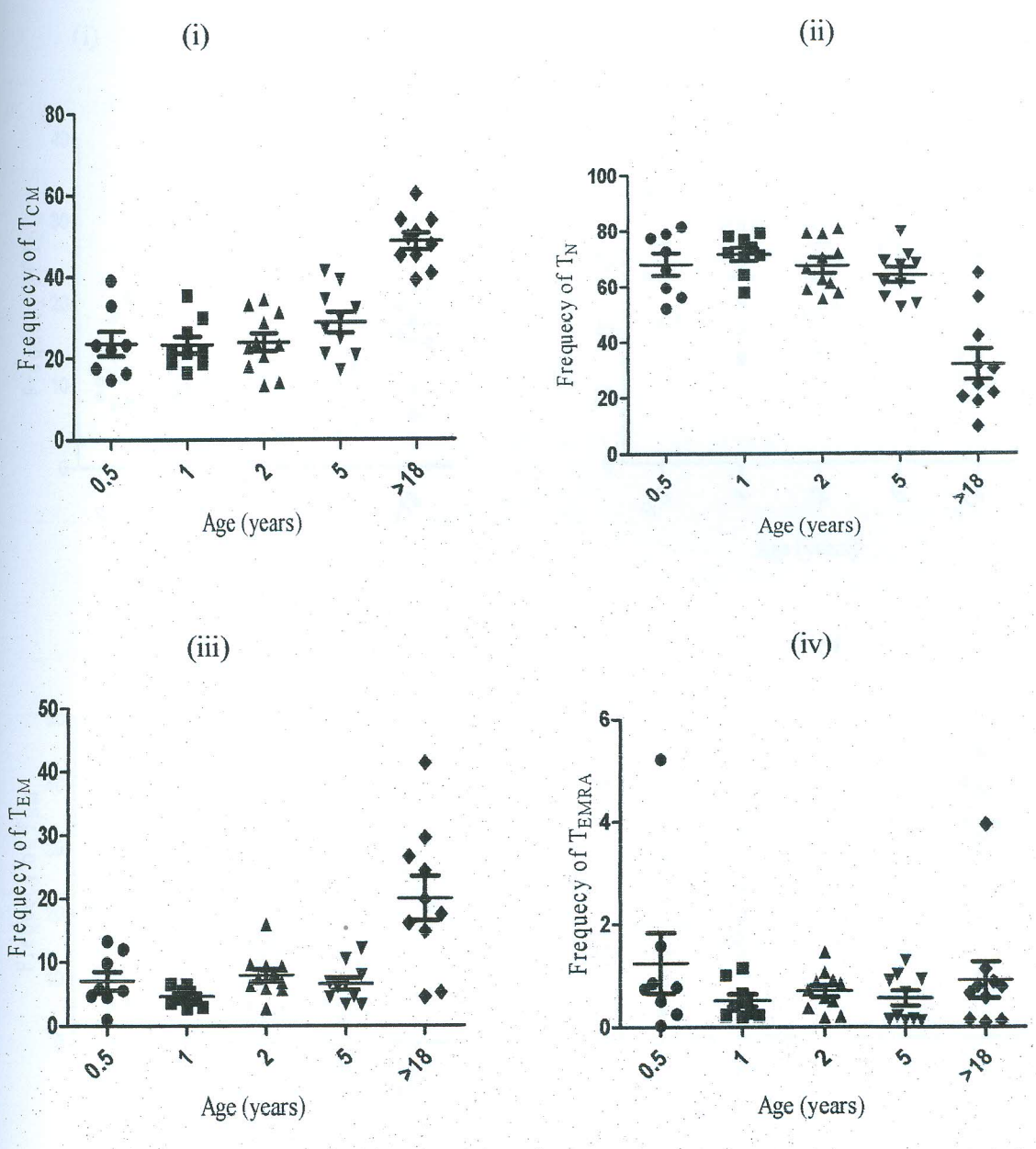


Figure 4. Proportion of *ex vivo* CD4 T cell subsets in different age groups. The frequency of CD4 T_{CM} and T_{EM} varied between different age groups (p =0.0001 and p = 0.0002, Kruskal Wallis, respectively) and were significantly higher in adults than children ($p < 0.05$, Dunn's post hoc test) while T_N were significantly lower in adults than children aged 0.5 years (p = 0.0003). The proportion T_{EMRAs}) were very low and similar in all age groups.

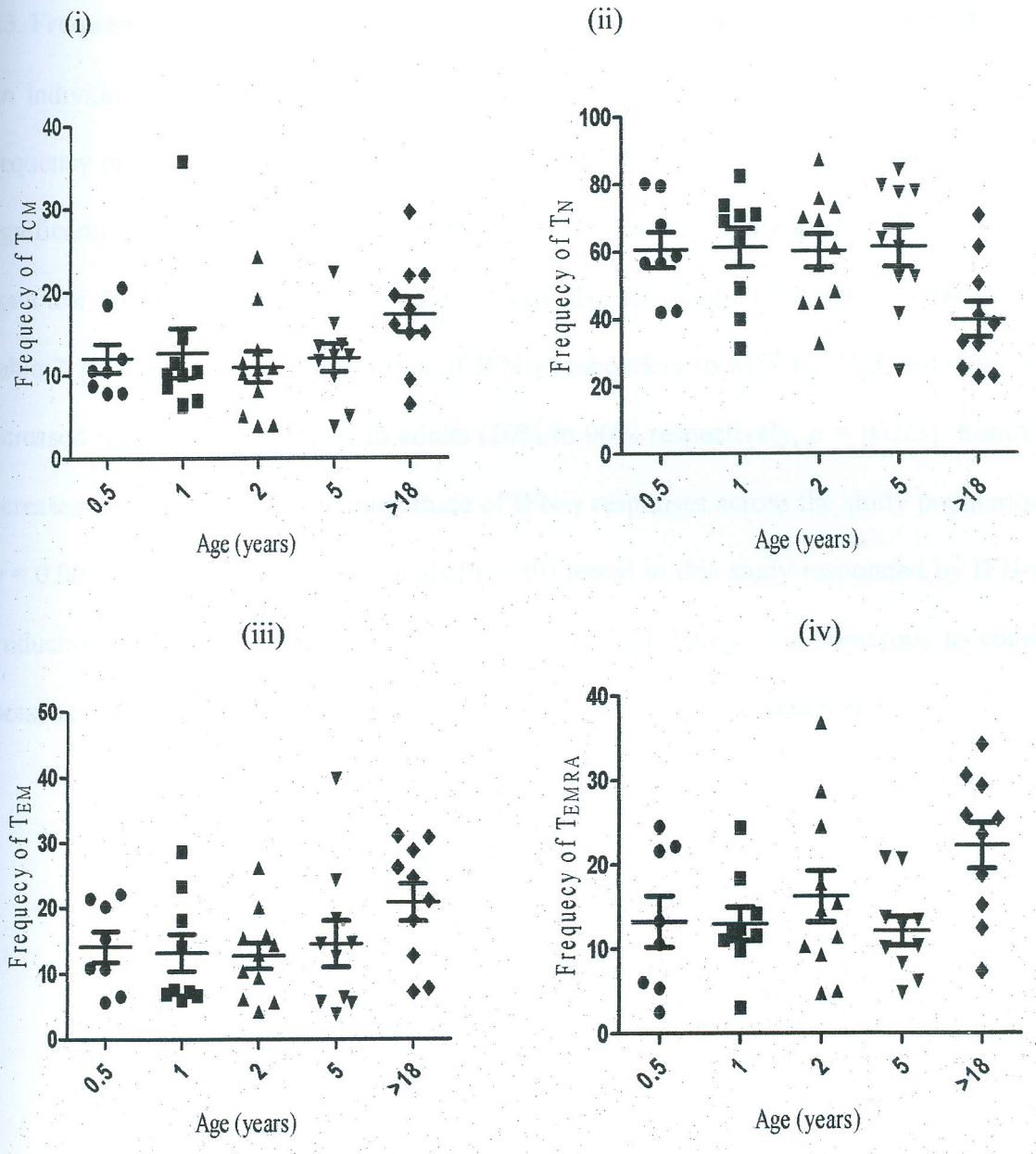


Figure 5. . Proportion of *ex vivo* CD8 T cell subsets across age groups. The frequency of CD4 T_{CM}, T_{EM} and T_{EMRAs} were similar in all age categories (All, $p > 0.05$, Kruskal Wallis) while T_N varied between age groups ($p = 0.0046$, Kruskal Wallis) and were significantly lower in adults than children aged 0.5 years ($p < 0.05$, Dunn's post hoc).

4.3. Frequency and Magnitude of IFN-γ Responses to MSP1423d7 by ELISPOT

An individual was defined as an IFN-γ responder to recombinant $MSP1_{42}$-3D7 if the frequency of the spot forming units (SFUs) per $1x10^6$ PBMCs in the stimulated well was significantly greater compared to the unstimulated PBS well using a Chi-square Fishers exact test. Most children below the age of 1 year did not secrete IFN-γ as summarized in Table 2 and Figure 6. The proportion of IFN-γ responders to $MSP1_{42}$-3D7 significantly increased with age from infants to adults (20% to 90% respectively, $p = 0.023$). Similar increases were observed in the magnitude of IFN-γ responses across the study population ($p = 0.0011$) (Figure 6). All individuals (n = 49) tested in this study responded by IFN-γ production to bacterial super antigen, SEB at very high levels (too numerous to count spots) demonstrating that both children and adults were immunocompetent.

Table 2. Proportion of IFN-γ responders to $MSP1_{42}3D7$ in each age group by ELISPOT.

Age (years)	Proportion (%) of responders	*p*
0.5	2/10 (20)	**0.023**
1	4/9 (44)	
2	5/10 (50)	
5	5/10 (50)	
>18	9/10 (90)	

Comparison of the proportion of individuals (n = 49) responding by IFN-γ production to $MSP1_{42}3D7$ in each age group varied with age (P = 0.023, Chi-square) and were significantly higher in adults than children aged 0.5 years ($P < 0.05$, Dunn's post hoc test)

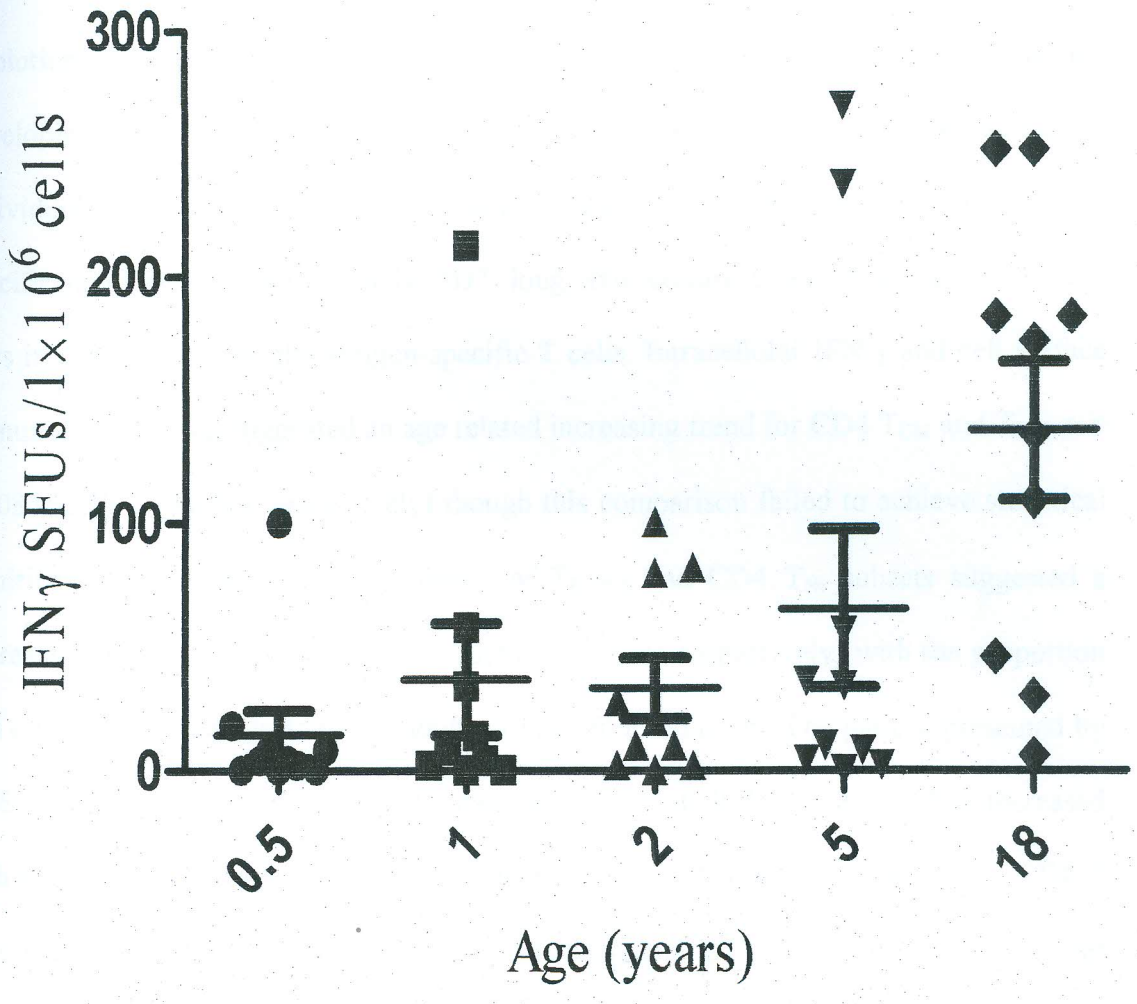


Figure 6. Magnitude of IFN-γ ELISPOT response to $MSP1_{42}$-3D7 by age. The magnitude of IFN-γ production within each age group was measured by counting the number of spot forming units (SFUs)/precursor cells per one million cells from each individual after stimulation with $MSP1_{42}$-3D7. The median SFUs were significantly different between age groups (P = 0.0011, Kruskall Wallis) and higher in adults compared to children aged 0.5 years ($p < 0.05$, Dunn's post hoc)

4.4. Development of Antigen Specific Memory T Cell Subsets

Evolution and maintenance of antigen-specific memory cells is the ultimate goal for developing a vaccine to effectively control or prevent an infectious agent. To address if individuals chronically exposed to natural *P. falciparum* infections develop antigen-specific memory T cells to $MSP1_{42}$-3D7, long term culture of PBMCs was done for 7 days in order to expand the antigen-specific T cells. Intracellular IFN-γ and cell surface immunophenotyping suggested an age related increasing trend for CD4 T_{CM} and T_{EM} (p = 0.5085 and p = 0.0999, respectively) though this comparison failed to achieve statistical significance. In contrast, the proportion of T_{EMRA} and CD4 T_{Ns} subsets suggested a decreasing trend with age (p = 0.1578 and p = 0.0119, respectively) with the proportion of T_N being significantly lower in adults compared to children. The pattern presented by CD8 T_{CM} was comparable across the age groups (p = 0.4678), however T_{EM} increased with age and approached borderline significance (p = 0.081). Both T_N and T_{EMRAs} CD8 T subsets appeared to decrease with age and the frequency of T_{EMRA} was generally higher than for CD4 across age groups (p = 0.2460 and p = 0.2565, respectivelly), however these observations failed to achieve statistical significance. These results are summarized in Figures 7 and 8.

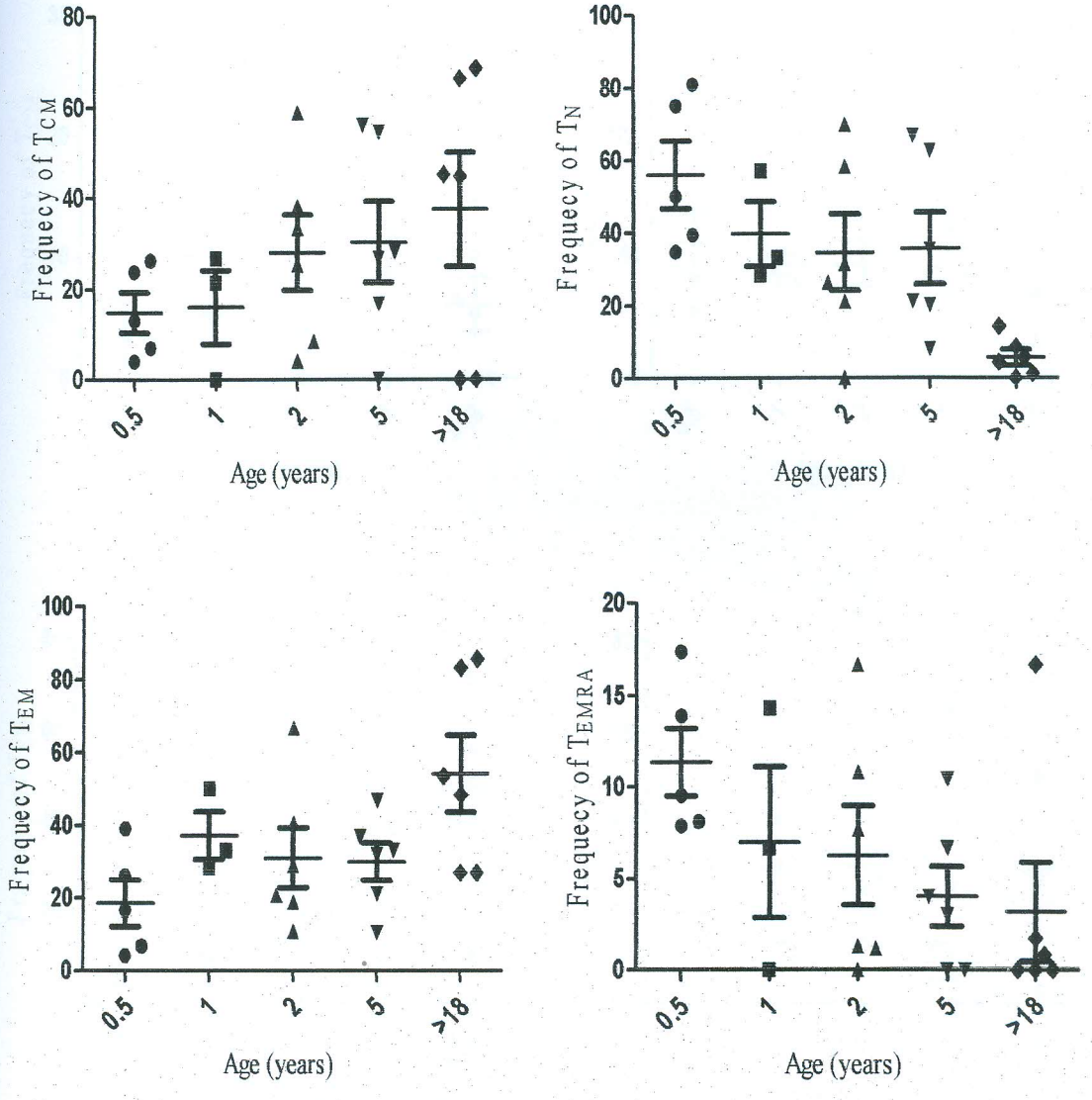


Figure 7. Antigen specific CD4 T cell response to $MSP1_{42}$-3D7. The percentage of CD4 T cell subsets responding by IFN-γ secretion to $MSP1_{42}$3D7 was measured in adults and children (n = 26) by surface and intracellular staining after 7 days of PBMC's culture. The median frequencies of IFN-γ response from T_{CM}, T_{EM}, and T_{EMRA} were similar ($p > 0.05$, Kruskal Wallis), but T_N varied ($p = 0.0119$, Kruskal Wallis) between different age groups. Adults had lower frequencies of T_N than children aged 0.5 years ($p > 0.05$, Dunn's post hoc).

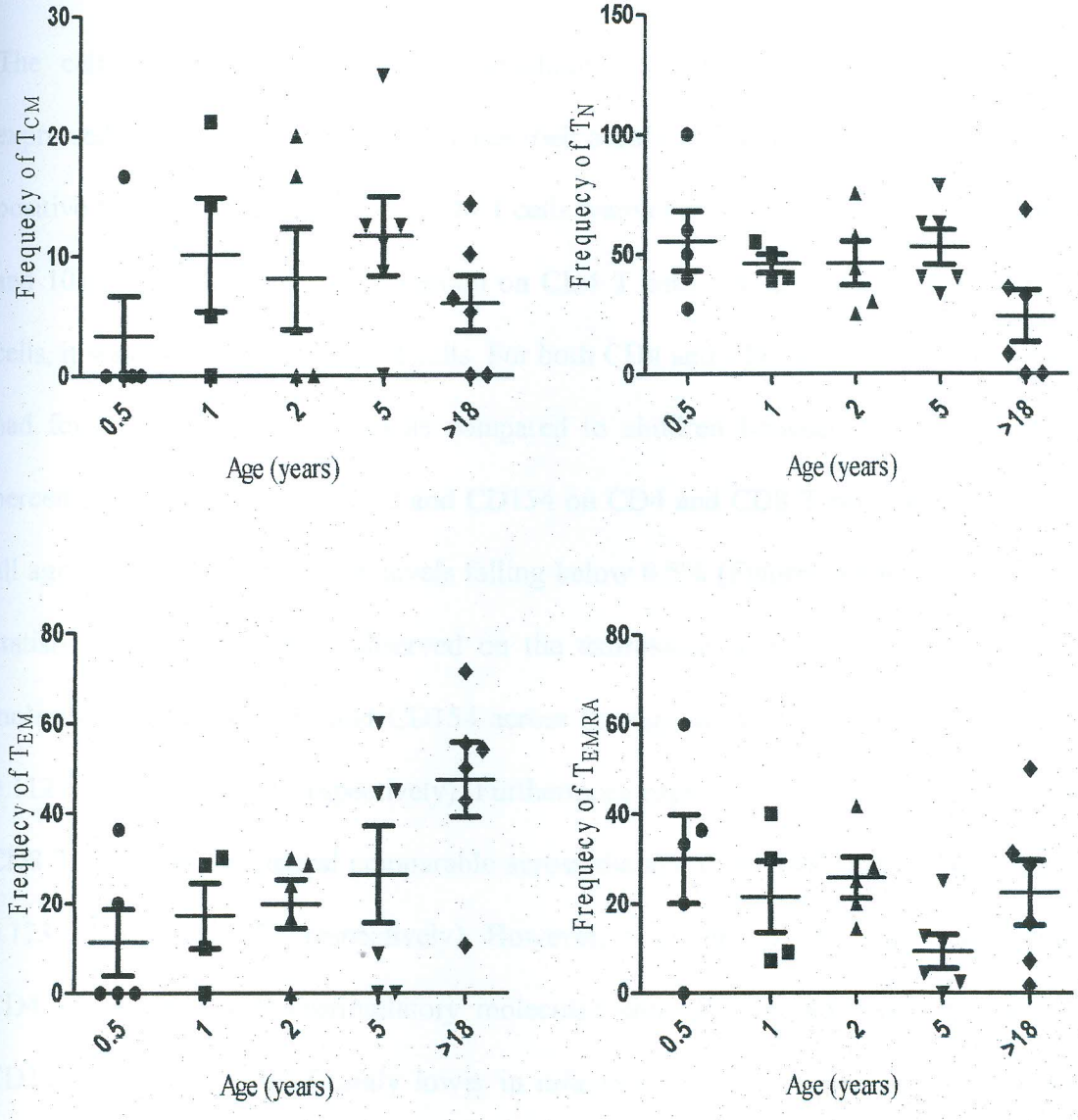


Figure 8. Antigen specific CD8 T cell response to $MSP1_{42}$-3D7. The percentage of CD8T cell subsets responding by IFN-γ secretion to $MSP1_{42}$3D7 was measured in adults and children (n = 26) by surface and intracellular staining after 7 days of PBMC's culture. The median frequencies of IFN-γ response from T_{CM}, T_{EM}, T_N, and T_{EMRA} were similar ($p > 0.05$, Kruskal Wallis) between different age groups.

4.5. Surface Expression of T Cell Costimulatory Molecules

The cell surface expression of costimulatory molecules (CD28, CD152, CD154) expressed on T cell subsets may influence their ability to respond to antigen. More CD28 positive CD4 T cells compared to CD8 T cells were observed in all age groups (Figures 9 and 10). The median CD28 expression on CD4 T cells was over 95% while in CD8 T cells, it was expressed by 50% of cells. For both CD4 and CD8 T cells, infants and adults had fewer CD28 positive cells as compared to children between 1 and 5 years. The percentage expression of CD152 and CD154 on CD4 and CD8 T cells were very low in all age groups with all median levels falling below 0.5% (Figures 9 and 10). Overall, no statistical differences were observed on the expression of CD4 T cell costimulatory molecules; CD28, CD152 and CD154 across the age groups (Kruskal Wallis test, p = 0.1121; 0.3374; 0.5262, respectively). Furthermore expression of CD28, 152 and 154 on CD8 T cells were statistical comparable across the age groups (Kruskal Wallis test, p = 0.1235; 0.5370; 0.5357, respectively). However, evaluation of the percentage ratios of CD4 and CD8 T cell costimulatory molecules showed that only the ratio of CD4 CD154/CD152 was significantly lower in infants and adults compared with other age groups (p = 0.032). No statistical difference was observed in the ratio of all other molecules (all, $p > 0.05$, Figures 11 and 12). Moreover, correlation between the three different costimulatory molecules revealed non-significant association across the age groups for all the molecules evaluated except for CD8, CD152 and CD154 (p = 0.0163) (Tables 3 and 4).

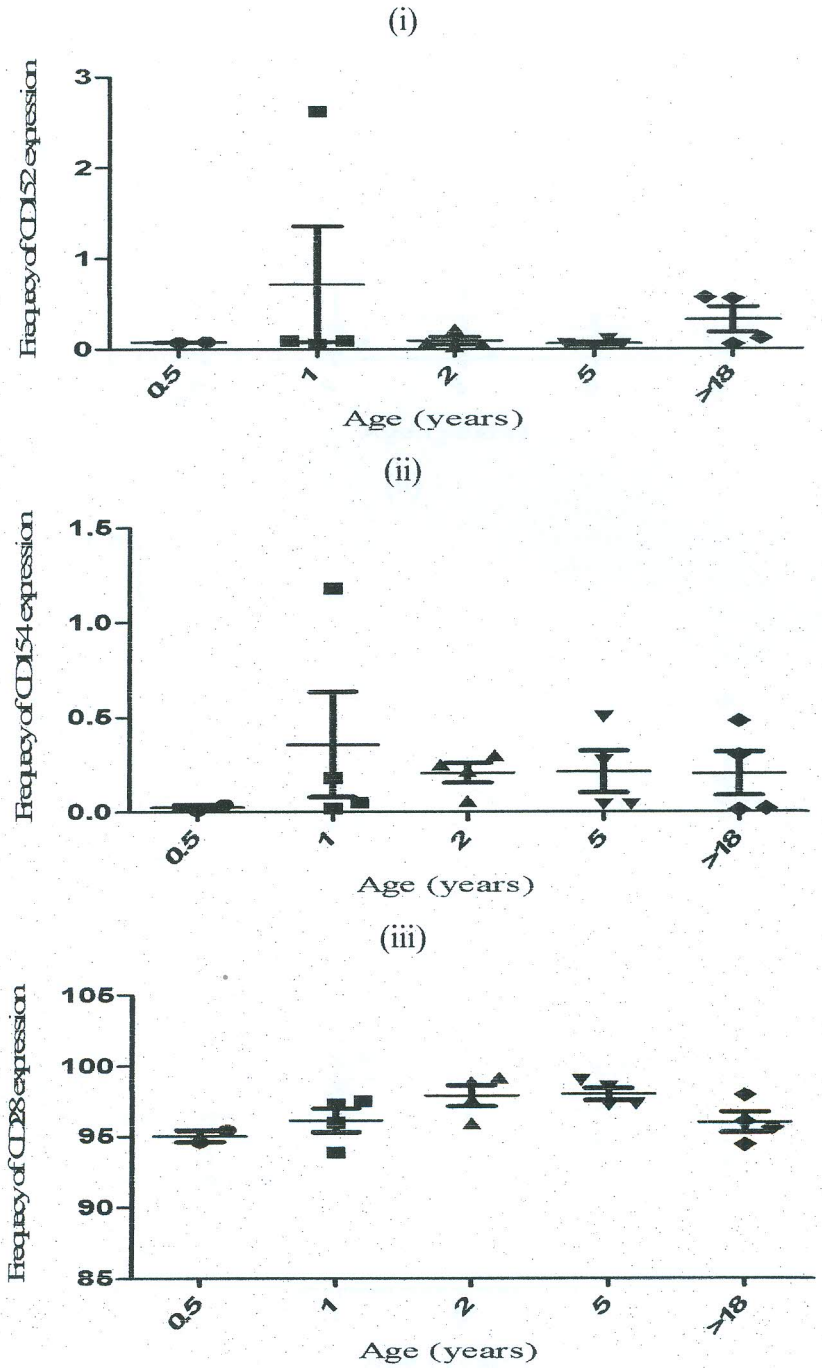
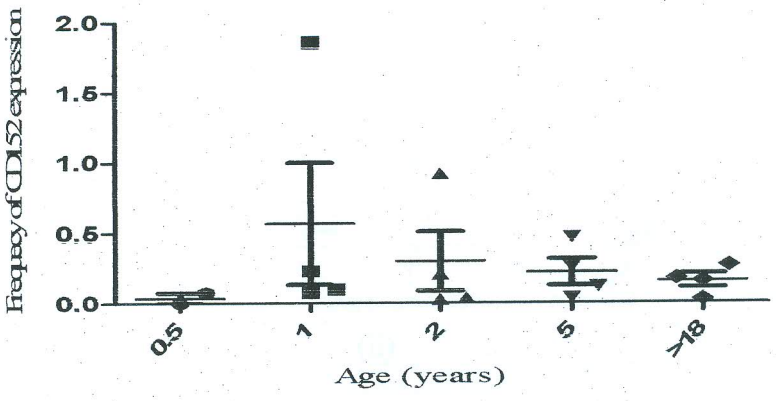
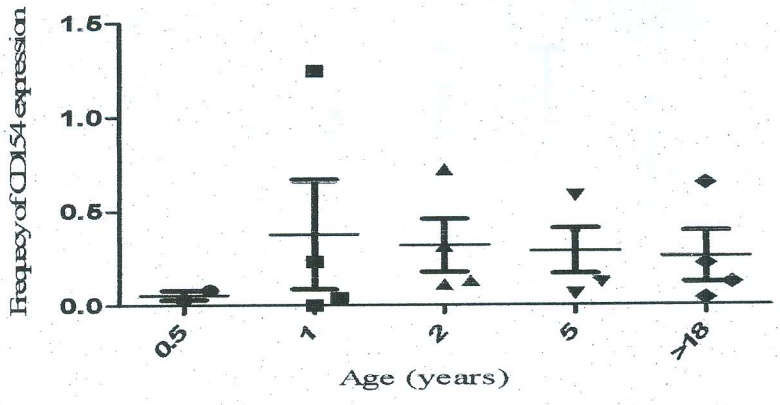


Figure 9. Antigen specific surface expression of CD4 T cell costimulatory molecules upon activation with $MSP1_{42}$3D7. Surface expression of CD28, CD154 and CD 154 was measured after stimulation of PBMC's with recombinant $MSP1_{42}$3D7 and were found to be similar ($P > 0.05$, Kruskal Wallis) between all age groups.

(i)



(ii)



(iii)

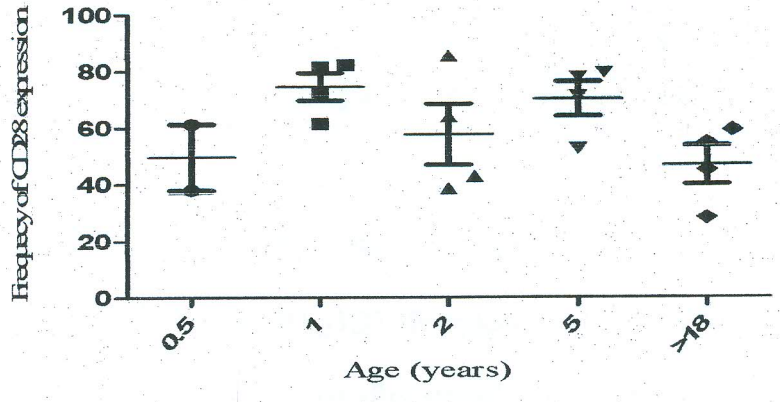
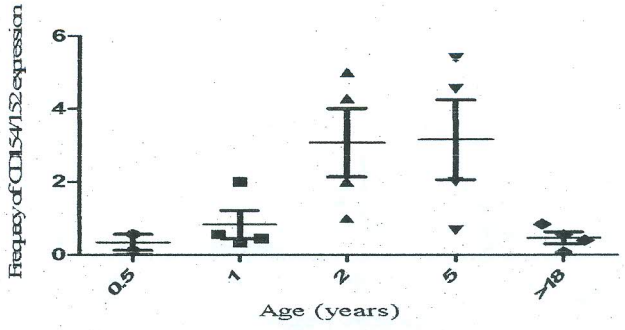
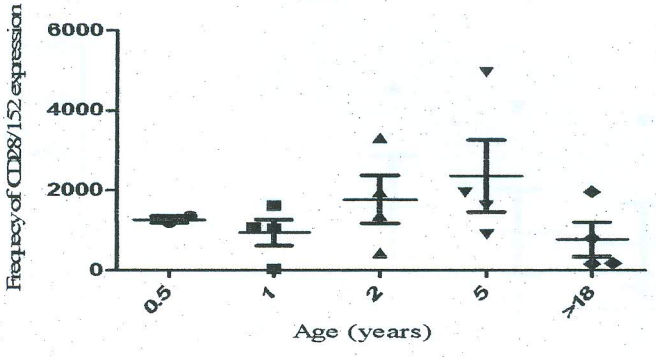


Figure 10. Antigen specific surface expression of CD8 T cell costimulatory molecules upon activation with MSP1$_{42}$3D7. Surface expression of CD28, CD154 and CD 154 was measured after stimulation of PBMC's with recombinant MSP1$_{42}$3D7 and were found to be similar ($P > 0.05$, Kruskal Wallis) between all age groups.

(i)



(ii)



(iii)

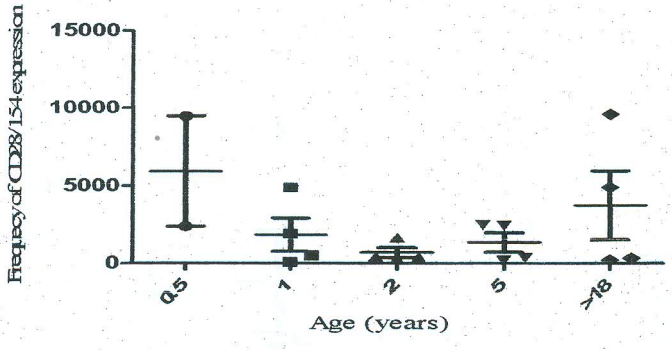


Figure 11. Ratios of antigen specific surface expression of CD4 T cell costimulatory molecules upon activation with $MSP1_{42}3D7$. Frequency ratios of surface expression of CD28, CD152 and CD 154 was measured after stimulation of PBMC's with recombinant $MSP1_{42}3D7$ and were found to be similar ($P > 0.05$, Kruskal Wallis) except for CD154 to CD 152 that varied ($p = 0.032$) between age groups. CD152 was highly expressed ($P < 0.05$, Dunn's post hoc) in adults and children aged 0.5 years compared to children between 1 and 5 years.

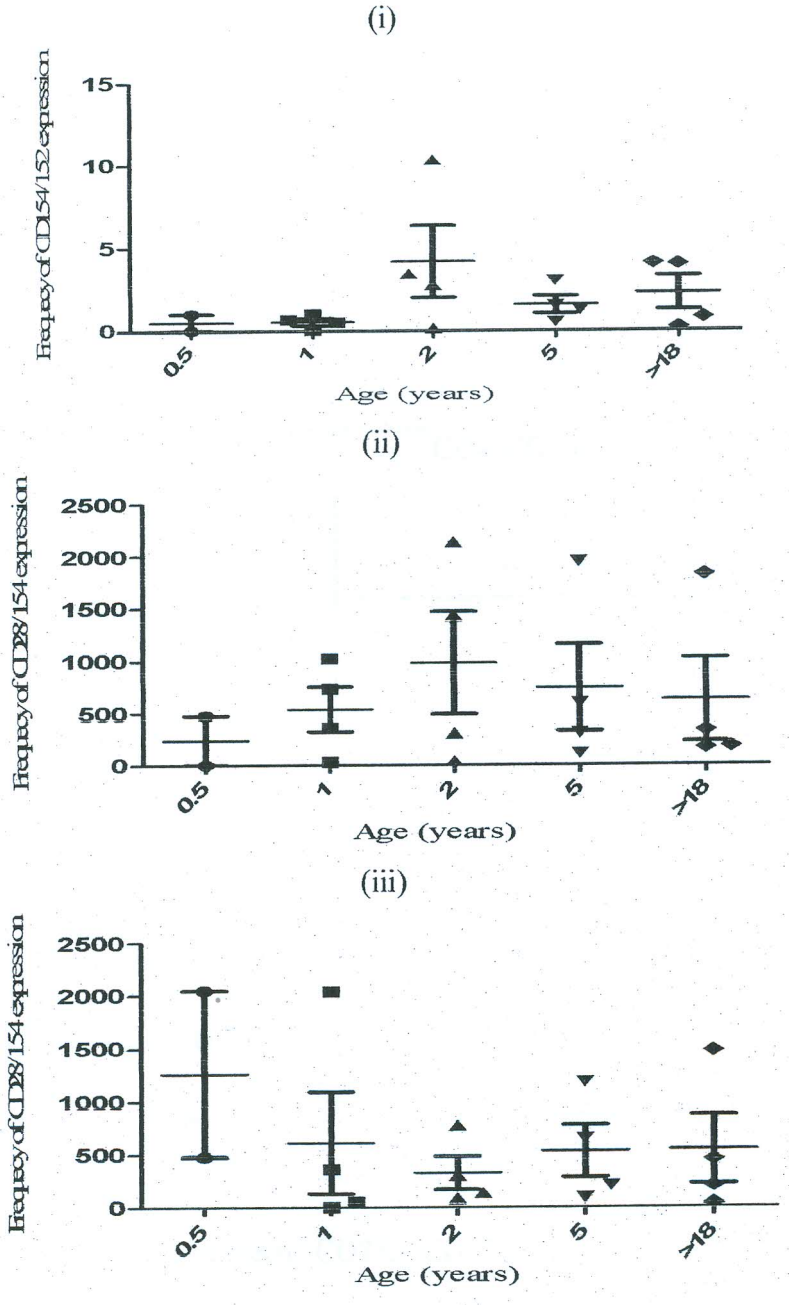


Figure 12. Ratios of antigen specific surface expression of CD8 T cell costimulatory molecules upon activation with $MSP1_{42}$3D7. Frequency ratios of surface expression of CD28, CD152 and CD 154 was measured after stimulation of PBMC's with recombinant $MSP1_{42}$3D7 and were found to be similar ($P > 0.05$, Kruskal Wallis) between all the age groups.

Table 3. Relationship in surface expression of CD4 T cell costimulatory molecules in children and adults (n = 26).

	CD4-CD152	CD4-CD154	CD4-CD28
CD4-CD152	r = 1.000		
CD4-CD154	r = 0.4293 p = 0.0754	r= 1.000	
CD4-CD28	r = -0.4164 p = 0.0857	r= 0.0963 p = 0.7038	r= 1.000

Coefficient of correlation between CD28, CD152 and CD154 was measured by Spearman's correlation and was found to be similar ($P > 0.05$) for all the molecules between the age groups.

Table 4. Relationship in surface expression of CD4 T cell costimulatory molecules in children and adults (n = 26).

	CD8-CD152	CD8-CD154	CD8-CD28
CD8-CD152	r = 1.000		
CD8-CD154	r = 0.5572 p = **0.0163**	r= 1.000	
CD8-CD28	r = -0.1075 p = 0.6712	r= -0.2252 p = 0.3689	r= 1.000

Coefficient of correlation between CD28, CD152 and CD154 was measured by Spearman's correlation and was found to be similar ($P > 0.05$) for all the molecules except CD154 and CD152 that positively correlated ($p = 0.0163$) between the age groups.

4.6. Th1 Cytokine Responses to *P. falciparum* MSP-1_{42}3D7

To evaluate the role of Th1 cytokines in the development of immunity to blood stage *P. falciparum* malaria, the frequency and levels of IL-2, IL-12, GM-CSF, IFN-γ and TNF-α were measured in culture supernatants in an age stratified population exposed to holoendemic malaria. Although the frequencies and levels of all the cytokines to MSP-1_{42}3D7 were similar across the age groups, individual cytokine levels demonstrated unique patterns across the age groups (Figure 13). For all Th1 cytokines, there was minimal response in children aged 6 months. Th1 cytokines levels were the highest in children aged 1 year. IFN-γ levels decreased by the age of 2 years but gradually increased from 3 years to adulthood. There was progressive decrease of TNF-α levels from 1 year to adulthood. Both IL-2 and IL-12 had similar but invariable patterns across the age groups.

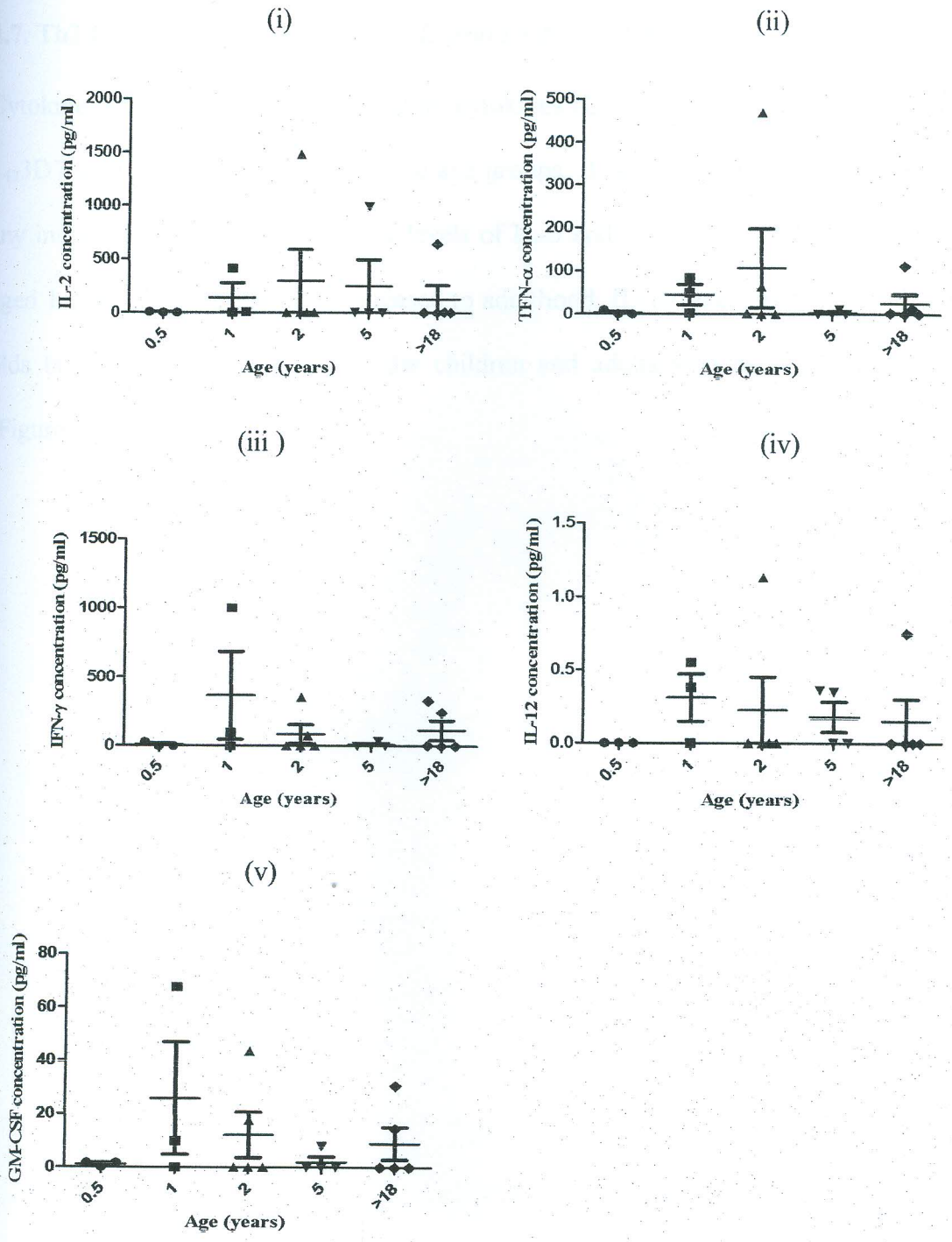


Figure 13. Th1 cytokine response to *P. falciparum* MSP-1_{42}3D7 in children of different age groups and adults. There were no significant differences in the levels of IL-2 (i), TNF-α (ii), IFN-γ (iii), IL-12 (iv) and GM-CSF (v), ($P > 0.05$, Kruskal Wallis), between different age groups.

4.7. Th2 Cytokine Responses to *P. falciprum* MSP-1_{42}3D7

Cytokine responses of anti-inflammatory cytokines IL-4, IL-5, IL-10 and IL-13 to MSP-1_{42}3D7 were also compared across the age groups. IL-4 responses were not detected in any individuals investigated. Higher levels of IL-5 and IL-13 were observed in children aged 1 year that progressively decreased to adulthood. IL-10 levels were lower in 2 year olds but gradually increased in older children and adults who had the highest levels (Figure 14).

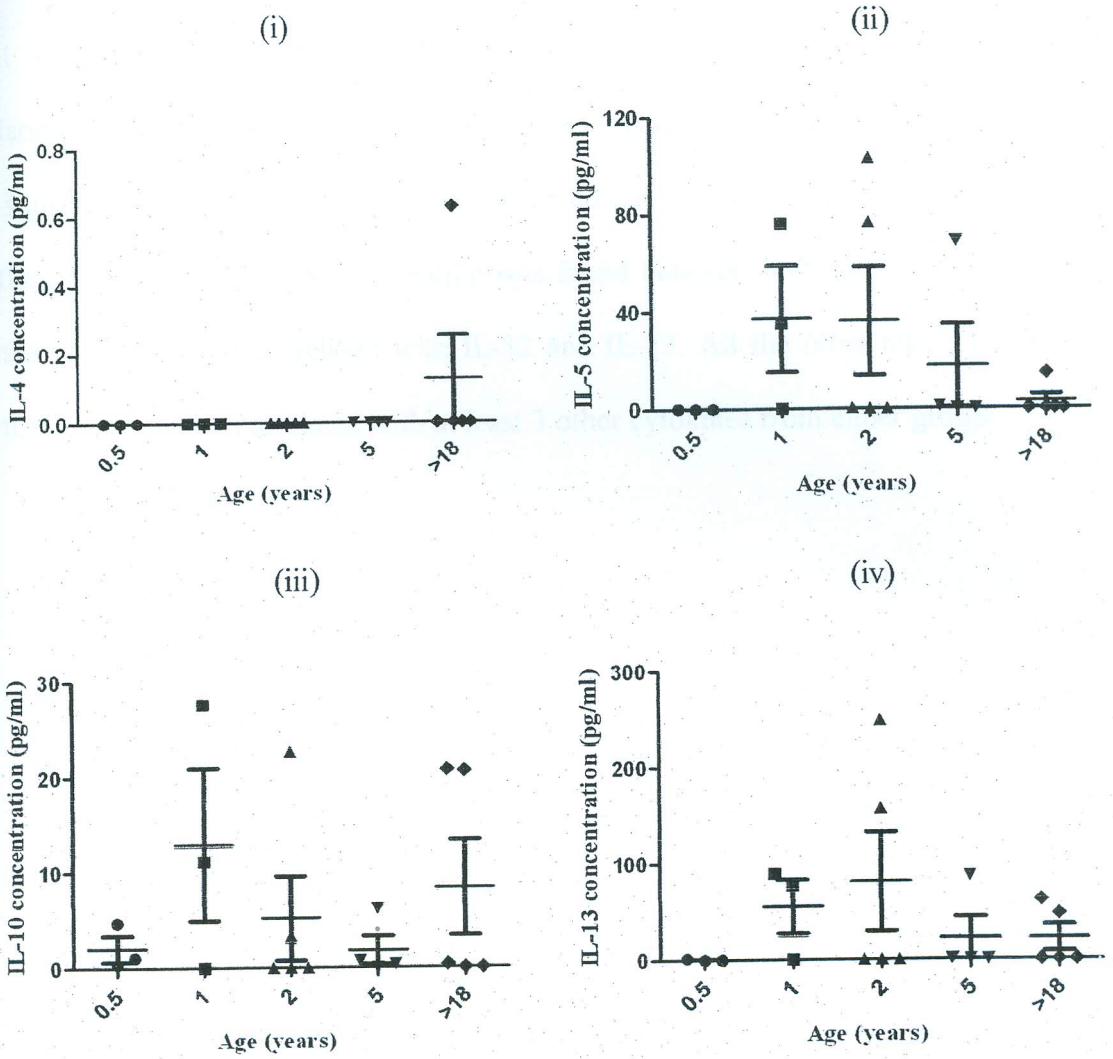


Figure 14. Th2 cytokine responses to *P. falciparum* MSP-1_{42}3D7 in children of different age groups and adults. There were no significant differences in the levels of IL-4 (i), IL-5 (ii), IL-10 (iii), and IL-13 (iv), (all, $P > 0.05$, Kruskal Wallis), between different age groups.

4.8. Correlation of Th1 and Th2 Cytokine Levels

All samples were pooled and Spearman's correlation was used to determine the relationship between different pro- and anti-inflammatory cytokines. As shown in Table 5, significant positive correlations were observed within and between some pro- and anti-inflammatory cytokines. No correlation was found between IL-4 and both Th1 and Th2 cytokines. IL-2 only correlated with IL-12 and IL-13. All the other nine Th1 and Th2 cytokines evaluated correlated with at least 3 other cytokines from either group.

Table 5. Correlation of Th1 and Th2 cytokine response to MSP-1_{42}3D7 across the age groups.

	IL-4	IL-2	TFN-α	IFN-γ	IL-10	IL-12	1L-13	GS-CSF	IL-5
-4	r=1.000								
-2	r=0.3188 *p* =0.1707	r=1.000							
FN-α	r=0.1878 *p* =0.4279	r=0.2823 *p* =0.2279	r=1.000						
N-γ	r=0.3266 *p* =0.1599	r=0.2647 *p* =0.2594	r=0.9202 ***p*=0.0000**	r=1.000					
-10	r=0.2646 *p* =0.2595	r=0.3667 *p* =0.1118	r=0.7344 ***p*=0.0002**	r=0.8473 ***p*=0.0000**	r=1.000				
-12	r=0.4169 *p* =0.0674	r=0.5160 ***p* =0.0199**	r=0.5639 ***p*=0.0096**	r=0.6829 ***p*=0.0009**	r=0.6043 ***p*=0.0048**	r=1.000			
-13	r=0.1816 *p* =0.4436	r=0.4555 ***p* =0.0436**	r=0.6287 ***p*=0.0030**	r=0.7112 ***p*=0.0004**	r=0.7484 ***p*=0.0001**	r=0.6670 ***p*=0.0013**	r=1.000		
M- SF	r=0.2395 *p* =0.3091	r=0.1996 *p* =0.3988	r=0.8477 ***p*=0.0000**	r=0.9135 ***p*=0.0000**	r=0.8793 ***p*=0.0000**	r=0.6149 ***p*=0.0039**	r=0.6870 ***p*=0.0008**	r=1.000	
-5	r=0.1839 *p*=0.4376	r=0.3417 *p* =0.1403	r=0.4229 *p*=0.0632	r=0.5648 ***p*=0.0095**	r=0.4854 ***p*=0.0301**	r=0.7257 ***p*=0.0003**	r=0.8030 ***p*=0.0000**	r=0.4964 ***p*=0.0260**	r=1.00

Cytokine levels from all the age groups were pooled together and the relationship between different cytokines was measured by Spearman's correlation. There was positive correlation ($P < 0.05$) between various cytokines as shown in bold in the table.

CHAPTER FIVE

5.0. DISCUSSION

Adults and older children residing in regions of perennial and intense *P. falciparum* transmission acquire immunologic protection after cumulative exposure as demonstrated by a decreasing incidence of the severe manifestations of malaria infections and lower parasite densities with age. Furthermore, adults acquire immunity more rapidly than children under the same exposure conditions (Baird, 1998). These epidemiologic observations leave the burden of malaria-associated morbidity and mortality to be carried by infants and young children in these areas (Rogier, 2000). Antibodies against malaria have been extensively studied and are associated with exposure and in some cases protection from infection and disease (Hogh, 1996). However, there remains a gap in our understanding about the role of T cell immunity, especially in protection against blood stage malaria infections.

5.1. Parasitemia and Hematologic Indices of the Study Population

Development of immunity to malaria is accompanied by progressive changes in the level of parasitemia with age and/or exposure. In the present study, there was very low parasite densities in children aged below 0.5 years and in adults compared to other groups of children. This could be due to ability of adult immune system to vigorously mount specific cell and antibody mediated immune responses. However young children may have been protected by maternal IgG antibodies specific to malaria antigens that crossed the placenta during pregnancy (Tena-Tomás *et al.*, 2007).

Although measurement of the hematologic indices was not a major goal of this study, it is well established that hematological indices are important laboratory parameters for clinical evaluation of health and disease progression. These variables may change with age, gender, race, environmental and genetic factors (Saxena and Wong, 1990). The available World Health Organization reference standard values for these parameters are mainly based on the established data collected from European and North American populations (Saxena and Wong, 1990). There have been attempts to measure and set up standard values in a few studies in African populations. In The Gambia, the Hb levels in children aged 0.5 to 6 years and in adults were 8.7 to 13.4 and, 9.8 to 16.6 respectively. In the present study, the median Hb levels in children aged 0.5 to 5 years ranged between 9.3 and 10.7, falling within the range of values established by W.H.O and other regional studies (Nicholson and Pesce, 2000; Lugada *et al.*, 2004). Moreover in the present study, levels of WBC, monocytes, and platelets were shown to significantly decrease with age corroborating results from other studies done in African populations (Lugada *et al.*, 2004; Adetifa *et al.*, 2008). It should be noted that in the present population, there was a limitation in measuring various nutritional deficiencies and other environmental factors that could have influenced the observed changes in the levels of various hematological indices.

5.2. Development of Global Memory T Cell Subsets

In order to better characterize the immune components that constitute protective immunity, this thesis first examined *ex vivo* T cells phenotypes in a cross-section of children and adults living in a holoendemic *P. falciparum* area in western Kenya. As

expected, adults had more mature central and effector T cell memory subsets as compared to children whose T cells were still immunologically naïve. The proportion of central and effector memory T cells increased with age and formed the majority of the T cell compartment in adults as compared to terminally differentiated and naïve T cells found in young children. Central and effector memory CD4 T cells significantly increased with age (p = 0.0001 and p = 0.002, respectively) while naïve T cells were infrequently found in adults (p = 0.0003). The observed differences in the proportion of *ex vivo* CD4 T subsets corroborate the results from other studies comparing adults and children above five years of age (Jackola *et al.*, 1994; Saule *et al.*, 2006). The decline in the T_N proportion with age could in part be due to attrition of the thymus with age leading to reduction in production of T_N. Differences in the actual rate of T cell maturation in infants and young children chronically or repeatedly exposed to numerous pathogens, such as malaria and schistosomiasis, compared to children not exposed to such infections remains unknown. However, we can speculate that persistent exposure to pathogens early in life could lead to earlier evolution of antigen-specific T cell maturation to aid in protection against infection and disease.

The role of memory CD4 T cell subsets in promoting other immune cells, such as antibody producing memory B cells, could be enhanced by T cell differentiation; whereas the role of CD8 T cell memory subsets may be involved in more direct cytotoxic activity in protective immunity. Unlike CD4 T cells, the frequencies of CD8 T_{CM} and T_{EM} were similar across the age groups but adults had significantly low T_N (p = 0.004). The difference in the patterns observed between CD4 and CD8 memory T cell subsets is suggestive of intrinsic variations in the regulatory mechanisms and functions of these

cells. The frequencies of T_{EMRAs} in the CD4 compartment were very low compared to higher proportions in the CD8 compartment which gradually increased with age. The higher frequencies of CD8 T_{EMRA} suggest that as people get exposed to a variety of infectious agents with age, CD8 T cells may be called upon for immediate effector function, compared to CD4 T cells, in order to fight the infection.

5.3. Interferon Gamma Specific Responses to *P. falciparum* MSP1$_{42}$3D7.

Interferon gamma is an essential cytokine in the resolution of *Plasmodium* infection in humans and animal models (Luty *et al.*, 1999; Taylor-Robinson and Phillips, 1992). It works synergistically with other cytokines and nitric oxide to eliminate parasites (Kern *et al.*, 1989). This study evaluated the age-dependent difference in the frequency and magnitude of IFN-γ secreted in response to MSP1$_{42}$-3D7, a leading blood stage malaria vaccine candidate. It was observed that adults had significantly higher levels and frequency ($p = 0.0011$ and $p = 0.023$ respectively) of IFN-γ secreting PBMC compared to children. The median SFU/1*10^6 PBMCs increased from 5 to 155 across the age groups. The magnitude of responses generated from natural exposure are comparable to those induced through immunization of naïve individuals with MSP-1$_{42}$ which detected mean values of 40 to 60 IFN-γ SFU/10^6 PBMCs (Huaman *et al.*, 2008). Age-related increases in IFN-γ have been observed in several other studies of individuals from malaria endemic areas. African children from malaria endemic areas presenting with mild disease were more efficient producers of IFN-γ when stimulated with sporozoite or merozoite antigen peptides, suggesting an association between antigen-specific IFN-γ production and reduced pathology (Luty *et al.*, 1999). Fewer CD4 IFN-γ producing cells in Gabonese children with acute malaria were associated with hyperparasitemia (Winkler *et al.*, 1999).

Prospective studies in young children from a malaria endemic areas in West Africa and Papua New Guinea reported age–related increases in IFN-γ secretion to MSP-1 (Riley *et al.*, 1992; al-Yaman *et al.*, 1997). Furthermore, higher prevalence and magnitude of IFN-γ were observed at the end of rainy season (transmission season) than dry season in seasonally endemic area of Gambia (Riley *et al.*, 1993). Together, these studies support a role for IFN-γ in the development of clinical immunity against malaria.

5.4. Development of *P. falciparum* $MSP1_{42}$ Specific Memory T Cell Subsets

Evidence to support the existence of immunologic memory to *P. falciparum* remains somewhat circumstantial and controversial. Epidemiologic studies describe a 'waning' of immunity within a year after people leave malaria endemic areas rendering them susceptible to infection and illness upon re-exposure (reviewed in Baird, 1998). This may be due to a lack of immunologic 'boosting' necessary to maintain malaria-specific T cells and supports the notion that malaria immunity may be short-lived (Achtman *et al.*, 2005). Demonstration of immunologic memory to malaria is mainly supported by serologic studies (Wang *et al.*, 2003; Woehlbier *et al.*, 2006; Yazdani *et al.*, 2006). However, antibody levels may be a more accurate measurement of malaria exposure history as opposed to the means by which protection is conferred. Although it is well accepted that memory T cells are an important component for protection against infection (Esser *et al.*, 2003; Tsuji *et al.*, 1994), there is limited information on memory T cells specific to malaria (Achtman *et al.*, 2005). Recently, one paper attempted to dissect the *Nature* of memory T cell population in malaria-naïve North Americans vaccinated with the 3D7 and FVO strains of $MSP1_{42}$ and demonstrated the induction of memory CD4 T

cells (Huaman *et al.*, 2008). Studies on *P. vivax* also show that individuals residing in *P. vivax* malaria endemic areas have an increased proportion of memory CD8 T cells subsets (Jangpatarapongsa *et al.*, 2006) supporting our premise that T cell immunity is as important as antibody responses for protection against malaria.

In order to address this gap in our knowledge, the present study assessed T cell immunity in individuals naturally exposed to *P. falciparum* infections focusing on responses to the dominant merozoite surface protein and a leading vaccine candidate, $MSP1_{42}$-3D7. Memory T cell population were defined based on intracellular detection of IFN-γ and surface expression of CD45RA and CD62 ligand. These molecules have been shown to be differentially expressed during T cell maturation and activation hence discriminates between T cell subsets (Sallusto *et al.*, 1999b). Results showed that CD4 T cells secrete IFN-γ in response to $MSP1_{42}$-3D7 and the frequencies of MSP1-specific central and effector memory phenotypes increased in an age-dependent manner. Accumulation of MSP-1 specific T_{CM} and T_{EM} within the CD4 compartment with age suggests that these subsets are associated with the development of protective immunity. As individuals age, there is general convolution of the thymus leading to reduced production of naïve T cells. The unexpected observation of IFN-γ being generated from phenotypically naïve T cells in response to MSP-1 stimuli from children but not adults could be an indication that malaria is driving an effector function from a population of transitional T cells. Whether these T cell differentiate into memory T cells or undergo apoptosis after parasitemia is cleared is unknown. Longitudinal cohort studies would be required to shed light on this phenomenon.

Previous studies have demonstrated the role of CD4 and CD8 T cells in regulation of asexual blood stages with clear indication that CD4 is the dominant effector cell (Podoba and Stevenson, 1991). The age-related inconsistencies observed in the frequencies of CD8 T_{CM} and T_{EM} in the present study lend support to the limited role of CD8 in development of immunity to blood stage infection. The observed trend of higher percentage of CD4 T_{EMRAs} in children compared to adults suggest that chronic exposure to blood stage *P. falciparum* infection induce greater T cell effector function in young children.

After 7 days of PBMC cultures, the frequencies of IFN-γ producing CD4 and CD8 T cells detected ranged between 0.5 to 1.1% and 0.3 to 1%, respectively. These frequencies fall within the ranges obtained from cultures of PBMC from naïve vaccinated individuals (Huaman *et al.*, 2008). The present study clearly demonstrated the presence of memory and naïve T cell subsets in a population exposed to natural holoendemic *P. falciparum* infection. The data obtained from evaluation of the functional and phenotypic *Nature* of T cell subsets in a population exposed to holoendemic malaria points to the characterization of the surrogate markers of natural immunity that will be important as clinical endpoints in critical evaluation of antimalarial blood stage vaccine candidates.

5.5. Surface Expression of T Cell Costimulatory Molecules

Development and differentiation of naive T cells to effector and memory subsets may depend partially on cellular interactions that transmit specific signals for maturation (Park *et al.*, 1997; MacLeod *et al.*, 2006). In the present study, the role of accessory molecules in development of specific T cell memory to *P. falciparum* was evaluated. To do this, cells were activated with malaria blood stage antigens and cell surface expression of

costimulatory molecules measured. In confirmation to other studies (Schlotmann *et al.*, 2000), over 95% of CD4 T cells and about 50% of CD8 T cells expressed CD28 molecule upon activation by MSP-1_{42} malaria antigen. CD152 and CD154 were expressed in less than 0.5% of both T cell subsets. Overall, the percentage expression of each of the three molecules was not altered by antigen stimulation since the frequencies after activation by PBS (negative control) were similar to antigen stimulated levels. Although this study was limited on its scope to investigate the presence and effects of other microbial pathogens, it is postulated that high levels of CD28 expression observed in this study population could be due to chronic activation of T cells by malaria and other persistent microbes.

The percentage of co-stimulatory molecules expressed on the surface of T cells is modulated during different disease conditions and depending on specific molecules, they could be upregulated or downregulated. Acute malaria infection results in up-regualtion of CD152 and downregulation of CD28 in clinical patients (Ayukawa *et al.*, 2004; Schlotmann *et al.*, 2000). Under normal conditions, the cell surface expression of CD152 and CD28 in CD4 T cells ranged between 0.5% to 2.1% and 81% to 92.9% respectively. However, acute malaria resulted to an increase of CD152 expression (11.9% to 20.5%), and reduction in CD28 expression (75.2% to 85.6%). Reduced CD28 expression may correlate with lower expression of other T cells activation molecules like HLA-DR (Borkow *et al.*, 2000). Consistent with these results (Borkow *et al.*, 2000), the present study observed similar levels to that of the normal population on surface expression of CD152 and CD28 in the CD4 T cell compartment. The study population for this project was exposed to holoendemic malaria but all the individuals in the study were clinically

normal and did not present with any acute malaria symptoms. Chronic T cell stimulation in the absence of CD28 may lead to reduction in early pro-inflammatory cytokine production and T cell anergy which could be detrimental to mounting a competent immune response.

There was no observed difference on the surface expression of these molecules across the age groups in the context of age-related evolution of immunologic memory to malaria in holoendemic areas. High cell surface turn over and intracellular translocation of the costimulatory molecules is known to occur (Higuchi *et al.*, 1997) and this could explain the low levels of CD152 and CD154 detected on the cells surface. Intracellular staining has revealed higher levels of these costimulatory molecules after polyclonal T cell activation (Higuchi *et al.*, 1997).

There was a significant reduction in expression of CD154/CD152 ratio in infants and adults compared to other age groups suggesting either upregulation of CD152 or down regulation of CD154 in individuals within these age groups. Reduced expression of CD152 is associated with induction of TGF-β, negative regulation of T cell proliferation, and IL-2 production (Chen *et al.*, 2007). Transient expression of high levels of CD152 in acute malaria has been observed in other studies and could be important in establishment and maintenance of peripheral tolerance (Shevach, 2002). Deficiency of CD152 in early years of life could lead to death due to autoimmune conditions (Tivol *et al.*, 1995). Hence malaria antigens could be polyclonal activators of T cells in infants and adults due to high antigenic exposures and tolerance may be acquired through upregulation of CD152 to avoid over activation of T cells that may lead to exhaustion and ultimate autoimmune conditions.

5.6. Cytokine Responses to *P. falciparum* MSP-1_{42}3D7

A gradual change in clinical malaria has been shown to vary with an increase in age and variation in transmission intensity and is thought to be accompanied by changes in cytokine profiles (John *et al.*, 2000). Both Th1 and Th2 cytokines measured tended to be higher in children aged 1 and 2 years but these differences were not statistically significant. Only samples positive for IFN-γ ICS and ELISPOT were evaluated for the presence of Th1 and Th2 cytokines. Using IFN-γ ICS as the primary molecule for differentiating the different T cell subsets could have partly contributed to low detection of other cytokines since different cytokines have variable time points for optimum secretion.

The balance between pro- and anti-inflammatory cytokines may be a measurement of cytokines induced in response to the infection and not necessarily ones that confer protection from infection. Early IL-12, IFN-γ and IL-2 and GM-CSF responses are acquired for primary resolution of parasites (Omer *et al.*, 2003) most likely through synergy with TNF-α to activate monocytes that directly kill parasites. Early pro-inflammatory cytokines are associated with clinical symptoms experienced during clinical malaria (Tchinda *et al.*, 2007). IFN-γ is associated with protective immunity hence the observed increase with age could be important for development of memory CD4 and CD8 T cells that are important in establishment of protective immunity. TNF-α responses are associated with fever and parasite clearance (Praba-Egge *et al.*, 2002) and the age related reduction of TNF-α could be a sign of reduction in parasite burden and establishment of clinical immunity.

Excess levels of pro-inflammatory cytokines may lead to immunopathologies when normal down-regulatory immune mechanisms are not able to control. High levels of Th2 cytokines, IL-10, IL-5 and IL-13 were observed in children aged 1-2 years possibly to neutralize the effects of excess Th1 cytokines. Although IL-10 levels were very low, there was an age related increase from 2 years to adulthood suggesting that this cytokine could be used as biomarker of evolution of immunologic memory to *P. falciparum* infection especially in the context of antibody production. Other studies have also suggested that IL-10 may protect from infection and clinical malaria (John *et al.*, 2000; Kurtis *et al.*, 1999). IL-5 and IL-13 are important for antibody production against parasitic diseases (McKenzie *et al.*, 1998; Nashed *et al.*, 2000). Reduced levels of these cytokines with age are suggestive of other factors that regulate development of clinical immunity. No individuals tested secreted detectable IL-4 which is in line with earlier studies that detected lower and similar IL-4 cytokine levels across the age groups (Fievet *et al.*, 1996; Rizos *et al.*, 2007). Therefore, the balance of Th1 and Th2 cytokines is tightly regulated at specific cytokine level hence differing patterns within and between cytokine groups.

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The data presented in this study demonstrated that in *P. falciparum* holoendemic areas, adults acquire mature memory T cells specific to malaria blood stage antigen MSP-1_{42} in contrast to infants and young children who have higher proportions of naïve T cells secreting IFN-γ in response to MSP-1. The age-related changes in T cell phenotypes are accompanied by a *P. falciparum* antigen-specific increase in the prevalence and levels of IFN-γ. Memory CD4 T cell subsets showed significant changes with age as compared to CD8 T cell subsets suggesting that CD4 subsets may play a greater role in development of immunity and regulation of blood stage *P. falciparum* infection. Hence, measurement of IFN-γ and memory CD4 T cell could be used as biomarkers of immunity to blood stage infections. Infants are exposed to maternal malaria antigens that may strongly sensitize their immune cells as occurs in adults chronically exposed to malaria. To avoid over reactivation of the immune system that may lead to autoimmune diseases, the body has to counter-regulate by upregulating certain immunosuppressive molecules. Therefore, the significantly lower levels observed in infants and adults in the ratio of CD154/CD152 CD4 T cell costimulatory molecules demonstrates that negative regulatory molecules are upregulated during the chronic exposure to blood stage antigens. Changes in the levels of Th1 and Th2 cytokines may not have been detected in this study population due to limited selection of optimum time point based on IFN-γ ICS as the primary molecule for differentiating T cell subsets.

6.2. Recommendation for Future Studies

1. Since this was a cross-sectional study, future studies should be designed to investigate a cohort of children from the time they are born to the age of 3 to 5 years. This would allow for evaluation of individual changes in memory T cell subsets and cytokine profiles that could be compared to those of putatively immune-protected adults and could be used as surrogate markers of immunity.
2. The prevalence, levels, and inhibitory capacity of immunoglobulin, mainly IgG and IgM subclasses, should be investigated in the same cohort of children. This would allow for an in-depth understanding of the role of memory T cell in directing affinity maturation of highly specific antibodies to *P. falciparum* blood stage antigens.
3. Investigation of *ex vivo* non-specific T cell phenotypes revealed a pattern of age-related increase in memory subsets suggesting that as people are exposed to a myriad of microbes antigen specific memory cells are generated. However, the impact of other microbial antigen on rate of T cell maturation needs to be investigated.

REFERENCES

Abdalla SH and Wickramasinghe SN (1988). A study of erythroid progenitor cells in the bone marrow of Gambian children with *falciparum* malaria. *Clin Lab Haematol* 10, 33-40.

Achtman AH, Bull PC, Stephens R, Langhorne J (2005). Longevity of the immune response and memory to blood-stage malaria infection. *Curr top microbiol immunol* 297, 71-102.

Adetifa IMO, Hill CP, Jeffries JD, Jackson-Sillah D, Ibanga BH, Bah G, Donkor S, Corrah T, Adegbola AR (2008). Haematological values from a Gambian cohort – possible reference range for a West African population. *Int. jnl. lab. hem* [Epub ahead of print].

al-Ramadi BK, Fernandez-Cabezudo MJ, Ullah A, El-Hasasna H, Flavell RA (2006). CD154 is essential for protective immunity in experimental *Salmonella* infection: evidence for a dual role in innate and adaptive immune responses. *J Immunol* 176, 496-506.

al-Yaman F, Genton B, Taraika J, Alpers MP (1997). Naturally acquired cellular immune responses to the synthetic malarial peptide SPf66 in children in Papua New Guinea. *Trans R Soc Trop Med Hyg* 91, 709-712.

Arakawa T, Tachibana M, Miyata T, Harakuni T, Kohama H, Matsumoto Y, Tsuji N, Hisaeda H, Stowers A, Torii M, Tsuboi T (2009). Malaria ookinete surface protein-based vaccination via the intranasal route completely blocks parasite transmission both in passive and active vaccination regimes in a rodent malaria infection model. *Infect Immun.* [Epub ahead of print].

Arreaza GA, Cameron MJ, Jaramillo A, Gill BM, Hardy D, Laupland KB, Rapoport MJ, Zucker P, Chakrabarti S, Chensue SW, Qin HY, Singh B, Delovitch TL (1997). Neonatal activation of CD28 signaling overcomes T cell anergy and prevents autoimmune diabetes by an IL-4-dependent mechanism. *J Clin Invest* 100, 2243-2253.

Artavanis-Tsakonas K, Eleme K, McQueen KL, Cheng NW, Parham P, Davis DM, Riley EM (2003). Activation of a subset of human NK cells upon contact with *Plasmodium falciparum*-infected erythrocytes. *J Immunol* 171, 5396-5405.

Audran R, Cachat M, Lurati F, Soe S, Leroy O, Corradin G, Druilhe P, Spertini F (2005). Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. *Infect Immun* 73, 8017-8026.

Ayukawa H, Matsubara T, Kaneko M, Hasegawa M, Ichiyama T, Furukawa S (2004). Expression of CTLA-4 (CD152) in peripheral blood T cells of children with influenza virus infection including encephalopathy in comparison with respiratory syncytial virus infection. *Clin Exp Immunol* 137, 151-155.

Baird JK (1998). Age-dependent characteristics of protection v. susceptibility to *Plasmodium falciparum*. *Ann Trop Med Parasitol* 92, 367-390.

Banchereau J and Steinman RM (1998). Dendritic cells and the control of immunity. *Nature* 392, 245-252.

Beltran E (1981). Commemoration of the discovery of the pathogenic germs of malaria. I. Charles Louis Alphonse Laveran (1845-1922). His 1880 discovery. *Gac Med Mex* 117, 195-201.

Bergmann ES, Ballou RW, Krzych U (1997). Detection of $CD4^+CD45RO^+$ T lymphocytes producing IL-4 in response to antigens on *Plasmodium falciparum* erythrocytes: an *in vitro* correlate of protective immunity induced with attenuated *Plasmodium falciparum* sporozoites. Cell Immunol 180, 143-152.

Blackman MJ and Holder AA (1992). Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1. *Mol Biochem Parasitol* 50, 307-315.

Bloland PB, Boriga DA, Ruebush TK, Mccormick BJ, Roberts MJ, Oloo JA, Hawley W, Lal A, Nahlen B, Campbell CC (1999). Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission II.

Descriptive epidemiology of malaria infection and disease among children. *Am J Trop Med Hyg* 60, 641-648.

Bojang KA, Olodude F, Pinder M, Ofori-Anyinam O, Vigneron L, Fitzpatrick S, Njie F, Kassanga A, Leach A, Milman J, Rabinovich R, McAdam KP, Kester KE, Heppner DG, Cohen JD, Tornieporth N, Milligan PJ (2005). Safety and immunogenicty of RTS,S/AS02A candidate malaria vaccine in Gambian children. *Vaccine* 23, 4148-4157.

Borkow G, Leng Q, Weisman Z, Stein M, Galai N, Kalinkovich A, Bentwich Z (2000). Chronic immune activation associated with intestinal helminth infections results in impaired signal transduction and anergy. *J Clin Invest* 106, 1053-1060.

Branch OH, Oloo AJ, Nahlen BL, Kaslow D, Lal AA (2000). Anti-merozoite surface protein-1 19-kDa IgG in mother-infant pairs naturally exposed to *Plasmodium falciparum*: subclass analysis with age, exposure to asexual parasitemia, and protection against malaria. V. The Asembo Bay Cohort Project. *J Infect Dis* 181, 1746-1752.

Brown A, Lutton JD, Nelson J, Abraham NG, Levere RD (1987). Microenvironmental cytokines and expression of erythroid heme metabolic enzymes. *Blood Cells* 13, 123-136.

Bynum WF (1999). Ronald Ross and the malaria-mosquito cycle. *Parassitologia* 41, 49-52.

Campbell JD and HayGlass KT (2000). T cell chemokine receptor expression in human Th1- and Th2-associated diseases. *Arch Immunol Ther Exp (Warsz)* 48, 451-456.

Carme B and Venturin C (1999). Malaria in the Americas. *Med Trop (Mars)* 59, 298-302.

Carter R and Mendis KN (2002). Evolutionary and historical aspects of the burden of malaria. *Clin Microbiol Rev* 15, 564-594.

Chalumeau M, Holvoet L, Chéron G, Minodier P, Foix-L'Hélias L, Ovetchkine P, Moulin F, Nouyrigat V, Bréart G, Gendrel D (2006). Delay in diagnosis of

imported *Plasmodium falciparum* malaria in children. *Eur J Clin Microbiol Infect Dis* 25, 186-189.

Chang SP, Case SE, Gosnell WL, Hashimoto A, Kramer KJ, Tam LQ, Hashiro CQ, Nikaido CM, Gibson HL, Lee-Ng CT, Barr PJ, Yokota BT, Hut GS (1996). A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 protects *Aotus* monkeys against malaria. *Infect Immun.* 64(1):253-61.

Chelimo K, Ofulla AV, Narum DL, Kazura JW, Lanar DE, John CC (2005). Antibodies to *Plasmodium falciparum* antigens vary by age and antigen in children in a malaria-holoendemic area of Kenya. *Pediatr Infect Dis J* 24, 680-684.

Chen J, Schmitt A, Giannopoulos K, Chen B, Rojewski M, Dohner H, Bunjes D, Schmitt M (2007). Imatinib impairs the proliferation and function of $CD4^+CD25^+$ regulatory T cells in a dose-dependent manner. *Int J Oncol* 31, 1133-1139.

Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM (2003). Conversion of peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198, 1875-1886.

Chidgey A, Dudakov J, Seach N, Boyd R (2007). Impact of niche aging on thymic regeneration and immune reconstitution. *Semin Immunol.* 19(5):331-40.

Clark IA, Rockett KA, Cowden WB (1991). Proposed link between cytokines, nitric oxide and human cerebral malaria. Parasitol Today 7, 205-207.

Colantonio L, Recalde H, Sinigaglia F, D'Ambrosio D (2002). Modulation of chemokine receptor expression and chemotactic responsiveness during differentiation of human naive T cells into Th1 or Th2 cells. *Eur J Immunol* 32, 1264-1273.

Coluzzi M (1993). Advances in the study of Afrotropical malaria vectors. *Parassitologia* 35 Suppl, 23-29.

Cot M, Brutus L, Le Goff G, Rajaonarivelo V, Raveloson A (2001). The campaign against malaria in central western Madagascar: comparison of lambda-cyhalothrin

and DDT house spraying. II--Parasitological and clinical study. *Parasite.* 8(4):309-16.

D'Alessandro U, Leach A, Drakeley CJ, Bennett S, Olaleye BO, Fegan GW, Jawara M, Langerock P, George MO, Targett GA (1995). Efficacy trial of malaria vaccine SPf66 in Gambian infants. *Lancet* 346, 462-467.

D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G (1993). Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 178, 1041-1048.

Darko CA, Angov E, Collins WE, Bergmann-Leitner ES, Girouard AS, Hitt SL, McBride JS, Diggs CL, Holder AA, Long CA, Barnwell JW, and Lyon JA (2005). The clinical-grade 42-kilodalton fragment of merozoite surface protein 1 of *Plasmodium falciparum* strain FVO expressed in Escherichia coli protects *Aotus nancymai* against challenge with homologous erythrocytic-stage parasites. *Infec Immun* 73(1):287-97.

Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, Chau TT, Mai NT, Phu NH, Sinh DX, White NJ, Ho M (1999). The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. *J Infect Dis* 180, 1288-1297.

De Zulueta J (1973). Malaria and Mediterranean history. *Parassitologia* 15, 1-15.

Deloron P and Chougnet C (1992). Is immunity to malaria really short-lived? *Parasitol Today* 8, 375-378.

Doolan DL and Martinez-Alier N (2006). Immune Response to Pre-Erythrocytic Stages of Malaria Parasites. *Current Molecular Medicine,* 6 (2), 1566-5240.

Druilhe P, Spertini F, Soesoe D, Corradin G, Mejia P, Singh S, Audran R, Bouzidi A, Oeuvray C, Roussilhon C (2005). A malaria vaccine that elicits in humans antibodies able to kill *Plasmodium falciparum*. PLoS Med 2, e344.

Engelhardt JJ, Sullivan TJ, Allison JP (2006). CTLA-4 overexpression inhibits T cell responses through a CD28-B7-dependent mechanism. *J Immunol* 177, 1052-1061.

Esser C and Radbruch A (1990). Immunoglobulin class switching: molecular and cellular analysis. *Annu Rev Immunol* 8, 717-735.

Esser MT, Marchese RD, Kierstead LS, Tussey LG, Wang F, Chirmule N, Washabaugh MW (2003). Memory T cells and vaccines. *Vaccine* 21, 419-430.

Ezhov MN, Zvantsov AB, Sergiev VP (2004). Malaria as a re-emerging disease in the countries of the WHO European Region: lessons of history and the present day situation in Trans-Caucasian Region and Turkey. *Med Parazitol (Mosk)*, 4, 16-19.

Fanslow WC, Clifford KN, Seaman M, Alderson MR, Spriggs MK, Armitage RJ, Ramsdell F (1994). Recombinant CD40 ligand exerts potent biologic effects on T cells. *J Immunol* 152, 4262-4269.

Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF (2004). Cutting edge: TGF-beta induces a regulatory phenotype in $CD4^+CD25^-$ T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 172, 5149-5153.

Farouk SE, Shen J, Tangteerawatana P, Bolad A, Berzins K, Troye-Blomberg M (2006). Analysis of T-cell responses in malaria-exposed and non-exposed donors using *Plasmodium falciparum* asexual blood stages enriched by a simple centrifugation method. *Acta Trop* 97, 42-49.

Faul F, Erdfelder E, Lang AG, Buchner, A (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, 39, 175-191.

Fell AH, Currier J, Good MF (1994). Inhibition of *Plasmodium falciparum* growth *in vitro* by $CD4^+$ and $CD8^+$ T cells from non-exposed donors. *Parasite Immunol* 16, 579-586.

Ferreira-da-Cruz MF, Deslandes DC, Oliveira-Ferreira J, Montenegro-James S, Tartar A, Druilhe P, Daniel-Ribeiro CT (1995). Antibody responses to

Plasmodium falciparum sporozoite-, liver- and blood-stage synthetic peptides in migrant and autochthonous populations in malaria endemic areas. *Parasite* 2, 23-29.

Fievet N, Ringwald P, Bickii J, Dubois B, Maubert B, Le Hesran JY, Cot M, Deloron P (1996). Malaria cellular immune responses in neonates from Cameroon. *Parasite Immunol* 18, 483-490.

Fievet N, Moussa M, Tami G, Maubert B, Cot M, Deloron P, Chaouat G (2001). *Plasmodium falciparum* induces a Th1/Th2 disequilibrium, favoring the Th1-type pathway, in the human placenta. *Journal of Infectious Diseases*, 183, 1530–1534

Fix AG (2003). Simulating hemoglobin history. *Hum Biol* 75, 607-618.

Flanagan KL, Plebanski M, Odhiambo K, Sheu E, Mwangi T, Gelder C, Hart K, Kortok M, Lowe B, Robson KJ, Marsh K, Hill AV (2006). Cellular reactivity to the *P. falciparum* protein TRAP in adult Kenyans: novel epitopes, complex cytokine patterns, and the impact of natural antigenic variation. *Am J Trop Med Hyg* 74, 367-375.

French LE, Huard B, Wysocka M, Shane R, Contassot E, Arrighi JF, Piguet V, Calderara S, Rook AH (2005). Impaired CD40L signaling is a cause of defective IL-12 and TNF-alpha production in Sezary syndrome: circumvention by hexameric soluble CD40L. *Blood* 105, 219-225.

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419, 498-511.

Genton B, Al-Yaman F, Anders R, Saul A, Brown G, Pye D, Irving DO, Briggs WR, Mai A, Ginny M, Adiguma T, Rare L, Giddy A, Reber-Liske R,

Stuerchler D, Alpers MP (2000). Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua New Guinea. *Vaccine* 18, 2504-2511.

Genton B, Al-Yaman F, Betuela I, Anders RF, Saul A, Baea K, Mellombo M, Taraika J, Brown GV, Pye D, Irving DO, Felger I, Beck HP, Smith TA, Alpers MP (2003). Safety and immunogenicity of a three-component blood-stage malaria vaccine (MSP1, MSP2, RESA) against *Plasmodium falciparum* in Papua New Guinean children. *Vaccine* 22, 30-41.

Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV, Pye D, Irving DO, Smith TA, Beck HP, Alpers MP. (2002). A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. *J Infect Dis* 185, 820-827.

Gil LH, Tada MS, Katsuragawa TH, Ribolla PE, da Silva LH. (2007). Urban and suburban malaria in Rondônia (Brazilian Western Amazon) II. Perennial transmissions with high *Anopheline* densities are associated with human environmental changes. *Mem Inst Oswaldo Cruz.* 102(3):271-6.

Good MF, Stanisic D, Xu H, Elliott S, Wykes M (2004). The immunological challenge to developing a vaccine to the blood stages of malaria parasites. *Immunol Rev* 201, 254-267.

Good MF, Xu H, Wykes M, Engwerda CR (2005). Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. *Annu Rev Immunol* 23, 69-99.

Gray D and Matzinger P (1991). T cell memory is short-lived in the absence of antigen. *J Exp Med* 174, 969-974.

Greenwood B and Mutabingwa T (2002). Malaria in 2002. *Nature* 415, 670-672.

Gruarin P, Primo L, Ferrandi C, Bussolino F, Tandon NN, Arese P, Ulliers D, Alessio M (2001). Cytoadherence of *Plasmodium falciparum*-infected erythrocytes

is mediated by a redox-dependent conformational fraction of CD36. *J Immunol* 167, 6510-6517.

Guevara Patino JA, Holder AA, McBride JS, Blackman MJ (1997). Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies. *J Exp Med.* 186(10):1689-99.

Guillemin J (2002). Choosing scientific patrimony: Sir Ronald Ross, Alphonse Laveran, and the mosquito-vector hypothesis for malaria. *J Hist Med Allied Sci* 57, 385-409.

Guinovart C, Navia MM, Tanner M, Alonso PL (2006). Malaria: burden of disease. *Curr Mol Med* 6, 137-140.

Halawani A and Shawarby AA (1957). Malaria in Egypt; history, epidemiology, control and treatment. *J Egypt Med Assoc* 40, 753-792.

He B (1988). A sketch of history of malarial epidemiology in China (Chi). *Zhonghua Yi Shi Za Zhi* 18, 1-8.

Helbok R, Dent W, Nacher M, Lackner P, Treeprasertsuk S, Krudsood S, Wilairatana P, Silachamroon U, Looareesuwan S, Schmutzhard E (2005). The use of the multi-organ-dysfunction score to discriminate different levels of severity in severe and complicated *Plasmodium falciparum* malaria. *Am J Trop Med Hyg* 72, 150-154.

Higuchi M, Nagasawa K, Horiuchi T, Oike M, Ito Y, Yasukawa M, Niho Y (1997). Membrane tumor necrosis factor-alpha (TNF-alpha) expressed on HTLV-I-infected T cells mediates a costimulatory signal for B cell activation--characterization of membrane TNF-alpha. *Clin Immunol Immunopathol* 82, 133-140.

Hirunpetcharat C, Tian JH, Kaslow DC, van Rooijen N, Kumar S, Berzofsky JA, Miller LH, Good MF (1997). Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1[19]) of *Plasmodium yoelii* expressed in *Saccharomyces cerevisiae*: correlation of protection with antigen-specific antibody titer, but not with effector $CD4^+$ T cells. *J Immunol* 159, 3400-3411.

Hirunpetcharat C, Vukovic P, Liu XQ, Kaslow DC, Miller LH, Good MF (1999). Absolute requirement for an active immune response involving B cells and Th cells in immunity to *Plasmodium yoelii* passively acquired with antibodies to the 19-kDa carboxyl-terminal fragment of merozoite surface protein-1. *J Immunol* 162, 7309-7314.

Hisaeda H, Yasutomo K, Himeno K (2005). Malaria: immune evasion by parasites. *Int J Biochem Cell Biol* 37, 700-706.

Hoffman BU and Chattopadhyay R (2008). *Plasmodium falciparum*: effect of radiation on levels of gene transcripts in sporozoites. *Exp Parasitol* 118, 247-252.

Hogh B (1996). Clinical and parasitological studies on immunity to *Plasmodium falciparum* malaria in children. *Scand J Infect Dis Suppl* 102, 1-53.

Holder AA (1994). Proteins on the surface of the malaria parasite and cell invasion. *Parasitology* 108 Suppl, S5-18.

Huaman MC, Martin LB, Malkin E, Narum DL, Miller LH, Mahanty S, Long CA. (2008). *Ex vivo* cytokine and memory T cell responses to the 42-kDa fragment of *Plasmodium falciparum* merozoite surface protein-1 in vaccinated volunteers. *J Immunol.* 1;180(3):1451-61.

Hviid L (1998). Clinical disease, immunity and protection against *Plasmodium falciparum* malaria in populations living in endemic areas. *Expert Rev Mol Med* 1998, 1-10.

Ing R, Segura M, Thawani N, Tam M, Stevenson MM (2006). Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. *J Immunol* 176, 441-450.

Jackola DR, Ruger JK, Miller RA (1994). Age-associated changes in human T cell phenotype and function. *Aging (Milano)* 6, 25-34.

Jacobs P, Radzioch D, Stevenson MM (1996). *In vivo* regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice. *Infect Immun* 64, 44-49.

Jangpatarapongsa K, Sirichaisinthop J, Sattabongkot J, Cui L, Montgomery SM, Looareesuwan S, Troye-Blomberg M, Udomsangpetch R (2006). Memory T cells protect against *Plasmodium vivax* infection. *Microbes Infect.* 3, 680-6

John CC, Moormann AM, Sumba PO, Ofulla AV, Pregibon DC, Kazura JW (2004). Gamma interferon responses to *Plasmodium falciparum* liver-stage antigen 1 and thrombospondin-related adhesive protein and their relationship to age, transmission intensity, and protection against malaria. *Infect Immun* 72, 5135-5142.

John CC, O'Donnell RA, Sumba PO, Moormann AM, de Koning-Ward TF, King CL, Kazura JW, Crabb BS (2004). Evidence that invasion-inhibitory antibodies specific for the 19-kDa fragment of merozoite surface protein-1 (MSP-1 19) can play a protective role against blood-stage *Plasmodium falciparum* infection in individuals in a malaria endemic area of Africa. *J Immunol.* 1;173(1):666-72.

John CC, Sumba PO, Ouma JH, Nahlen BL, King CL, Kazura JW (2000). Cytokine responses to *Plasmodium falciparum* liver-stage antigen 1 vary in rainy and dry seasons in highland Kenya. *Infect Immun* 68, 5198-5204.

Kabilan L (1997). T cell immunity in malaria. *Indian J Med Res* 106, 130-148.

Kaneko A (1998). Malaria on the global agenda: control and chemotherapy of malaria in Vanuatu. *Rinsho Byori* 46, 637-644.

Kemp K, Akanmori BD, Kurtzhals JA, Adabayeri V, Goka BQ, Hviid L (2002). Acute *P. falciparum* malaria induces a loss of $CD28^-$ T IFN-gamma producing cells. *Parasite Immunol* 24, 545-548.

Kern DE, Grabstein KH, Okuno K, Schreiber RD, Greenberg PD (1989). Identification of a unique T cell-derived lymphokine that primes macrophages for tumor cytotoxicity. *J Immunol* 143, 4308-4316.

Khan HI, Krishnan VV, Ziman M, Janatpour K, Ted Wun T, Paul A. Luciw AP, Tuscano J (2009). A comparison of multiplex suspension array large-panel kits for profiling cytokines and chemokines in rheumatoid arthritis patients *Cytometry B Clin Cytom*, 76(3):159-68

Kitua AY (1997). Field trials of malaria vaccines. *Indian J Med Res* 106, 95-108.

Korbel DS, Newman KC, Almeida CR, Davis DM, Riley EM (2005). Heterogeneous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. *J Immunol* 175, 7466-7473.

Krishnan A and Karnad DR (2003). Severe *falciparum* malaria: an important cause of multiple organ failure in Indian intensive care unit patients. *Crit Care Med* 31, 2278-2284.

Krishnegowda G, Hajjar AM, Zhu J, Douglass EJ, Uematsu S, Akira S, Woods AS, Gowda DC (2005). Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J Biol Chem* 280, 8606-8616.

Krzych U, Lyon JA, Jareed T, Schneider I, Hollingdale MR, Gordon DM, Ballou WR (1995). T lymphocytes from volunteers immunized with irradiated *Plasmodium falciparum* sporozoites recognize liver and blood stage malaria antigens. *J Immunol* 155, 4072-4077.

Kumar S, Yadava A, Keister DB, Tian JH, Ohl M, Perdue-Greenfield KA, Miller LH, Kaslow DC (1995). Immunogenicity and *in vivo* efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. *Mol Med* 1, 325-332.

Kurtis JD, Lanar DE, Opollo M, Duffy PE (1999). Interleukin-10 responses to liver-stage antigen 1 predict human resistance to *Plasmodium falciparum*. *Infect Immun* 67, 3424-3429.

Labaied M, Harupa A, Dumpit FR, Coppens I, Mikolajczak AS, Stefan H I, Kappe HIS (2007). *Plasmodium yoelii* Sporozoites with Simultaneous deletion of P52 and P36 are completely attenuated and confer sterile immunity against Infection. *Infection and Immunity*. 75 (8), 3758-3768.

Langhorne J (1994). The immune response to the blood stages of *Plasmodium* in animal models. *Immunol Lett* 41, 99-102.

Langhorne J, Simon-Haarhaus B, Meding SJ (1990). The role of $CD4^+$ T cells in the protective immune response to *Plasmodium chabaudi in vivo*. *Immunol Lett* 25, 101-107.

Lenschow DJ, Su GH, Zuckerman LA, Nabavi N, Jellis CL, Gray GS, Miller J, Bluestone JA (1993). Expression and functional significance of an additional ligand for CTLA-4. *Proc Natl Acad Sci U S A*, 90, 11054-11058.

Lugada E.S., Mermin J., Kaharuza F., Ulvestad E., Were W., Langeland N., Asjo B., Malamba S. & Downing R. (2004). Population-based hematologic and immunologic reference values for a healthy Ugandan population. *Clinical and Diagnostic Laboratory Immunology* 11, 29–34.

Lusingu JP and Von Seidlein L. (2008). Challenges in malaria control in sub-Saharan Africa: the vaccine perspective. *Tanzan J Health Res.* 10(4):253-66.

Luty AJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, Matousek P, Herbich K, Schmid D, Migot-Nabias F, Deloron P, Nussenzweig RS, Kremsner PG (1999). Interferon-gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *J Infect Dis* 179, 980-988.

MacLeod M, Kwakkenbos MJ, Crawford A, Brown S, Stockinger B, Schepers K, Schumacher T, Gray D (2006). CD4 memory T cells survive and proliferate but fail to differentiate in the absence of CD40. *J Exp Med* 203, 897-906.

Magill AJ (2006). Malaria: diagnosis and treatment of *falciparum* malaria in travelers during and after travel. *Curr Infect Dis Rep* 8, 35-42.

Malhotra I, Mungai P, Muchiri E, Ouma J, Sharma S, Kazura JW, King CL (2005). Distinct Th1- and Th2-type prenatal cytokine responses to *Plasmodium falciparum* erythrocyte invasion ligands. *Infect Immun* 73, 3462-3470.

McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN (1998). A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr Biol* 8, 339-342.

Meding SJ and Langhorne J (1991). $CD4^+$ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi chabaudi. Eur J Immunol* 21, 1433-1438.

Mendis K, Rietveld A, Warsame M, Bosman A, Greenwood B, Wernsdorfer WH (2009). From malaria control to eradication: The WHO perspective.*Trop Med Int Health.* 7, 802-9.

Miller KL, Silverman PH, Kullgren B, Mahlmann LJ (1989). Tumor necrosis factor alpha and the anemia associated with murine malaria. *Infect Immun* 57, 1542-1546.

Miller LH, Roberts T, Shahabuddin M, McCutchan TF (1993). Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). *Mol Biochem Parasitol* 59, 1-14.

Morrot A and Zavala F (2004). Regulation of the $CD8^+$ T cell responses against *Plasmodium* liver stages in mice. *Int J Parasitol* 34, 1529-1534.

Mullbacher A (1994). The long-term maintenance of cytotoxic T cell memory does not require persistence of antigen. *J Exp Med* 179, 317-321.

Nardin E, Zavala F, Nussenzweig V, Nussenzweig RS (1999). Pre-erythrocytic malaria vaccine: mechanisms of protective immunity and human vaccine trials. *Parassitologia* 41, 397-402.**Nashed BF, Maekawa Y, Takashima M, Zhang T, Ishii K, Dainichi T, Ishikawa H, Sakai T, Hisaeda H, Himeno K** (2000). Different cytokines are required for induction and maintenance of the Th2 type response in DBA/2 mice resistant to infection with *Leishmania major. Microbes Infect* 2, 1435-1443.

Nebl T, De Veer MJ, Schofield L (2005). Stimulation of innate immune responses by malarial glycosylphosphatidylinositol via pattern recognition receptors. *Parasitology* 130 Suppl, S45-62.

Nicholson JF and Pesce MA (2000). Reference ranges for laboratory tests and procedures. In: Nelson Textbook of Paediatrics (Eds R.E. Behrman, R.M. Kliegman and H.B. Jenson), 16th Edn, pp. 2181–2229. W.B. *Saunders Company, Philadelphia*, PA.

Nikodem D and Davidson E (2000). Identification of a novel antigenic domain of *Plasmodium falciparum* merozoite surface protein-1 that specifically binds to human erythrocytes and inhibits parasite invasion, *in vitro*. *Mol Biochem Parasitol* 108, 79-91.

Norton SD, Zuckerman L, Urdahl KB, Shefner R, Miller J, Jenkins MK (1992). The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells. *J Immunol* 149, 1556-1561.

Nussenzweig RS, Vanderberg J, Most H, Orton C (1967). Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. *Nature* 216, 160-162.

O'Donnell RA, de Koning-Ward TF, Burt RA, Bockarie M, Reeder JC, Cowman AF, Crabb BS (2001). Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. *J Exp Med* 193, 1403-1412.

O'Garra A, Hosken N, Macatonia S, Wenner CA, Murphy K (1995). The role of macrophage- and dendritic cell-derived IL12 in Th1 phenotype development. *Res Immunol* 146, 466-472.

Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, Tucker K, Waitumbi JN, Diggs C, Wittes J, Malkin E, Leach A, Soisson LA, Milman JB, Otieno L, Holland CA, Polhemus M, Remich SA, Ockenhouse CF, Cohen J, Ballou WR, Martin SK, Angov E, Stewart VA, Lyon JA, Heppner DG, Withers MR (2009). MSP-1 Malaria Vaccine Working Group. Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. *PLoS One*. 4(3):e4708.

Okech B, Mujuzi G, Ogwal A, Shirai H, Horii T, Egwang TG (2006). High titers of IgG antibodies against *Plasmodium falciparum* serine repeat antigen 5 (SERA5) are associated with protection against severe malaria in Ugandan children. *Am J Trop Med Hyg* 74, 191-197.

Omer FM, de Souza JB, Riley EM (2003). Differential induction of TGF-beta regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal *Plasmodium yoelii* infections. *J Immunol* 171, 5430-5436.

Park W, Weyand CM, Schmidt D, Goronzy JJ (1997). Co-stimulatory pathways controlling activation and peripheral tolerance of human $CD4^{+}CD28^{-}$ T cells. *Eur J Immunol* 27, 1082-1090.

Perraut R, Diatta B, Marrama L, Garraud O, Jambou R, Longacre S, Krishnegowda G, Dieye A, Gowda DC (2005). Differential antibody responses to *Plasmodium falciparum* glycosylphosphatidylinositol anchors in patients with cerebral and mild malaria. *Microbes Infect* 7, 682-687.

Pied S, Nussler A, Pontent M, Miltgen F, Matile H, Lambert PH, Mazier D (1989). C-reactive protein protects against preerythrocytic stages of malaria. *Infect Immun* 57, 278-282.

Plebanski M, Flanagan KL, Lee EA, Reece WH, Hart K, Gelder C, Gillespie G, Pinder M, Hill AV (1999). Interleukin-10 mediated immunosuppression by a variant CD4 T cell epitope of *Plasmodium falciparum*. *Immunity* 10, 651-660.

Podoba JE and Stevenson MM (1991). $CD4^{+}$ and $CD8^{+}$ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi AS*. *Infect Immun* 59, 51-58.

Pouniotis DS, Proudfoot O, Bogdanoska V, Apostolopoulos V, Fifis T, Plebanski M (2004). Dendritic cells induce immunity and long-lasting protection against blood-stage malaria despite an *in vitro* parasite-induced maturation defect. *Infect Immun* 72, 5331-5339.

Praba-Egge AD, Montenegro S, Cogswell FB, Hopper T, James MA (2002). Cytokine responses during acute simian *Plasmodium cynomolgi* and *Plasmodium knowlesi* infections. *Am J Trop Med Hyg* 67, 586-596.

Prudhomme O'meara W, Mckenzie EF, Magill JA, Forney RJ, Permpanich B, Lucas C, Gasser AR, Wongsrichanalai C (2005). Sources of variability in

determining malaria parasite density by microscopy, *Am. J. Trop. Med. Hyg.*, 73(3), 593-598.

Reece HHW, Pinder M, Gothard KP, Milligan P, Bojang K, Doherty T, Plebanski M, Akinwunmi P, Everaere S, Watkins RK, Voss G, Tornieporth N, Alloueche A, Greenwood MB, Kester EK, McAdam PWJK, Cohen J, Hill VS (2004). A $CD4^+$ T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease. *Nat Med* 10, 406-410.

Reinhardt RL, Khoruts A, Merica R, Zell T, Jenkins MK (2001). Visualizing the generation of memory CD4 T cells in the whole body. *Nature* 410, 101-105.

Riley EM, Allen SJ, Bennett S, Thomas PJ, O'Donnell A, Lindsay SW, Good MF, Greenwood BM (1990). Recognition of dominant T cell-stimulating epitopes from the circumsporozoite protein of *Plasmodium falciparum* and relationship to malaria morbidity in Gambian children. *Trans R Soc Trop Med Hyg* 84, 648-657.

Riley EM, Allen SJ, Wheeler JG, Blackman MJ, Bennett S, Takacs B, Schonfeld HJ, Holder AA, Greenwood BM (1992). Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity. *Parasite Immunol* 14, 321-337.

Riley EM, Morris-Jones S, Blackman MJ, Greenwood BM, Holder AA (1993). A longitudinal study of naturally acquired cellular and humoral immune responses to a merozoite surface protein (MSP1) of *Plasmodium falciparum* in an area of seasonal malaria transmission. *Parasite Immunol* 15, 513-524.

Rizos D, Protonotariou E, Malamitsi-Puchner A, Sarandakou A, Trakakis E, Salamalekis E (2007). Cytokine concentrations during the first days of life. *Eur J Obstet Gynecol Reprod Biol* 131, 32-35.

Rogier C (2000). Natural history of *Plasmodium falciparum* malaria and determining factors of the acquisition of antimalaria immunity in two endemic areas, Dielmo and Ndiop (Senegal). *Bull Mem Acad R Med Belg* 155, 218-226.

Roy A, Ansari MA, Kabilan L (1998). A longitudinal study of sero-reactivity to *Plasmodium falciparum* antigen in children and adult living in an endemic area of U.P. *Indian J Malariol* 35, 48-56.

Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, Foucault C, Chongsuphajaisiddhi T, Druilhe P (1991). Parasitologic and clinical human response to immunoglobulin administration in *falciparum* malaria. *Am J Trop Med Hyg* 45, 297-308.

Sallusto F, Kremmer E, Palermo B, Hoy A, Ponath P, Qin S, Forster R, Lipp M, Lanzavecchia A (1999a). Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur J Immunol* 29, 2037-2045.

Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A (1999b). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708-712.

Saule P, Trauet J, Dutriez V, Lekeux V, Dessaint JP, Labalette M (2006). Accumulation of memory T cells from childhood to old age: central and effector memory cells in $CD4^+$ versus effector memory and terminally differentiated memory cells in $CD8^+$ compartment. *Mech Ageing Dev* 127, 274-281.

Saxena S and Wong ET. (1990). Heterogeneity of common hematologic parameters among racial, ethnic, and gender subgroups. *Archives of Pathology and Laboratory Medicine* 114, 715–719.

Schlotmann T, Waase I, Julch C, Klauenberg U, Muller-Myhsok B, Dietrich M, Fleischer B, Broker BM (2000). CD4 alphabeta T lymphocytes express high levels of the T lymphocyte antigen CTLA-4 (CD152) in acute malaria. *J Infect Dis* 182, 367-370.

Schluns KS and Lefrancois L (2003). Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* 3, 269-279.

Schofield L, Bushell GR, Cooper JA, Saul AJ, Upcroft JA, Kidson C (1986) A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes

recognized by inhibitory monoclonal antibodies. *Mol Biochem Parasitol* 18, 183-195.

Serghides L, Smith TG, Patel SN, Kain KC (2003). CD36 and malaria: friends or foes? Trends Parasitol 19, 461-469.

Shevach EM (2002). $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2, 389-400.

Sims PF and Hyde JE (2006). Proteomics of the human malaria parasite *Plasmodium falciparum*. *Expert Rev Proteomics* 3, 87-95.

Singh S, Miura K, Zhou H, Muratova O, Keegan B, Miles A, Martin LB, Saul AJ, Miller LH, Long CA (2006). Immunity to recombinant *Plasmodium falciparum* merozoite surface protein 1 (MSP1): protection in *Aotus nancymai* monkeys strongly correlates with anti-MSP1 antibody titer and *in vitro* parasite-inhibitory activity. *Infect Immun*.74(8):4573-80.

Snapper CM, McIntyre TM, Mandler R, Pecanha LM, Finkelman FD, Lees A, Mond JJ (1992). Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J Exp Med* 175, 1367-1371.

Snow RW, Gouws E, Omumbo J, Rapuoda B, Craig MH, Tanser FC, le Sueur D, Ouma J (1998). Models to predict the intensity of *Plasmodium falciparum* transmission: applications to the burden of disease in Kenya. *Trans R Soc Trop Med Hyg* 92, 601-606.

Snow RW, Bastos de Azevedo I, Lowe BS, Kabiru EW, Nevill CG, Mwankusye S, Kassiga G, Marsh K, Teuscher T (1994). Severe childhood malaria in two areas of markedly different *falciparum* transmission in East Africa. *Acta Trop*. 57(4):289-300

Speake C and Duffy PE. (2009). Antigens for pre-erythrocytic malaria vaccines: building on success. *Parasite Immunol*. 31(9):539-46

Stephens R and Langhorne J (2006). Priming of CD4+ T cells and development of $CD4^+$ T cell memory; lessons for malaria. *Parasite Immunol* 28, 25-30.

Stevenson MM and Riley EM (2004). Innate immunity to malaria. *Nat Rev Immunol* 4, 169-180.

Stevenson MM and Zavala F (2006). *Immunology* of malaria infections. *Parasite Immunol* 28, 1-4.

Su Z, Fortin A, Gros P, Stevenson MM (2002). Opsonin-independent phagocytosis: an effector mechanism against acute blood-stage *Plasmodium chabaudi AS* infection. *J Infect Dis* 186, 1321-1329.

Su Z and Stevenson MM (2002). IL-12 is required for antibody-mediated protective immunity against blood-stage *Plasmodium chabaudi AS* malaria infection in mice. *J Immunol* 168, 1348-1355.

Takala SL, Coulibaly D, Thera MA, Dicko A, Smith DL, Guindo AB, Kone AK, Traore K, Ouattara A, Djimde AA, Sehdev PS, Lyke KE, Diallo DA, Doumbo OK, Plowe CV (2007). Dynamics of polymorphism in a malaria vaccine antigen at a vaccine-testing site in Mali. *PLoS Med.* 4(3):e93.

Taylor-Robinson AW (2002). A model of development of acquired immunity to malaria in humans living under endemic conditions. *Med Hypotheses* 58, 148-156.

Taylor-Robinson AW and Phillips RS (1992). Functional characterization of protective $CD4^+$ T cell clones reactive to the murine malaria parasite *Plasmodium chabaudi. Immunology* 77, 99-105.

Taylor-Robinson AW, Smith EC. (1999). Modulation of experimental blood stage malaria through blockade of the B7/CD28 T-cell costimulatory pathway. *Immunology* 96(3):498-504.

Tchinda VH, Tadem AD, Tako EA, Tene G, Fogako J, Nyonglema P, Sama G, Zhou A, Leke RG (2007). Severe malaria in Cameroonian children: correlation between plasma levels of three soluble inducible adhesion molecules and TNF-alpha. *Acta Trop* 102, 20-28.

Tena-Tomás W, Marielle K, Bouyou-Akotet KM, Kendjo E, Kombila M, Kremsner GP, and Jürgen FJ, Kun FJJ (2007). Prenatal immune responses to

Plasmodium falciparum erythrocyte membrane protein 1 DBL-alpha domain in Gabon. *Parasitol Res.* 101(4):1045-50.

Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3, 541-547.

Todryk SM and Walther M (2005). Building better T-cell-inducing malaria vaccines. *Immunology* 115, 163-169.

Troye-Blomberg M, Riley EM, Kabilan L, Holmberg M, Perlmann H, Andersson U, Heusser CH, Perlmann P (1990). Production by activated human T cells of interleukin 4 but not interferon-gamma is associated with elevated levels of serum antibodies to activating malaria antigens. *Proc Natl Acad Sci U S A* 87, 5484-5488.

Truneh A, Reddy M, Ryan P, Lyn DS, Eichman C, Couez D, Hurle RM, Sekaly PR, Daniel Olive D, Sweet R (1996). Differential recognition by CD28 of its cognate counter receptors CD80 (B7.1) and B70 (B7.2): analysis by site directed mutagenesis. *Mol Immunol* 33, 321-334.

Tsuji T, Nibu R, Iwai K, Kanegane H, Yachie A, Seki H, Miyawaki T, Taniguchi N (1994). Efficient induction of immunoglobulin production in neonatal naive B cells by memory $CD4^+$ T cell subset expressing homing receptor L-selectin. *J Immunol* 152, 4417-4424.

Uneke CJ (2007). Impact of placental *Plasmodium falciparum* malaria on pregnancy and perinatal outcome in sub-Saharan Africa: I: introduction to placental malaria. *Yale J Biol Med.* 80(2):39-50.

Urban BC, Ing R, Stevenson MM (2005). Early interactions between blood-stage *Plasmodium* parasites and the immune system. *Curr top microbiol immunol* 297, 25-70.

Urban BC, Willcox N, Roberts DJ (2001). A role for CD36 in the regulation of dendritic cell function. *Proc Natl Acad Sci U S A* 98, 8750-8755.

Vanham G, Bisalinkumi E (1995). Immunology of human *Plasmodium falciparum* malaria. *Ann Soc Belg Med Trop* 75, 159-178.

von der Weid T, Langhorne J (1993). The roles of cytokines produced in the immune response to the erythrocytic stages of mouse malarias. *Immunobiology* 189, 397-418.

Wagner G, Koram K, McGuinness D, Bennett S, Nkrumah F, Riley E (1998). High incidence of asymptomatic malara infections in a birth cohort of children less than one year of age in Ghana, detected by multicopy gene polymerase chain reaction. *Am J Trop Med Hyg* 59, 115-123.

Walther M, Tongren J, Andrews L, Korbel D, King E, Fletcher H, Andersen R, Bejon P, Thompson F, Dunachie S (2005). Upregulation of TGF-beta, FOXP3, and $CD4^{+}CD25^{+}$ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 23, 287-296.

Wang L, Crouch L, Richie TL, Nhan DH, Coppel RL (2003). Naturally acquired antibody responses to the components of the *Plasmodium falciparum* merozoite surface protein 1 complex. *Parasite Immunol* 25, 403-412.

Wernsdorfer G and Wernsdorfer WH (2003). Malaria at the turn from the 2nd to the 3rd millenium. *Wien Klin Wochenschr* 115 Suppl 3, 2-9.

WHO (1992). World malaria situation 1990. Division of Control of Tropical Diseases. World Health Organization *World Health Stat O*, Geneva. 45(2-3):257-66.

Williamson WA and Greenwood BM (1978). Impairment of the immune response to vaccination after acute malaria. *Lancet* 1, 1328-1329.

Winkler S, Willheim M, Baier K, Schmid D, Aichelburg A, Graninger W, Kremsner PG (1999). Frequency of cytokine-producing T cells in patients of different age groups with *Plasmodium falciparum* malaria. *J Infect Dis* 179, 209-216.

Woehlbier U, Epp C, Kauth CW, Lutz R, Long CA, Coulibaly B, Kouyate B, Arevalo-Herrera M, Herrera S, Bujard H (2006). Analysis of antibodies directed

against merozoite surface protein 1 of the human malaria parasite *Plasmodium falciparum*. *Infect Immun* 74, 1313-1322.

Wroczynska A, Nahorski W, Bakowska A, Pietkiewicz H (2005). Cytokines and clinical manifestations of malaria in adults with severe and uncomplicated disease. *Int Marit Health* 56, 103-114.

Xu H, Hodder AN, Yan H, Crewther PE, Anders RF, Good MF (2000). $CD4^+$ T cells acting independently of antibody contribute to protective immunity to *Plasmodium chabaudi* infection after apical membrane antigen 1 immunization. *J Immunol* 165, 389-396.

Yang X, Ye RG, Kong QY, Yang QQ, Gao Y, Zhong JH, Wang T (2000). CD40 ligand expression on macrophages during peritonitis in continuous ambulatory peritoneal dialysis patients. *Adv Perit Dial* 16, 213-215.

Yazdani SS, Mukherjee P, Chauhan VS, Chitnis CE (2006). Immune responses to asexual blood-stages of malaria parasites. *Curr Mol Med* 6, 187-203.

Yoshida S, Kobayashi T, Matsuoka H, Seki C, Gosnell WL, Chang SP, Ishii A (2003). T-cell activation and cytokine production via a bispecific single-chain antibody fragment targeted to blood-stage malaria parasites. *Blood* 101, 2300-2306.

Young SL, Wilson M, Wilson S, Beagley KW, Ward V, Baird MA (2006). Transcutaneous vaccination with virus-like particles. *Vaccine*. 24(26):5406-12

Zhu Y, Ljunggren H, Mix E, Li HL, van der Meide P, Elhassan AM, Winblad B, Zhu J (2001). CD28-B7 costimulation: a critical role for initiation and development of experimental autoimmune neuritis in C57BL/6 mice. *J Neuroimmunol* 114, 114-121.

Zuberek K, Ling V, Wu P, Ma HL, Leonard JP, Collins M, Dunussi-Joannopoulos K (2003). Comparable *in vivo* efficacy of CD28/B7, ICOS/GL50, and ICOS/GL50B costimulatory pathways in murine tumor models: IFN gamma-dependent enhancement of CTL priming, effector functions, and tumor specific memory CTL. *Cell Immunol* 225, 53-63.