IMMUNE RESPONSE TO NEW *Plasmodium falciparum* LIVER STAGE ANTIGENS IN CHILDREN NATURALLY EXPOSED TO MALARIA

BY

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY MEDICINE OF SOKOINE UNIVERSITY OF AGRICULTURE.

MOROGORO, TANZANIA.

2009

ABSTRACT

Interferon gamma (IFN-γ) responses to Human Leukocyte Antigen (HLA) class 1 restricted peptide antigens have been shown to be protective against malaria in different populations where malaria is endemic. The present study examined whether newly discovered liver stage *Plasmodium falciparum (P. falciparum)* protein antigens, which were matched to different HLA class 1 alleles predicted to be expressed by Tanzanians, can stimulate T cells with subsequent production of IFN-γ. A crossectional study from an ongoing cohort of children between the age of 0-5 years in malaria endemic area of Morogoro municipality, Tanzania, was undertaken to test IFN-γ responses to the new liver stage antigens. To examine whether the these liver stage peptides stimulated T cell proliferation and IFN-γ production, an enzyme linked immunospot (ELISPOT) assay was used, and results were compared to parasitological and haematological parameters of the children investigated. HLA B15 predicted specific responses were most frequent 63.6%, (21/33) when compared to responses to peptides predicted to be restricted by other HLA class 1 alleles such as HLA B35 22% (4/18) and HLA A02 15.2% (7/49) ($P<0.05$) by a chi square test. Children below 6 months were found to respond to the peptide antigens less frequently (7.2%, 5/70) than children above 6 months (92.8%, 65/70) ($P=0.0001$). Moreover, responding children above 6 months (78.8%, 26/33) were found to be protected from malaria parasitemia within two months follow up period, $P=0.0003$. This study confirms the presence of adaptive cell-mediated immunity to the liver stage malaria antigens in children from Tanzania and demonstrates that alleles of the HLA-B15 can effectively present antigenic epitopes. These antigens therefore provide suitable candidates for inclusion into the pool of pre-erythrocytic antigens for malaria vaccine candidates.

DECLARATION

I, Catherine Gerald Mkindi, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and has never been submitted nor concurrently being submitted for a higher degree award in any other University.

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ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my supervisor, Dr Patrick E. Duffy, Seattle Biomedical research Institute (SBRI), USA, for his detailed and constructive comments, and for his important support throughout this work. His wide knowledge and logical way of thinking have been of great value to me. I wish to express my warm and sincere thanks to my supervisor, Professor Paul S. Gwakisa, Sokoine University of Agriculture (SUA). His extensive discussions around my work and interesting explorations in operations have been very helpful. His understanding, encouragement and personal guidance have provided a good basis for the present Thesis. I am deeply grateful to my supervisor Dr Joshua Malago, (SUA), for his valuable advice and friendly help. His kind support and guidance have been of great value. My warm thanks are due to Dr Christian Okafor (SBRI laboratory), who directed me on cell culture procedures and ELISPOT assays. I also wish to thank Dr Moses Gwamaka for his guidance in statistical analysis.

I would also like to thank the Mother Offspring Malaria Studies (MOMS) project for the granting me an opportunity to work in their laboratory and those who agreed to be enrolled to the project, for without their time and cooperation, this work would not have been possible. During this work I have collaborated with many colleagues for whom I have great regard, and I wish to extend my warmest thanks to all those who have helped me with my work in the MOMS project and SBRI laboratory.

I owe my loving thanks to my husband Dr. Ladslaus Mnyone, my daughters, Nambua and Nankondo. They lost a lot due to my absence in a busy research schedule. Without their

encouragement and understanding it would have been impossible for me to finish this work.

The financial support from the Programme for Agricultural and Natural Resources Transformation for Improved Livelihoods (PANTIL), through SUA is gratefully acknowledged.

To each of the above, I extend my deepest appreciation.

DEDICATION

This Dissertation is dedicated to my wonderful parents, Gerald and Leonarda, who have raised me to be the person I am today. You have been with me every step of the way, through good times and bad. Thank you for all the unconditional love, guidance, and support that you have always given me, helping me to succeed and instilling in me the confidence that I am capable of doing anything I put my mind to. Thank you for everything. I love you!

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ABBREVIATIONS

AMA	_	Apical membrane antigen
CSP	_	Circumsporozoite protein
CTLs	_	Cytotoxic T lymphocytes
EBA	_	Erythrocyte-binding antigen
ELISPOT	_	Enzyme-linked immunospot
EMPE	_	Erythrocyte membrane protein
ER	_	Endoplasmic reticulum
Exp-1	_	Exported protein 1
FMP1	_	Falciparum malaria protein 1
GLURP	_	Glutamate-rich protein
Hb	_	Haemoglobin
HLA	_	Human leukocyte antigens
IFN-γ	_	Interferon gamma
IL	_	Interleukin
LS	_	Liver stage
LSA-1	_	Liver stage antigen 1
LSA-3	_	Liver stage antigen 3
ME	_	Mercaptoethanol
ME–TRAP	_	Multiple epitope–thrombospondin-related adhesive protein
MHC	_	Major histocompatibility complex
MIP	_	Macrophage inflammatory protein
MOMS	_	Mother Offspring Malaria Studies
MSP	_	Merozoite surface protein
NO	_	Nitric oxide

PBMC	_	Peripheral blood mononuclear cells
PBS	_	Phosphate buffer saline
Pf	_	*Plasmodium falciparum* protein
PMA	_	Phorbol 12-myristate 13-acetate
Pv	_	*Plasmodium vivax* protein
PVDF	_	Polyvinylidene difluoride
RANTES	_	Regulated on activation, normal T-cell expressed and secreted
RAP	_	Rhoptry-associated protein
RESA	_	Ring-infected erythrocyte surface antigen
SALSA	_	Sporozoite- and liver-stage antigen
SBRI	_	Seattle Biomedical Research Institute
SERA	_	Serine repeat antigen
SFU	_	Spot-forming units
SP	_	Sulfadoxine/ pyrimethamine
SPf66	_	Synthetic *P. falciparum* 66
SSP	_	Sporozoite surface protein
STARP	_	Sporozoite Threonine and Asparagines Rich Protein
STARP	_	Sporozoite threonine- and asparagine-rich protein
STAT	_	Transducers associated with transcription
SUA	_	Sokoine University of Agriculture
TAP	_	Transporters associated protein
TNF-α	_	Tumour necrosis factor alpha
TRAP	_	Thrombospondin Related Adhesion Protein
TRAP	_	Thrombospondin-related adhesive protein
WHO	_	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Malaria is one of the most common infectious diseases and an enormous public-health problem affecting between 300 and 500 million people every year, causing between 1 and 3 million deaths annually (Rowe *et al.*, 2006). Most of the deaths occur in children below 5 years of age and pregnant women (Adam *et al.*, 2005; Roca-Feltrer, 2008). WHO (2000, 2005) reports that one African child dies from malaria every 30 seconds. Malaria is caused by a protozoan parasite of genus *Plasmodium* in which 75% is caused by *P. falciparum*. It is spread by an insect vector, a female Anopheles mosquito, *Anopheles gambiae (A. gambiae)* being the major vector in Africa (Dimopoulos, 2002; Dong, 2006).

A human host gets infected following a bite by a *P. falciparum* infected female Anopheles when taking a blood meal, which injects sporozoites in the process. These sporozoites are carried through blood vessels to the liver where they develop in the liver cells (hepatocytes) into infective blood stage merozoites. Each bite inoculates about 15-123 sporozoites (Rosenberg *et al.*, 1990; Prudencio, 2006). Sporozoites invade hepatocytes and undergo asexual multiplication resulting in the release of 20 000-30 000 merozoites per original sporozoite (Todryk and Hill, 2007). This liver stage of the life cycle is symptomless. After the rupture of an infected hepatocyte, merozoites which are released infect red blood cells resulting to the pathology of malaria such as fever, loss of renal function, malarial anemia, cerebral malaria and comma (Rosenthal, 2008).

Efforts for global eradication of malaria are being deployed in several approaches including vector (mosquito) control measures, anti-parasite drug innovations and vaccine research and development. However, treatment and vector control approaches are currently dependent on few compounds and thus are vulnerable to emergence of resistant parasites and vectors (Todryk and Hill, 2007; Gesase *et al.*, 2009). Moreover, new malarial drugs such as chemical derivatives of artemisin are too expensive to be afforded by poor countries and still in limited supply (Ro *et al.*, 2006). Further, the development of therapeutic and potentially preventive vaccine for malaria is hampered by the complex biology of the parasite, making it a difficult, challenging and a long process. It has been found that *Plasmodium* parasite has more than 5200 genes that could code for protective antigens (Duffy *et al.*, 2005) making identification of candidate vaccine antigens difficult. This scenario renders it more complex because these antigen genes are differentially expressed during the life cycle of the parasite and that many antigens display a high degree of variability.

Both pre-erythrocytic and erythrocytic stage vaccines have been under intense investigation. However for prophylactic purposes, a pre-erythrocytic vaccine is considered advantageous as it aims at preventing or reducing the acquisition of clinical infection (Girard *et al.*, 2007).

Studies on the erythrocytic stage vaccine candidate antigen, falciparum malaria protein 1 (FMP1), which is a fragment of merozoite surface protein-1 (MSP-1), showed that antibodies to FMP1 were associated with protection of malaria in the Gambian population (Corran *et al.*, 2004). However a recent study in Kenya (Ogutu *et al.*, 2009) has shown no evidence that FMP1 protects against *P. falciparum* malaria in an area of holoendemic

transmission, suggesting that the antigen is no longer a promising candidate for the development of monovalent blood stage malaria vaccine. On the other hand, a clinical trial of a pre-erythrocytic stage *P. falciparum* malaria vaccine RTS, S which is derived from circumsporozoite protein (CSP) showed reductions of clinical disease by 35% and 49% after a follow up of 18 months in Mozambican children (Alonso *et al.*, 2004, 2005). This study affirms the feasibility of pre erythrocytic malaria vaccines and their potential impact to the burden of malaria disease in children.

Epitopes from liver stage Plasmodium proteins may be presented by HLA class I molecules on the hepatocyte, rendering the parasitized hepatocyte susceptible to killing by cytotoxic T lymphocytes (CTLs). However HLA polymorphisms such as the presence of heterozygous class 1 alleles among individuals in different populations resulting into variation in their binding affinities to protein peptides, leads to complexity of developing a successful malaria vaccine (Lyke *et al.*, 2005). Thus understanding common HLA class 1 alleles in the population in question is of paramount importance in the process of malaria vaccine development.

Considerable evidence has implicated CD8+ T-cells as critical effector cells in protective immunity against pre-erythrocytic stage malaria (Hill *et al.*, 1992; Hoffman *et al.*, 1996; Doolan *et al.*, 1996, 2001). $CD8^{+}$T cells specific for parasite–delivered peptide/class 1 Major Histocompatibility Complex (MHC) molecule complexes on the surface of infected hepatocytes are primary immune effectors of cell mediated immunity (Hoffman, 2000). CD8+ T-cells recognize parasite-derived peptides that are presented in association with class I HLA on the surface of infected hepatocytes. The infected hepatocytes may be the primary target of cell-mediated immune responses (Doolan and Hoffmann, 1997) due to

the reason that they are the only host cells expressing MHC encountered by the parasite during the course of its life cycle (Kurtis *et al.*, 2001).

Many studies have investigated on the pre-erythrocytic antigens which can be used to induce CTL immune response necessary to prevent the development of Plasmodium sporozoites to deadly blood stage merozoites. Aidoo *et al.* (1995, 2000) studied CTL epitopes by HLA class I molecules from pre-erythrocytic antigens, including circumsporozoite protein (CSP), Thrombospondin Related Adhesion Protein (TRAP), Sporozoite Threonine and Asparagines Rich Protein (STARP), Liver Stage Antigen 3 (LSA 3) and Exported Protein 1 (Exp-1). These antigens are expressed in the sporozoites and in the liver stage of the parasite. Liver stage antigen 1 (LSA 1), on the other hand was reported to be the only pre-erythrocytic antigen which is exclusively expressed in the liver (Hillier *et al.*, 2005). Earlier on, Connelly (1997) had reported that LSA1 was associated with T cell proliferation in more than 90% of adults in Papua New Guinea where malaria in holoendemic.

Several studies in other African countries such as Gabon (Luty, 1999), Kenya (John *et al.*, 2004) have shown that IFN-γ responses to LSA1 are associated with delayed time to re-infection with *falciparum* malaria. These well characterized pre-erythrocytic antigens apart from the LSA 1 are detected in decreasing amounts as the parasite develops in the liver (Kurtis, 2001). CSP and TRAP which are involved in the invasion of liver cells, are present on the surface of infective sporozoites (Moorman *et al.*, 2006), while LSA 3 is found in the sporozoites and in the liver stage (Aidoo, 2000). Being the only liver stage antigen that is increasingly produced in the liver as the parasite develops, LSA 1 seems to be the only

suitable candidate for the liver stage vaccine development strategies, thus creating a need for more liver stage antigens.

Efforts are being made to search for more liver stage antigens which can be incorporated in the pre-erythrocytic liver stage vaccine. The present study aimed at studying novel liver stage antigens as targets of IFN-γ immune responses in children living in a malaria endemic area of Morogoro, Tanzania.

1.2 Justification

Pre erythrocytic stage vaccine includes antigens from the sporozoite and liver stages of the Plasmodium parasite. Taylor-Robinson (2002) reported that a vaccine that targets pre-erythrocytic parasites in the liver could potentially control malaria by blocking development of the pathogenic erythrocytic stage and subsequent parasite transmission. There are reports which have concluded that pre-erythrocytic stage vaccines can induce a state of strong sterile immunity both in human and animal models (Druilhe and Barnwel, 2007). Pre-erythrocytic stage antigens such as CSP, TRAP, LSA-3 and Exp 1 have been shown to induce considerable T cell responses which may be protective against malaria, but most of these antigens are detected in decreasing amount as the parasites develop, which might limit their efficacy as vaccines.

This prompts us to search for more suitable liver stage antigens which will be potentially effective in the development of pre-erythrocytic malaria vaccine.

1.3 Objectives

1.3.1 General Objective

To evaluate the immune response of children to HLA class I-restricted peptides derived from genes/proteins that are expressed or up-regulated during the liver stage of *P. falciparum* infection.

1.3.2 Specific objectives

1. To quantify the number of IFN-γ secreting T cells in response to stimulation with novel liver stage antigens, matched or unmatched by HLA type.
2. To evaluate the T cell responses in relation to parasitological and haematological parameters of the children under study.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Life cycle of *Plasmodium falciparum*

Malaria is a disease caused by parasites of the genus *Plasmodium*. It is initiated when an infectious female Anopheles mosquito inoculates sporozoites into the circulation of the host during a blood meal. An average of 15-123 sporozoites is injected during a blood meal (Prudencio *et al.*, 2006). Sporozoites migrate to the liver, where they invade hepatocytes within 30-60 minutes of inoculation and develop as pre-erythrocytic forms for about 5-10 days depending on the species, approximately 7 days for *P. falciparum* (Todryk and Hill, 2007). About 10 000-30 000 merozoites are produced for each infected hepatocyte (Moorthy and Hill, 2002). Infected hepatocytes eventually rupture to release merozoites which enter the circulation, invade erythrocytes and undergo another asexual replication, producing as many as 36 merozoites per mature schizont. Many red blood cells are destroyed in the process, contributing to malarial anaemia. This erythrocytic stage is responsible for the symptoms and pathology of malaria (Todryk and Hill, 2007). *P. falciparum* infected cells often sequester and adhere to uninfected erythrocytes and to the vascular endothelium resulting into occlusion of microvasculature (Mackintosh *et al.*, 2004; Rosenthal, 2008). Some parasites do not undergo asexual replication; instead they develop into the sexual forms (male and female gametocytes). These are taken up by mosquitoes during a blood meal and emerge from within the red blood cells as gametes, which then fertilize and mature within the mosquito midgut. New sporozoites migrate to the salivary gland, and the life cycle recommences (Fig. 1).

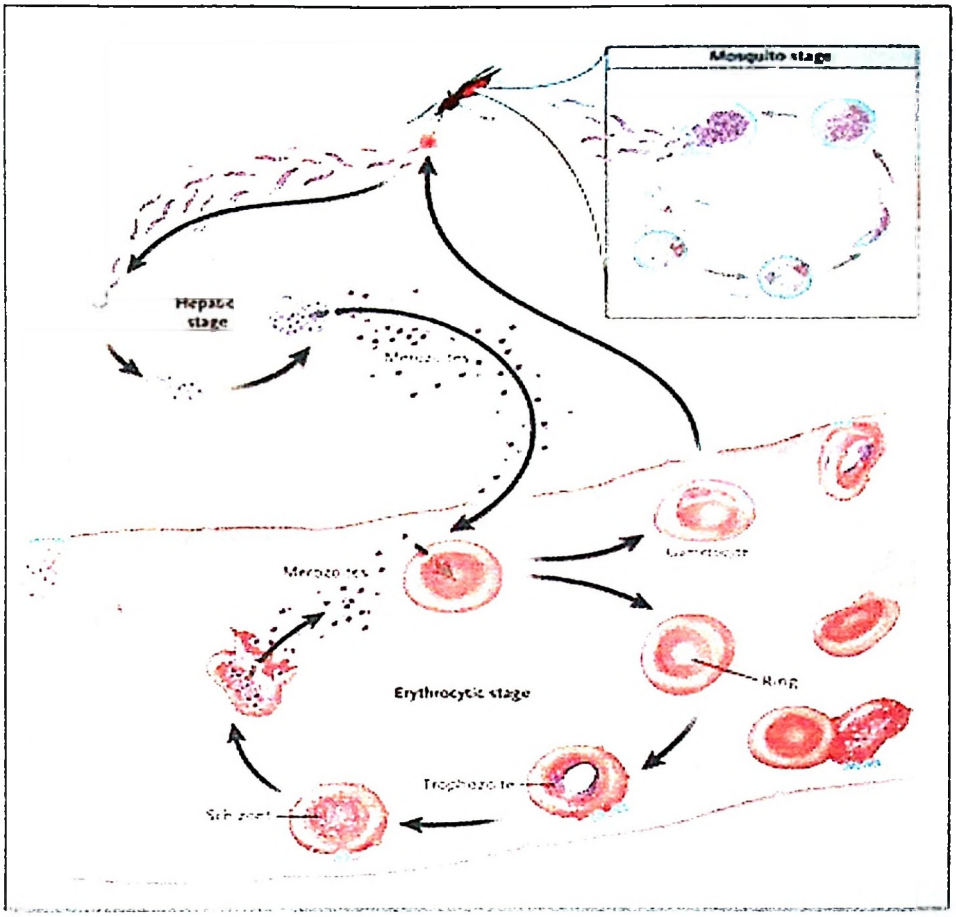


Figure 1: Life Cycle of *Plasmodium falciparum.*

Erythrocytes containing P. falciparum in mature intraerythrocytic stages (trophozoites and schizonts) adhere to vascular endothelium, thereby avoiding clearance by the spleen. High numbers of circulating parasites and elaboration of host and parasite factors in the vasculature of various organs lead to the manifestations of severe malaria. Source: Rosenthal (2008).

2.2 Plasmodium Antigens

Studies have revealed several Plasmodium antigens both in pre-erythrocytic and erythrocytic stage of the *P. falciparum* lifecycle (Tongren *et al.*, 2004), all of which can serve a protective role against malaria. Highly expressed pre-erythrocytic antigens are CSP, TRAP, LSA-3 and Exp 1 (Fig. 2). Once within the hepatocytes additional antigens are expressed, LSA-1 being one that has been characterized to date. These antigens have been well studied both in humans and experimental animals and have been shown to induce protective immune responses and therefore are considered to be the major antigens for malaria vaccine development.

The liver plays a crucial role in the *Plasmodium* life cycle, as hepatocytes are an obligatory site for schizogony, a process of amplification and molecular changes for the parasite (Taylor-Robinson and Heal, 2001). Although only a small number of extracellular sporozoites invade the liver, schizogony increases the parasite load and gives rise to a richer pool of antigens (Fig. 2). Blood stage antigens include Merozoite surface protein (MSP) (MSP 1, MSP 2 MSP3, Glutamate-Rich Protein (GLURP) and (Apical membrane antigen 1) AMA-1.

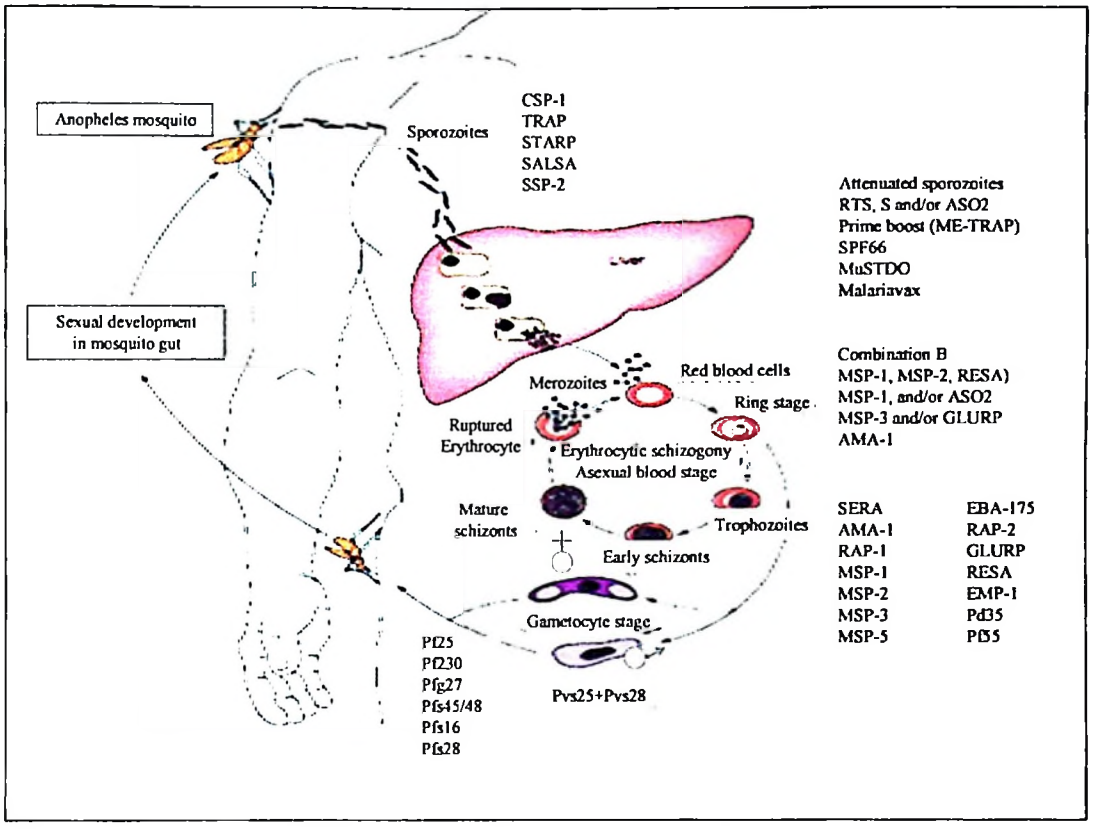


Figure 2: Plasmodium antigens for malaria vaccine development.

Life cycle of P. falciparum showing individual antigens that are being or have been evaluated as vaccine candidates, and vaccine constructs that are currently being or have been evaluated in clinical trials for each stage of the life cycle. Source: Tongren (2004).

2.3 Malaria Vaccines Strategies

Malaria vaccine development aims to significantly reduce mortality and morbidity in the two high-risk groups: young children and pregnant women in sub-Saharan Africa. Pre-erythrocytic vaccines target the sporozoites by generating inhibitory antibodies which will block sporozoites from infecting hepatocytes (Matuschewski, 2006). Moreover they target the infected hepatocytes by generating IFN-γ-secreting effector T cells against the antigens expressed by infected hepatocytes, which prevent merozoite release by killing infected

hepatocytes or interfering with parasite development (Todryk and Hill, 2007). On the other hand blood-stage vaccines aim to reduce the asexual parasite burden by antibody-mediated inhibition of merozoite invasion of the red blood cells or of adhesion of the endothelium by infected erythrocytes. Some important developments towards anti malaria vaccines are shown in Table 1.

Table 1: Comparison of some malaria vaccine development strategies

Strategy	Immune response	Example candidate	Pros	Cons	Latest development
Subunit					
Pre-erythrocytic stage					
Protein immunization	Humoral and some cellular	RTS,S/AS02	Demonstrated efficacy in field	Transient immunity	Partial protection in African children for 18 months
Prime-boost	Humoral and cellular	DNA-MVA[c] -ME-TRAP[d]	Strong T-cell responses	No field efficacy	FP9[e]-MVA-ME-TRAP Phase II b field study underway
Erythrocytic stage	Humoral	MSP1, MSP2[f] and RESA[g]	Prevent disease/death	Antigenic polymorphism	In field study 3-component vaccine reduces parasitemia
Sexual stage	Humoral	Pvs25[i]	Prevent transmission	No disease protection	Phase Ia clinical study underway
Whole organism					
Sporozoite	Cellular	Attenuated sporozoite	Protective in animal and human models	Logistic concerns	Encouraging data with knockout sporozoites
Low dose blood stage	Cellular	*P. falciparum* and *P. chabaudi*	Small antigenic dose	Safety concerns	Heterologous protection with low dose parasite in mice

Source: Pinzon-Charry *et al.* (2006)

2.4 Cellular Immune Response

The cellular immune response is mediated by T cells that can be subdivided into two subsets, T helper cells (Th) which express the surface marker CD4+ and cytotoxic T cells (CTLs) which express the surface marker CD8+. Both CD4+ and CD8+ lymphocytes are

critical for the protection against infectious microbes. Studies by Tsuji and Zavala (2003) showed that T cells from different subsets play a major role in protective immunity against pre-erythrocytic stage of malaria parasites by inhibiting parasite development in the liver. CD4+ T cells play a crucial role in regulation of virtually all immune responses, providing help for antibody production by B cells, and growth factors for B cells. In malaria infection, CD4+ T cells play a triple role in protective immunity against the liver stages of the malaria parasite: (1) CD4+ T cells help B cells to induce a high level of antimalaria humoral response, (2) they assist in the induction of CD8+ T-cell responses; and (3) they directly inhibit the development of liver stage parasites (Tsuji and Zavala, 2003). On the other hand CD8+ T cells are important in production of cytokines (IL-2, IFN-γ, TNF-α), chemokines (RANTES, MIP-I α, MIP-I β) and antigen specific lysis of MHC class 1 matched target cells (Ochiel *et al.,* 2005). Target killing by the CD8+ T cells can be mediated by the release of granules containing cytolytic effector molecules such as perforins, granzymes and lysins (Janeway *et al.*, 2005). CD8+ cells are implicated in killing the *P. falciparum* while in the hepatocytes, hence providing a protective role against malaria.

2.5 T cell Immunity in Malaria

The potent protective immunity against malaria induced by immunization of mice and humans with radiation-attenuated *Plasmodium* sporozoites is thought to be mediated primarily by T-cell responses directed against infected hepatocytes (Doolan and Hoffman, 1997). There are now data indicating that CD8+ T cells, CD4+ T cells, cytokines, and nitric oxide (NO) can all mediate elimination of infected hepatocytes *in vitro* and *in vivo* (Mellouk, 1994). It has been demonstrated that different T cell-dependent immune responses mediate protection by dissecting the protection induced by immunization with

irradiated sporozoite, DNA and synthetic peptide adjuvant vaccines. Hoffman *et al.* (2002) summarized data showing that immunization of human with radiation-attenuated *Plasmodium* parasites confers sterile protection against sporozoite challenge. Several studies in rodents and nonhuman primate models had first reported the same (Nussenzweig *et al.*, 1967). The critical effector mechanism in the radiation attenuated sporozoite model is thought to be CD8+ T-cell responses directed against parasite antigens expressed in the liver stage (Doolan and Martinez, 2006).

2.6 Role of Interferon Gamma in Protection of Intracellular Parasites

Interferons (IFNs) are natural cell signalling proteins produced by the cells of the immune system in response to challenges by viruses, parasites and tumour cells. They are classified into three types based on the type of receptors through which they signal. Type I interferons bind to specific cell surface receptor complex called Interferon alpha receptor (IFN-α). In humans they include IFN-α and IFN-β. Type II interferons bind to Interferon gamma receptors (IFNGR) and in human is the IFN-γ. Type III interferons signal through a receptor complex consisting of IL10R2 and IFNLR1.

IFN-γ or type II interferon is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumour control. IFN-γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4+ and CD8 +cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops.

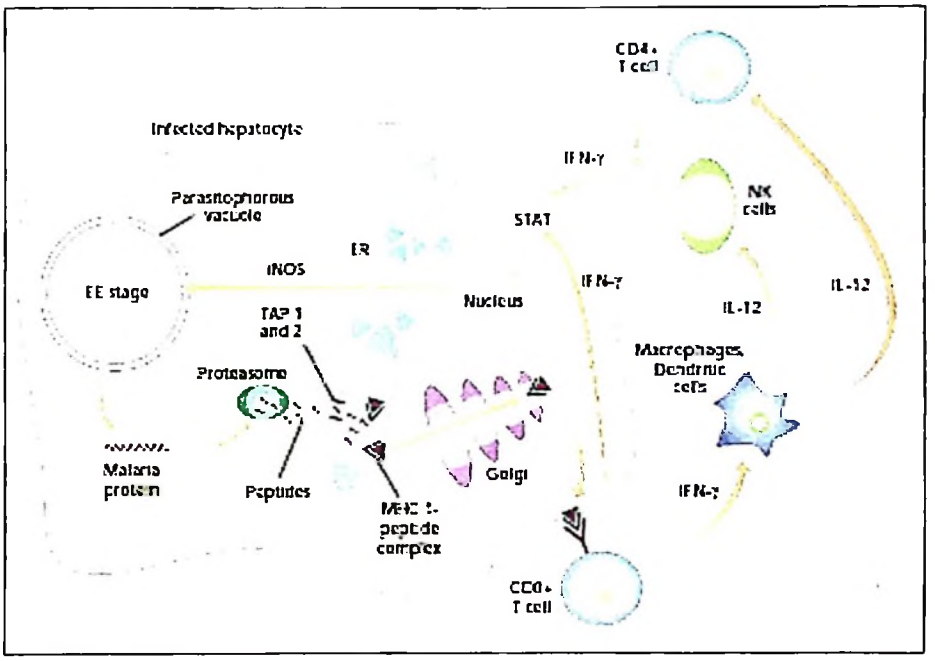
IFN-γ production enhances cytolytic mechanisms by increasing class I molecules on the surface of hepatocytes, making those hepatocytes better targets for CTL lysis, and

activating subsidiary effector cells such as NK cells and macrophages. These effector cells are also capable of killing target cells through CTL-like mechanisms, tumour necrosis factor-α (TNF- α) induction and NO production. Studies in animal models (Ferreira, 1986; Doolan, 2000) reported that IFN-γ produced by CD8+ T cells induce infected hepatocytes to produce L-arginine-derived NO which is toxic to intracellular parasites. It has been shown that parasite-derived Plasmodium antigens are recognized by CD8+ T-lymphocytes on the surface of infected hepatocytes presented in the context of MHC class 1 molecules. This triggers different effector mechanisms such as direct lysis of malaria infected hepatocytes by activated CD8+ T-lymphocytes and inhibition of parasite growth mediated by cytokines such as IFN-γ released by stimulated T-lymphocytes (Mellouk *et al.*, 1994).

2.7 Proposed Mechanism of Protective Immunity Directed Against the Plasmodium Infected Hepatocyte

In animal models, protective immunity directed against pre-erythrocytic stage malaria is primarily mediated by antigen-specific CD8+ T cells that recognize parasite-derived peptides presented in association with MHC class 1 molecules on the surface of infected hepatocytes (Hoffmann and Doolan, 2000). Short peptides are derived from the cytoplasmic malaria protein by the proteolytic action of proteasomes. The peptides are imported into the endoplasmic reticulum (ER) via the transporters associated with antigen processing, TAP1 and TAP2. In the ER, the peptides associate with the MHC class I molecule, and the peptide/MHC complex passes through the Golgi apparatus to the cell surface where it can be recognized by the T cell receptor on the surface of CD8+ T cells. IFN-γ is produced as a direct consequence of the CD8+ T cell activation, and subsequent production of IFN-γ may be upregulated by a positive feedback loop involving interleukin (IL)-12 (produced by dendritic cells, macrophages or other cells), NK cells and/or CD4+ T

cells, depending on the host. IFN-γ, via signal transducers associated with transcription (STAT), activates nitric oxide synthase (iNOS) and induces the L-arginine dependent NO pathway to eliminate the infected hepatocyte or the intrahepatic schizonts.



Source: Hoffman (2000)

Figure 3: Mechanisms of protective immunity directed against the *Plasmodium* infected hepatocyte.

2.8 Interferon Gamma ELISPOT Assays

The enzyme-linked immunospot (ELISPOT) assay was established in the late 1980's for assessment of cytokine release at a single cell level (Czerkinsky *et al.*, 1988). The assay is increasingly being used for the quantitative assessment of peptide reactive T lymphocytes from peripheral blood mononuclear cells (PBMC) in infectious disease (Yang *et al.*, 2000).

It is also used in the course of vaccination trials aimed at the induction of tumour-specific T cells in patients with cancer (Scheibenbogen, 2000).

The ELISPOT assay detects individual T cells present in peripheral blood that release IFN-γ on stimulation with specific antigen. It thus provides an immediate reflection of the precursor frequencies of peptide-specific IFN-γ producing cells in peripheral blood (Lanagan *et al.*, 2003). It is simple to perform and provides a highly sensitive method for assessing cytokine production in malaria exposed populations. Thus for measuring natural immune responses or response following vaccination, the technique of IFN-γ ELISPOT has proven very useful. This assay is able to give absolute numbers of antigen-specific IFN-γ secreting cells (CD8+ and CD4+) in a rapid (18 hours) or long cultured (15 days) assay involving cytokine capture on a cellulose membrane and antibody-mediated colour development revealing a spot for every cytokine-secreting cell. The ELISPOT has been successfully used to assess CD8+ T-lymphocyte responses to tumours and viral infections in humans or the response to malaria infection by CD4+ cells (Elghazali *et al.*, 1997). Furthermore, CD8+ T-lymphocyte responses in rodent malaria model have been also studied by this method (Miyahira *et al.*, 1995). ELISPOT allows the detection of the IFN-γ production at the single cell level and permits the enumeration of antigen specific T-lymphocytes present at relatively low frequencies in peripheral blood lymphocytes.

2.9 Human Leukocyte Antigens and Malaria

The MHC is a dense complex of genes with immunological and non-immunological functions and is present in all vertebrates. In humans it is known as HLA system (Marsh *et al.*, 2000). HLA allele frequencies exhibit ethnic variation, with some alleles found widely distributed among populations and others almost exclusively within particular ethnic

groups. Infection by *P. falciparum* is one of the major driving forces for the selection of various HLA genes and it has been shown that in malaria endemic areas, there is selection of some HLA alleles which are involved in protection (Ghosh, 2008). There have been large HLA association studies with malaria infections. In the Gambia HLA-B53 was found to efficiently present certain malaria antigens to generate CTLs which were associated with a reduced risk of severe malaria in childhood (Hill *et al.*, 1991).

In Thailand, HLA B15 was found to be highly associated with protection from malaria disease when compared with other HLA B alleles (Hananantachai *et al.*, 2005). HLA-B35 has also been shown to be associated with resistance to severe malaria in several populations in which it recognizes the highly polymorphic Th3R region of the CSP (Gilbert, 1998). Four allelic variants of these CSP antigens exist in Gambia where variants (cp 26 and cp29) bind HLA-B35 and elicit a CTL response, whereas two others (cp27 and cp28) fail to bind to HLA-B35 (Hill, 1994; Gilbert, 1998).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Study Population

This study was carried out as part of the Mother Offspring Malaria Studies (MOMS) project at Morogoro regional hospital in Tanzania. In this area, malaria transmission occurs throughout the year, with two peak transmission seasons from March to May and October to December. Children less than five years of age experience the greatest morbidity and mortality from malaria, with severe anaemia as the major cause of malaria-related mortality in this area. In the MOMS project, blood samples from infants from birth onwards and children under five years of age are collected routinely for the detection of malaria parasites and the determination of haemoglobin (Hb) concentrations. Children are recruited from the villages in and outside Morogoro municipality. Children found febrile (axillary temperature 37.5°C) with any density of parasitemia, or afebrile with parasitemia more than 5000/L of blood were treated with standard doses of sulfadoxine/ pyrimethamine (SP).

3.2 Ethical Clearance

The MOMS project has been reviewed and accepted by Institutional Review Boards in Tanzania (National Institute for Medical Research (NIMR) and Medical Research Coordinating Committee (MRCC): Identification of natural ligands and soluble mediators involved in malaria during early life, Permit number; NIMR/HQ/R.8a/Vol.IX/394of 29 September 2005 and updated on January 2008) and U.S.A (US Western Institutional Review Board (WIRB): Identification of natural ligands and soluble mediators involved in malaria during early life; Permit number WIRB Pro.Num 20040974).

Informed consent was obtained from all parents/guardians bringing the kids to the clinic prior to the donation of 5ml of venous blood into EDTA anti-coagulant.

3.3 Peptides

HLA typing of a sample population from Tanzania was previously done by the MOMS project (unpublished data) to determine the alleles that occur with high frequency in the population (Fig. 4).

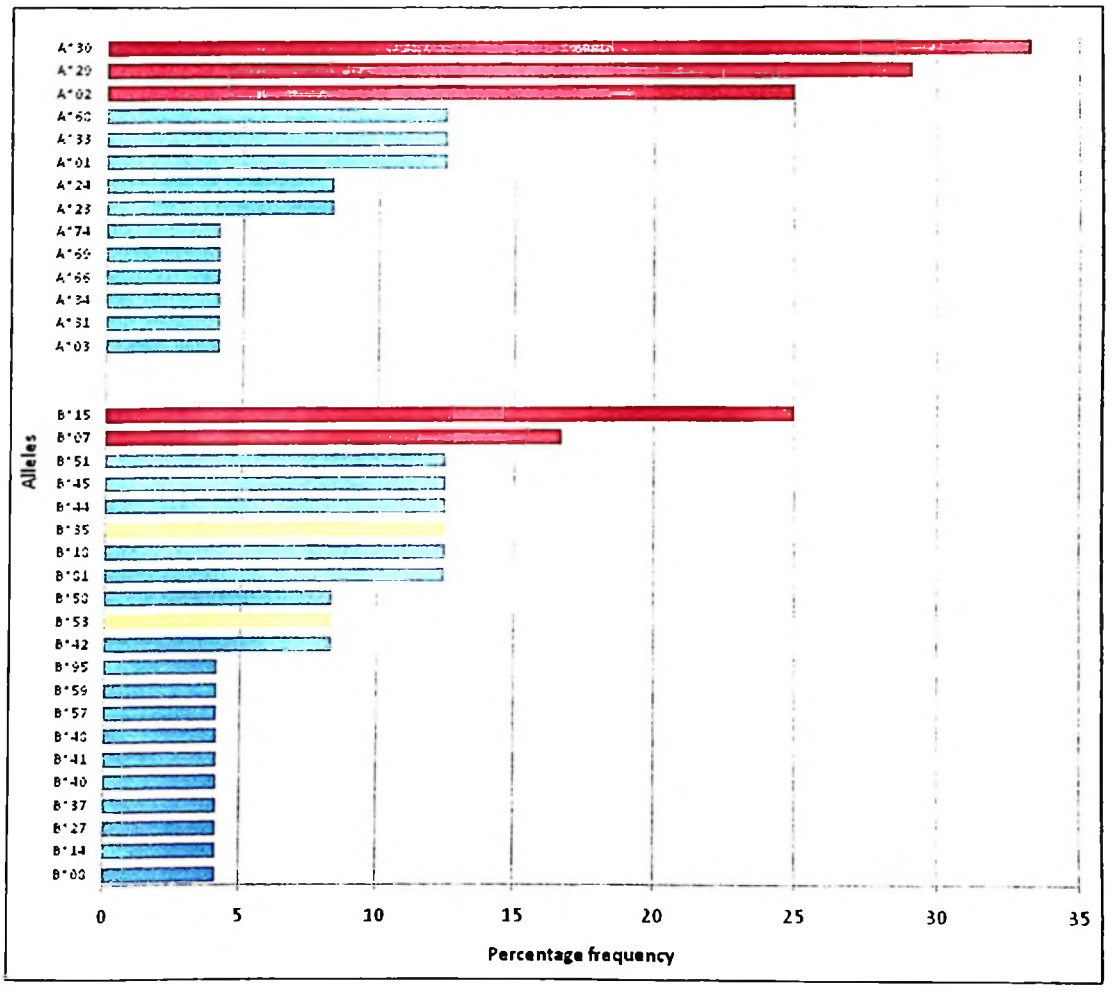


Figure 4: HLA Class 1 Typing Data for a subset of Tanzanian population.

Proteins expressed in the pre-erythrocytic stages of *P. falciparum* were then screened for the presence of the peptide-binding motif to the common HLA class I molecule alleles that occur with high frequencies in Tanzanians. A total of 541 peptides were designed from 30 genes that were thought to be expressed or upregulated specifically in the liver stage of the *P. falciparum* and the peptide design was done using bioinformatics approach (Table 2). In this approach, 9 amino acid long peptides were detected from the liver stage genes that can bind HLA alleles A02, A29, A30, B07, B15, B35 and B53 using the Microsoft Research Corporation Epitope prediction software (http://atom.research.microsoft.com/ bio/epipred. aspx).

Table 2: Liver stage specific peptides

Pf gene name	aa size	Putative function
PFLSA 4	857 aa	adapter-related protein
PF08_0054	677aa	Heat Shock 70 KDa Protein, (HSP70)
PFLSA 5	594 aa	repeat organellar protein
PFLSA 6	164 aa	conserved hypothetical protein
PFLSA 7	2940 aa	S22; conserved hypothetical protein
PFLSA 8	74 aa	conserved hypothetical protein
PFLSA 9	943 aa	conserved hypothetical protein
PFLSA 10	3597 aa	conserved hypothetical protein
PFLSA 11	462 aa	serine hydroxymethyltransferase, putative
PFLSA 12	1620 aa	putative zinc carboxypeptidase
PFLSA 13	1410 aa	conserved hypothetical protein
PFLSA 14	1946 aa	conserved hypothetical protein
PFLSA 15	224 aa	conserved hypothetical protein
PFLSA 16	327 aa	transcription initiation factor TFiid, TATA-binding protein, putative
PFLSA 17	1894 aa	RNA-binding protein, putative
PFLSA 18	420 aa	synthetic antigen of P.falciparum, putative (PbUIS8)
PFLSA 19	387 aa	conserved hypothetical protein
PF10_0164	108 aa	UIS4
PF13_0012	229 aa	UIS3
PFLSA 20	230 aa	beta-hydroxyacyl-ACP dehydratase precursor
PFLSA 21	553 aa	cysteine desulfurase, putative (PyUIS5)
PFLSA 22	671 aa	conserved hypothetical protein (PbUIS27)
PFLSA 23	395 aa	conserved hypothetical protein (PyS14)
PF11_0351	663 aa	Heat Shock 70 KDa Protein (HSP70) (PbUIS24)
PFL0800c	421 aa	CelTOS / Antigen 2
PFC0210c	397 aa	CSP
PF10_0356	251 aa	LSA-1
PFLSA 24	359 aa	2-phosphoglycerate kinase, putative
PFLSA 25	301 aa	conserved hypothetical protein
PFLSA 26	603 aa	WD repeat protein, putative

3.4 PBMCs Separation From Whole Blood

PBMCs were separated from whole blood by Ficoll-Hypaque density gradient centrifugation. In brief, whole blood sample was diluted 1:4 with RPMI media and carefully blood suspension was overlaid on top of lymphocyte separation media which was firstly put in a 15ml conical tube. The conical tube was span at 2200 rpm for 15 minutes (without brakes). Then a white layer of cells was transferred to another 15ml conical and the cells were washed with PBS by spinning at 1500 rpm for 15 minutes (with brakes). The Supernatant was removed and cells were re-suspended in 5ml RPMI and span at 1500 rpm for 5 minutes. The Supernatant was again removed and cells were re-suspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% human AB serum, 50g of gentamicin 1ml/L, streptomycin 10ml/L, amphotericin B, sodium bicarbonate 2g/L and 2mM glutamine10ml/L, Non essential amino acids 10ml/L, Sodium citrate 10ml/L and Mercaptoethanol (ME) 3µl/L, the mixture referred to as R10 media. PBMCs were resuspended at a concentration of $2x10^6$ cells/ml.

3.5 PBMCs Stimulation

Isolated PBMC were plated in 96 well flat bottom culture plates (Millipore, Inc., Billerica, Mass) at $2x10^5$ cells/well (150µl of cell suspension) in duplicate wells, and test peptides at 5µg/ml (50 µl) were added. Media alone was used as negative control and represented the background level of IFN-γ produced by non stimulated cells. Cells were incubated at 37°C in 5% CO_2 for 12-15 days, and then transferred to ELISPOT plates for determination of the quantity of IFN-γ producing cells.

3.6 IFN-γ ELISPOT Assay

In the second stimulation for quantifying the IFN-γ producing cells, sterile 96-well polyvinylidene difluoride PVDF-backed plates (referred to as ELISPOT plates) (Millipore, Inc., Billerica, Mass) were pre coated at 4°C overnight with 50 µl of 1µg/ml of anti-human-IFN-γ 1 DIK monoclonal antibodies in phosphate-buffered saline (PBS) (Mabtech USA). The following day plates were washed six times with sterile PBS ready for the second stimulation. PBMCs from the first stimulation were span down for 5 minutes at 1500 rpm, supernatant discarded, then washed twice by adding 200µl sterile PBS, span and discarded the supernatant. They were then re-suspended in 150µl R10 medium. $2x10^5$ cells/well (150µl of cell suspension) cells were transferred to the coated ELISPOT plates, and 25µl peptides were added per well at a final concentration of 5µg/ml with a positive control and PMA, IONOMYCIN as mitogen control. Media alone was used as the negative control and represented the background level of IFN-γ produced by non stimulated cells.

Cells were incubated at 37°C in 5% CO_2 for 24 hours. Plates were then washed six times with 1×PBS in the presence of 0.5% Tween 20 (PBS-T), 100µl/well of 1 µg/ ml biotinylated anti-IFN-γ (clone 7-B6-1, MabTech, Sweden) added per well, and the plates incubated for 1 hour at room temperature. Plates were washed three times with 1×PBS-T and 100 µl/well streptavidin-alkaline phosphatase conjugate (MabTech, Sweden) was added at 1:1000 dilutions in PBS. After 1 hour incubation at room temperature, plates were washed three times with 1×PBS-Tween , and developed with BCIP/NBT SOLUTION (SIGMA) conjugate substrate, (BioRad Laboratories, Hercules, CA) according to the manufacturer's instructions. After 3 minutes, the plates were rinsed extensively with distilled water to stop the colorimetric reaction, dried and stored in the dark. The number of

spot-forming units (SFU) per well was counted by using ImmunoSpot scanning and imaging software (Pharmingen).

Criteria for positive stimulant wells included (i) >8 spots per well (ii) the number of stimulant wells to be more than twice media control (iii) the mean number of spots in stimulant wells to differ significantly from media control by a paired t test (one tailed, $P<0.05$) as described by Lyke *et al.* (2005).

3.7 Statistical Analysis

Contingency table based on chi-square test was used to compare proportions of IFN-γ responders to the new liver stage specific antigens after grouping the data by age or parasitemia. The level of significance in all cases was set at $P<0.05$. Statview, SAS institution Inc, was used for all statistical analysis.

CHAPTER FOUR

4.0 RESULTS

This study investigated children in terms of IFN-γ secretion by T cells, haematological and parasitological parameters. The T cell responses were induced by pools of liver stage specific *P. falciparum* peptides predicted to be upregulated during the liver stage of the infection. Peptide pools were grouped from 541 peptide antigens that were predicted to be upregulated during the liver stage of *P. falciparum* and the pooling strategy was either by HLA type (HLA matched) or by protein pools (HLA unmatched). Each pool comprised an average of 12 peptides. In all cases, IFN-γ secreting T cells were quantified by the standard ELISPOT assay.

4.1 Response of Children to Stimulation with Peptides Matched by Different HLA class 1 Alleles

Peptides matched by HLA class one alleles, HLA A02, HLA B15 and HLA B35, which are among those known to occur with high frequency among Tanzanians (Fig. 3, unpublished data), and several HLA unmatched peptides were used in this study. Data shown in Table 3 depict that 63% of the children responded to HLA B15-matched peptide antigens when compared to 27% and 15% of the children responding to HLA B35-and HLA A02-restricted peptides, respectively. These differences in number of children responding and those not responding to peptides based on specific HLA alleles were shown to be statistically significant (P=0.0001).

Table 3: IFN-γ response to HLA class 1 matched peptides

HLA Type	Responders	Non Responders	P value
A02	15.2 (7/46)	84.8 (39/46)	<0.0001
B15	63.6 (21/33)	36.4 (12/33)	
B35	22.2 (4/18)	77.8 (14/18)	

4.2 IFN-γ Responses to Liver Stage Specific Peptides Differ Between Age Groups

The liver stage peptides induced different levels of IFN-γ responses in children. In order to determine whether the responses to the peptides were influenced by age, the responses were analysed separately for children aged less than six months and those above six months of age. Results shown in Table 4 indicate that children aged less than 6 months had significantly lower IFN-γ responses upon stimulation as compared to older children (P=0.0001). Thus, while in the group of children aged less than six months, there was a 92.8% non response (and 7.2% response), the group of children above six months of age showed only 40.3% non responses compared to 59.7% responses.

Table 4: IFN-γ responses in children as stratified in two age groups : 0-6 months and >6 months

Age	Response	Non Response	P value
0-6 Months	7.1 (5/70)	92.8 (65/70)	0.0001
> 6 Months	59.7 (46/77)	40.2 (31/77)	

Moreover, when stratified in three age groups of 0-6 months, 7-12 months and >12 months, children above 12 months showed significantly higher responses (63.6%) as opposed to younger children, aged less than six months (8.1%) and those between 7 -12 months (18.2%), respectively (Table 5).

Table 5: IFN-γ responses in children as stratified in three age groups:<6 months, 7-12 months and >12 months

Age	Response	Non Response	P value
0-6 Months	8.1 (5/62)	91.9 (57/62)	0.0001
7-12 Months	18.2 (6/33)	81.2 (27/33)	
>12 Months	63.6 (35/55)	34.4 (19/55)	

4.3 Relationship Between IFN-γ Responses and Parasitemia

In the present study, parasitemia was recorded in 39 out of 46 (84.8%) children not responding to the peptides, although the difference between the two groups was not statistically significant. Also, results have revealed that only 29.5% (31/105) children who responded to the peptide antigens experienced no parasitemia during the two months follow up period (P=0.06, Table 6). Moreover, children with normal Hb levels at the time of sampling and two months later (Hb level of >10g/dl) were significantly more likely to respond (71.9%, 87/121) when compared to those with low Hb levels in which only 7.6%, (2/26) responded to the peptide antigens. (P=0.0281) as depicted in Table 6.

Table 6: Relationship between malaria infection status and Hb levels with IFN-γ responses to peptides

	Response	Non Response	P value
With parasitemia	15.2 (7/46)	84.8 (39/46)	0.06
Without parasitemia	29.5 (31/105)	70.5 (74/105)	
Low haemoglobin	7.6 (2/26)	92.4 (24/26)	0.0281
Normal haemoglobin	71.9 (87/121)	28.1 (43/121)	

4.4 IFN-γ Responses to the Peptides Predicted Resistance to Malaria Among the Older Children

Among the older children (>6 months) that responded, 26 out of 33 (78.8%) did not experience malaria parasitemia (P=0.0003) compared to non responders 7/33 (21.2%) within two months of the follow-up period after the test (Table 7).

Table 7: Relationship between response and parasitemia among older children

Response	With parasites	Without parasites	P value
Responders	21.2 (7/33)	78.8 (26/33)	0.0003
Non Responders	54.2 (26/48)	45.8 (22/48)	

CHAPTER FIVE

5.0 DISCUSSION

The goals of this study were to investigate whether healthy and aparasitemic children at the time of sampling, in malaria endemic areas of Morogoro region respond to newly synthesized liver stage specific peptides derived from *P. falciparum* antigens. Due to the importance of understanding HLA restricted adaptive immune responses to defined epitopes of malaria antigens for development of a successful malaria vaccine, peptide antigens were pooled according to the HLA class one alleles commonly expressed by Tanzanians. Attention was focused on IFN-γ producing T cells since many studies have shown that this cytokine is associated with protection from malaria infection (Luty *et al.*, 1999; Kurtis *et al.*, 2001).

This study has shown that children responded significantly more to the HLA B15 matched peptides (63%) as compared to the peptides matched to other HLA class 1 alleles, HLA B35 and HLA A02. This is finding consistent with the study conducted in north west Thailand (Hananantachai *et al.*, 2005), which investigated 19 HLA B alleles in mild and severe malaria patients and revealed that HLA B15 was the most common allele expressed with a frequency of 20.5 % as compared to other HLA B alleles (1%). Studies by Contu *et al.* (1998) found that HLA B35 was associated with protection from malaria in individuals living in the highland areas of the Sardinia Island in the Mediterranean Sea where malaria is endemic when compared to the low land areas. On the other hand, studies of immune responses to LSA3 and Exp-1, the pre-erythrocytic antigens, revealed HLA A02-restricted CTL responses in the Gambian and Tanzanian naturally exposed to malaria and that the responses were associated with protection (Aidoo, 2000). Lyke *et al.* (2005) investigated

on HLA A02 PBMCs from Malian children if there were responses against epitopes from TRAP, Exp-1 and CSP and reported presence of protective immunity to these pre-erythrocytic antigens. The study concluded that 43% of the Malian individuals have HLA A02 supertypes especially A0202, A0205and A0201.

This study has shown that IFN-γ responses to the new liver stage specific antigens in malaria endemic areas increase with age. Thus, children below 6 months of age responded poorly when compared to older children above six months. This could partly be due to immaturity of the immune system in younger children less than six months (Chelimo *et al.*, 2003) or due to the fact that they have not had a frequent exposure to the parasite antigens. These findings are consistent with the study conducted in Kenya (John *et al.*, 2004, 2009) and Papua New Guinea (Connelly *et al.*, 1997) on liver stage antigen 1 (LSA 1), which revealed that response to LSA 1 was dependent on age and /or repeated exposure to malaria.

The general relationship between liver stage specific T cell responses and parasitemia risk did not achieve significance (P=0.06), although a trend towards decreasing parasitemia was observed in responders. When only children above 6 months were analysed, the response was significantly associated with protection against parasitemia (P=0.003). A study with appropriately powered larger sample size might be able to better define this relationship. The relationship between IFN-γ responses and absence of parasitemia has been shown earlier in a study on LSA 1 and TRAP antigens in Kenyan children by John *et al.* (2004). The mechanism of IFN-γ protection against malaria parasitemia has been suggested to be by elimination of infected hepatocytes through induction of the NO pathway (Hoffmann and Dolan, 2000).

The present results also showed that children responding upon stimulation with the new liver stage antigens had significantly higher Hb levels than those who did not respond. Earlier studies by Ong'echa *et al.* (2003) investigated on the IFN-γ responses to six pre-erythrocytic antigens, which were recognized in the contest of HLA class 1 alleles, HLA A02, HLA A03, HLA B07 and HLA B35 by the Kenyan children and similarly found that Hb levels one month after testing was significantly higher among the responding children than non responders. Higher Hb levels among the responders can be explained by the fact that IFN-γ mediates protection from malaria parasitemia and thus decreased red blood cell lysis. Moreover, Jones *et al.* (2002) has shown that malaria parasitemia induces anaemia through suppression of bone marrow. In addition, IFN γ mediated upregulation of IL-12 increases bone marrow and splenic erythropoiesis (Mohan and Stevenson, 1998), which seems to support the reason for higher Hb levels among responders.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

From this study it can be concluded that the novel liver stage antigens potentially induce considerable IFN-γ responses. Since IFN-γ responses are associated with protection against malaria, the results of this study affirms that these *P. falciparum* liver stage peptides are competent to induce protective immune responses against malaria in children living in malaria endemic areas. These antigens therefore provide suitable candidates for inclusion into the pool of pre-erythrocytic antigens for malaria vaccine candidates.

Due to small quantity of blood donated by children, the analysis was limited to the measurement of the immune responses to pools of epitopes rather than individual epitopes. It would however be recommended to measure response to specific epitopes in order to identify those specifically involved in the protection within each pool.

REFERENCE

Adam, I., Khamis, A. H. and Elbashir, M. I. (2005). Prevalence and risk factors for *Plasmodium falciparum* malaria in pregnant women of eastern Sudan. *Malaria Journal* 4: 1475-2875

Aidoo, M., Lalvani, A., Allsopp, C. E., Plebanski, M., Meisner, S. J., Krausa, P., Browning, M., Jones, S. M., Gotch, F., Fidock, D., Takiguchi, M., Robson, K. J., Greenwood, B. M., Druilhe, P., Whittle, H. C. and Hill, A. V. S. (1995). Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria. *Lancet* 345: 1003-1007.

Aidoo, M., Lalvani, A., Gilbert, S. C., Hu, J. T., Daubersies, P., Hurt, N., Whittle, H. C., Druihle, P. and Hill, A. V. S. (2000). Cytotoxic T lymphocyte epitopes for HLA-B53 and other HLA types in the malaria vaccine candidate liver-stage Antigen 3. *Infection and Immunity* 68: 227-232.

Alonso, P. L., Sacarlal, J., Aponte, J. J., Amanda L. and Macete, E. (2004). Efficacy of the RTS, S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised, controlled trial. *Lancet* 364: 1411-1420.

Alonso, P. L., Sacarlal, J., Aponte, J. J., Amanda, L., and Macete E. (2005). Duration of protection with RTS, S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet* 366: 2012-2018.

Chelimo, K., Peter O., Sumba, P. O., Kazura, J. W., Ofula, A. V. and Chandy. C. J. (2003). Interferon-gamma responses to *Plasmodium falciparum* liver-stage antigen-1 and merozoite-surface protein-1 increase with age in children in a malaria holoendemic area of western Kenya. *Malaria Journal* 2: 37.

Connelly, M., King, C., Bucci, K., Walters, S., Genton, B., Alpers, M. P., Hollingdale, M. and Kazura J. W. (1997). T cell immunity to peptide epitopes of liver-stage antigen 1 in an area of Papua New Guinea in which malaria is holoendemic. *Infection and Immunity* 6: 5082-5087.

Contu, L., Carcassi, C., Orru, S., Mulargia, M., Arras, M., Boero, R., Gessa S., Loizedda A.L., Lai S. and Floris L. (1998). HLA-B35 frequency variations correlate with malaria infection in Sardinia. *Tissue Antigens* 52: 452-461.

Corran, P. H., O'Donnell, R. A., Todd, J., Uthaipibull, C. and Holder, A. A. (2004). The fine specificity, but not the invasion inhibitory activity, of 19-kilodalton merozoite surface protein 1-specific antibodies is associated with resistance to malarial parasitemia in a cross-sectional survey in The Gambia. *Infection and Immunity* 72: 6185–6189.

Czerkinsky, C. C., Anderson, G., Ekre, H. P., Nielsson, L. A., Klaereskog, L. and Ouchterlony, O. (1988). Reverse ELISPOT assay for clonal analysis of cytokine production: Enumeration of gamma-interferon secreting cells. *Journal of Immunological Methods* 110: 29-36

Dimopoulos, G., Christophides, G. K., Meister, S., Schultz, J., White, K. P., Carolina Barillas-Mury, C. and Kafatos, F. C. (2002). Genome expression analysis of *Anopheles gambiae*: Response to injury, bacterial challenge and malaria infection. *Proceedings of the National Academy of Sciences* 99: 8814–8819.

Dong, Y., Xi, R. A. Z., Mongin, E. and Dimopoulos, G. (2006). *Anopheles gambiae* immune responses to human and rodent Plasmodium parasite species. *Public Library of Science* 2: 52.

Doolan D. L. and Martinez-Alier N. (2006). Immune response to pre-erythrocytic stages of malaria parasites. *Current Molecular Medicine,* 6: 169-185.

Doolan, D. L. and Hoffman, S. F. (2000). The Complexity of Protective Immunity against Liver-Stage Malaria. *Journal of Immunology* 165: 1453-1462.

Duffy, P. E., Krzych, U., Francis, S. and Fried, M. (2005). Malaria vaccines: using models of immunity and functional genomics tools to accelerate the development of vaccines against *Plasmodium falciparum. Vaccine* 23: 2235-2242.

Druilhe, P. and Barnwell, J. W (2007). Pre-erythrocytic stage malaria vaccines: time for a change in path. *Current Opinion in Microbiology* 10: 371-378.

Elghazali, G., Perlmann, H., Rutta, A. S. M., Perlmann, P. and Troye-blomberg, M. (1997). Elevated plasma levels of IgE in *Plasmodium falciparum*-primed individuals reflect an increased ratio of IL-4 to interferon-gamma-producing cells. *Clinical and Experimental Immunology* 109: 84-89.

Ferreira, A., Schofield, L., Enea,V., Schellekens, H.,van der Meide, P., Collins, W. E., Nssenzweig, R. S. and Nussenzweig, V. (1986). Inhibition of development of exoerythrocytic forms of malaria parasites by gamma Interferon. *Science* 232: 881-884.

Flanagan, K. L., Mwangi, T., Plebanski, M., Odhiambo, K., Sheu, E., Kortok, M., Lowe, B., Marsh, K. and Hill, A. V. S. (2003). Ex vivo interferon-gamma immune response to thrombospondin-related adhesive protein in coastal Kenyans: longevity and risk of *Plasmodium falciparum* infection. *American Journal of Tropical Medicine and Hygiene* 68: 421–430.

Gesase, S., Gosling, R. D., Hashim, R., Ord, R., Naidoo, I, Madebe, R, Mosha, J F, Joho, A., Mandia, V., Mrema, H., Mapunda, E., Savael, Z., Lemnge, M., Mosha, F.W., Greenwood, B., Roper, C. and Chandramohan, D. (2009). High resistance of *Plasmodium falciparum* to sulphadoxine/pyrimethamine in northern Tanzania and the emergence of dhps resistance mutation at Codon 581. *Public Library of Science One*. 4: 4569.

Ghosh, K. (2008). Evolution and selection of human leukocyte antigen alleles by *Plasmodium falciparum* infection. *Human Immunology* 69: 856-860.

Gilbert, S. C., Plebanski, M., Gupta S., Morris, J., Cox, M., Aidoo, M., Kwiatkowski, D., Greenwood, B. M., Whittle H. C. and Hill A. V. (1998). Association of malaria parasite population structure, HLA, and immunological antagonism. *Science* 279: 1173–1177.

Girard, M. P., Reed, Z. H., Friede, M. and Kieny, M. P. (2007). A review of human vaccine research and development: Malaria. *Vaccine* 25: 1567-1580.

Hananantachai, H., Patarapotikul, J., Naka, J. O. I., Looareesuwan, S. and Tokunaga, K. (2005). Polymorphism of HLA B and HLA DR B1 genes in Thai malaria patients. *Japanese Journal of Infectious Diseases* 58: 25-28.

Hill, A. V. S., Allsopp, C. E., Kwiatkowski, D., Anstey, N. M., Twumasi, P., Rowe, P. A., Bennett, S., Brewster, D., McMichael, A. J. and Greenwood, B. M. (1991). Common West African HLA antigens are associated with protection from severe malaria. *Nature* 352: 595-600.

Hill, A. V. S., Yates, S. N., Allsopp, C. E. M., Gupta, S., Gilbert, S. C. and Lalvani, A. (1994). Human leukocyte antigens and natural selection by malaria. *Philosophical Transactions of the Royal Society Biology letters* 346: 379-385.

Hillier, C. J., Ware, L. A., Barbosa, A., Angov, E., Lyon, J. A., Heppner, D. G and Lanar, D. E. (2005). Process, Development and Analysis of Liver-Stage Antigen 1, a Preerythrocyte-Stage Protein-Based Vaccine for *Plasmodium falciparum. Infection and Immunity* 73: 2109–2115.

Hoffman, S. L. and Doolan, D. L. (2000). Malaria vaccines–targeting infected hepatocytes. *Nature Medicine* 6: 1258-1263.

Hoffman, S. L., Goh, L. M., Luke, T. C., Scheider I., Le, T. P., Doolan, D. L., Sacci, J., de la Vega, P., Dowler M., Paul, C., Gordon, D. M., Stoute J. A., Church L. W., Sedegah, M., Hepper, D. G., Ballou, W. R.and Richie, T. L. (2002). Protection of humans against malaria by immunization with radiation attenuated *Plasmodium falciparum* sporozoites. *Journal of Infectious Diseases* 185: 1155-1164.

Janeway, C.A., Travers, P., Walport, M. and Shlomchik, M. (2005). Immunobiology: The immune system in health and disease. 6th Edition. Garland Science Publishing. New York. pp 319-333.

John, C. C., Moormann, A. M., Sumba, P. O., Ofulla, A. V., Pregibon, D. C. and Kazura J. W. (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. *Infection and Immunity* 72: 5135-5142.

Jones, T. R., Stroncek, D. F., Gozalo, A. S., Obaldia III, N., Andersen, E. M., Lucas, C., Narum, D. L., Magill, A. J., Sim, B. K. and Hoffman, S. L.(2002). Anemia in parasite- and recombinant protein-immunized aotus monkeys infected with *Plasmodium falciparum. American Journal of Tropical Medicine and Hygiene* 66: 672-679.

Kappe, S. H., Gardener, M. J., Brown, M. S. M., Ross, M. J., Matuschewski, K., Riberio,M. J., Adams, J. H., Jennifer, Q. J., Cho, J. J. Carucci, D. J., Hoffman, S. L. and Nussenzweig, V. (2001). Exploring the transcriptome of malaria sporozoites stage. *Proceedings of the National Academy of Sciences* 19: 9895-9900.

Kurtis, J. D., Hollingdale, M. R., Luty, A. J. F., Lanar, D. E., Krzych, U. and Duffy, P. E. (2001). Pre-erythrocytic immunity to *Plasmodium falciparum*: The case for LSA vaccine. *Trends in Parasitology* 17: 219-223.

Luty, A. J. F., Lell, B., Schmidt-Ott, R., Lehman, L.G., Luckner D., Greve, B., Matousek, P., Herbich, K., Schmid, D., Migot-Nabias, F., Deloron, P., Nussenzweig, R.S. and Kremsner, P.G. (1999). Interferon gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *Journal of Infectious Disease* 179: 980-988.

Lyke, K. E., Burges, R. B., Cissoko, Y., Sangare, L., Kone, A., Dao, M., Diarra, I., Ferna´ndez-Vin, M. A., Plowe, C. V., Doumbo, O. K. and Sztein, M. B. (2005). HLA-A2 Supertype-Restricted Cell-Mediated Immunity by Peripheral Blood Mononuclear Cells Derived from Malian Children with Severe or Uncomplicated *Plasmodium falciparum* Malaria and Healthy Controls. *Infection and Immunity* 73: 5799-5808.

Mackintosh, C. L., Beeson, J. G, and Marsh, K. (2004). Clinical features and pathogenesis of malaria. *Trends in Parasitology* 20:597-603.

Marsh, S. G. E., Bodmer, J. G., Albert, E. D., Bodmer, W., Bontrop, R. E. and Dupont, B., (2000). Nomenclature for factors of the HLA system. *European Journal of Immunology* 28: 377.

Matuschewski, K. (2006). Vaccine development against malaria. *Current Opinion in Immunology* 18: 449-457.

Mellouk, S., Hoffman, S. L., Liu, Z. Z., de la Vega, P., Billiar, T. R. and Nussler, A. K., (1994). Nitric oxide-mediated anti-plasmodial activity in human and murine hepatocytes induced by gamma interferon and the parasite it self: enhancement by exogenous tetrahydrobiopterin. *Infection and Immunity* 62: 4043–4048.

Miyahira, Y., Kobayashi, S., Takeuchi, T., Kamiyama, T., Nara, T., Junko Nakajima-Shimada, J. and Takashi, T. (1995). Induction of $CD8^+$ T cell-mediated protective immunity against *Trypanosoma cruzi. International Immunology* 11: 133-141.

Mohan, K. and Stevenson, M. M. (1998). Dyserythropoiesis and severe anaemia associated with malaria correlate with deficient interleukin-12 production. *British Journal of Haematology* 103: 942–949.

Moormann, A. M., John, C. C., Sumba, P. O., Tisch, D., Embury, P. and Kazura, J. W. (2006). Stability of interferon gamma and interleukin-10 responses to *Plasmodium falciparum* liver stage antigen-1 and thrombospondin-related adhesive protein in residents of a malaria holoendemic area. *American Journal of Tropical Medicine and Hygiene* 74: 585–590.

Moormann, A. M., John, C. C., Sumba, P. O., Tisch, D., Embury, P. and Kazura, J. W. (2009). Stability of interferon gamma and interleukin-10 responses to *Plasmodium falciparum* liver stage antigen-1 and thrombospondin-related adhesive protein immunodominant epitopes in a highland population from Western Kenya. *American Journal of Tropical Medicine and Hygiene* 81: 489-495.

Moorthy, V. and Hill, A. V. S (2002). Malaria vaccine. *British Medical Bulletin* 62:59-72.

Mota, M. M. and Rodriguez, A. (2004). Migration through host cells: the first steps of Plasmodium sporozoites in the mammalian host. *Cellular Microbiology* 6: 1113-1118.

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

Ochiel, D. O, Awandare, G. A., Keller, C. C., Hittner, J. B., Kremsner, P. G., Weinberg, J. B. and Perkins D. J (2005). Differential Regulation of Chemokines in Children with *Plasmodium falciparum* Malaria. *Infection and Immunity* 73: 4190-4197.

Ogutu, B. R., Apollo, O. J., McKinney, D., Okoth W., Siangla J., Dubovsky F., Tucker K., Waitumbi J N., Diggs C and Wittes J. (2009). Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. *Public Library of Science* 4: 4780.

Ong'echa, J. M. O., Altaf, A., Lal, A. A., Terlouw, D. J., Ter Kuile, F. O., Kariuki, S. K., Udhayakumar, V., Orago, A. S. S., Hightower, A. W., Nahlen, B. L. and Shi, Y. P. (2003). Association of interferon gamma responses to pre-erythrocytic stage vaccine candidate antigens of *Plasmodium falciparum* in young Kenyan children with improved hemoglobin levels: xv. Asembo bay cohort project. *American Journal of Tropical Medicine and Hygiene* 68: 590–597.

Pinzon-Charry, A., Anderson, V., McPhun, V., Wykes, V. and Good, M. F. (2006). Malaria vaccines: New hope in old ideas. *Drug Discovery Today: Therapeutic Strategies* 3: 167-172.

Prudencio, M., Rodriguez, A. and Mota, M. M. (2006). The silent path to thousands of merozoites: the Plasmodium liver stage. *Nature Reviews Microbiology* 4: 849-856.

Rieckmann, K. H., Beaudoin, R. L., Cassells, J. S. and Sell, K. W. (1979.). Use of attenuated sporozoites in the immunization of human volunteers against *falciparum* malaria. *Bulletio of. World. Health. Organization.* 57(Supplimenrary. 1): 261-265.

Ro, D. K, Paradise, E, M, Ouellet, M., Fisher, K. J., Newman, K. L. and Ndungu, J. M. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440: 940–943.

Roca-Feltrer, A., Carneiro, I. and Schellenberg, J. R. M. A (2008). Estimates of the burden of malaria morbidity in Africa in children under the age of 5 years. *Tropical Medicine and International Health* 13: 771–783.

Rosenberg, R., Wirtz, R. A., Schneider, I. and Burge R. (1990). An estimation of the number of malaria sporozoites ejected by a feeding mosquito. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 84: 209–12.

Rosenthal, P. J. (2008). Artesunate for the treatment of severe *falciparum* malaria. *New England Journal of Medicine* 358: 1829-1836.

Rowe, A. K., Rowe, S. Y., Snow, R. W., Korenromp, E. L., Schellenberg, J. R. M. A., Stein, C., Nahlen, L., Bryce, J., Black, R. E. and Steketee R. W. (2006) The burden of malaria mortality among African children in the year 2000. *International Journal of Epidemiology* 35: 691-704.

Scheibenbogen, C., Schmittel, A., Keilholz, U., Allgauer, T., Hofmann, U., Max, R., Thiel, E. and Schadendorf, D. (2000). Phase II trial of vaccination with tyrosinase peptides and granulocyte-macrophage colony-stimulating factor in patients with metastatic melanoma. *Journal of Immunotherapy* 23: 275.

Taylor-Robinson, A. W. and Heal, K. G. (2001). Candidacy of liver stage antigen-1 for *Plasmodium falciparum* vaccine development. *Journal of Protozoology and. Research* 11: 1-18.

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

Todryk, S. T. and Hill, A. V. S. (2007). Malaria vaccines: the stage we are at. *Nature Reviews of Microbiology* 5: 487-489.

Tongren, J. E., Zavala, F., Roos, D. S. and Riley, E. M. (2004). Malaria vaccines: if at first you don't succeed. *Trends in Parasitology* 20: 604-610.

Tsuji, M. and Zavala, F. (2003). T cells as mediators of protective immunity against liver stage of *Plasmodium*. *Trends in Parasitology* 19: 88-93.

WHO, (2000). Malaria-a global crisis. Geneva: World Health Organization.

WHO, (2005). World malaria report. (http://www.rollbackmalaria.org/wmr2005/) site visited on 2 / 5 / 2009.

Yang, J., Lemas, V. M., Flinn, I. W., Krone, C. and Ambinder, R. F., (2000). Application of the ELISPOT assay to the characterization of CD8+ responses to Epstein–Barr Virus antigens. *Blood* 95: 241.

Young, K., Frodsham, A., Doumbo, O. K., Gupta, S., Dolo, A., Hu, J. T., Robson, K. J. H., Crisanti, A., Hill, A. V. S. and Gilbert, S. C. (2005). Inverse associations of human leukocyte antigen and malaria parasite types in two West African populations. *Infection and Immunity* 73: 953–955.