CYTOKINE LEVELS AND T CELL APOPTOSIS ASSOCIATED WITH CEREBRAL MALARIA IMMUNOPATHOLOGY DURING *PLASMODIUM BERGHEI* ANKA INFECTION IN A MOUSE MODEL

JULIUS NDIRANGU MUGWERU

MASTER OF SCIENCE

(Immunology)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2013

Cytokine Levels and T cell Apoptosis Associated With Cerebral Malaria Immunopathology During *Plasmodium berghei* Anka Infection In A Mouse Model

Julius Ndirangu Mugweru

A thesis submitted in partial fulfillment for the degree of Master of Science in Immunology in the Jomo Kenyatta University of Agriculture and Technology

2013

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature……. Date.....

Julius Ndirangu Mugweru

This thesis has been submitted for examination with our approval as University supervisors:

Signature……. Date.....

Dr. Rebecca Waihenya

JKUAT, KENYA

Signature……. Date.....

Dr. Francis W. Muregi

KEMRI, KENYA

Signature……. Date.....

Prof. Zipporah Ng'ang'a

JKUAT, KENYA

DEDICATION

This work is dedicated to my family, who encouraged and supported me all through to this level of education. Above all, to God, the creator of all beings, who provided strength, health and favor to enable me see this output.

ACKNOWLEDGEMENTS

I am deeply indebted to all my supervisors, Prof. Zipporah Ng'ang'a, Dr. Rebecca Waihenya, and Dr. Francis W. Muregi for the guidance, encouragement and correction that they provided throughout the study period. I sincerely thank the Kenya Medical Research Institute for hosting me during the duration of the project. I thank Mr. Francis Kimani, for his invaluable guidance on molecular work. Lastly, I thank all my colleagues at the Department of Zoology JKUAT and at KEMRI malaria and immunology laboratories for helping me by creating a friendly working environment in the laboratory during the carrying out of this work. To you all, I say God bless you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ACRONYMS AND ABBREVIATIONS

APC	Antigen Presenting Cell
CBA	Cytometric Bead Array
CCL	Chemokine (c-c) Motif, Ligand
CD	Cluster differentiation
CIDR-1	Cysteine-rich interdomain region-1
CM	Cerebral Malaria
CTL	Cytotoxic T cells
CXCR	Chemokine (cxc) Motif, Receptor
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
ECM	Experimental Cerebral Malaria
FCS	Fetal Calf Serum
FCS/HBSS	Fetal calf serum / Hanks buffered Saline solution
HLA	Human Leukocyte Antigen
ICAM -1	Intercellular Adhesion Molecule–1

IFN-γ	Interferon Gamma
IL	Interleukin
INOS	Inducible Nitric Oxide Synthase
IP -10	Interferon-Induced Protein of 10kda
IRBC	Infected red blood cells
LFA -1	Lymphocyte Function-Associated Antigen-1
LPS	Lipopolysaccharide
LTα	Lymphotoxin alpha
MACS	Magnetic Activated Cell Sorting
MHC	Major Histocompatibility Complex
MIG	Monokine Induced by Gamma interferon
NK	Natural Killer
NO	Nitric Oxide
PbA	*Plasmodium berghei* ANKA
PBS	Phosphate Buffered Saline
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein

PRBCS	Parasitized Red Blood Cells
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RBC	Red Blood Cell
RNI	Reactive Nitrogen Intermediate
ROI	Reactive Oxygen Intermediate
STAT1	Signal Transducer and Activator of Transcription 1
TBE	Tris Borate/EDTA
T-BET	T box expressed in T cells
TH	T helper
TLR	Toll like receptors
TNFR	Tumor Necrosis Factor Receptor
TNF-α	Tumor Necrosis Factor Alpha
VCAM -1	Vascular Cellular Adhesion Molecule – 1
WHO	World Health Organization
γ δ	Gamma delta

ABSTRACT

During the course of malaria infection, a range of pro- and anti-inflammatory cytokines are produced by the host immune system. Successful recovery from malaria involves striking a balance between these counteracting cytokines. The cytokine imbalance contributes to pathological features but their exact levels have not been elucidated. The present study aimed at investigating the role played by circulating cytokines in pathophysiology of cerebral malaria. Using an experimental cerebral malaria (ECM) model, the profile of five serum cytokines was determined by employing Cytometric Bead Assay. Seventy-two BALB/c mice (7-9 week/old) were intraperitoneally inoculated with approximately 1 x 10^5 parasitisized red blood cells at day 0 and randomized into six groups (six mice/group). Another set of non-infected mice was included to serve as control. The mice were sacrificed at day 4, 6, 8, 11 and 20 pi. The possible role of cytokines in inducing T-cell apoptosis associated with CM was investigated using the whole genomic DNA extracted from splenic and brain lymphocytes. Significantly higher systemic levels ($P<0.05$,) of IFN-γ (mean ±S.D 210.6±133, 169.8±80.5, 203.6±91.6, 22.0±3.5 pg/ml), were observed between day 8 and 20 p.i while TNF-α levels were significant at days 4, 8 11, 14 and 20 respectively (M ±S.D 2.9 ± 0.2, 33.9±17.5, 95.5 ±17.0, 22.1±3.6 pg/ml) in BALB/c mice that survived until day 20 pi with a higher parasitemia (up to 52.6%±0.8). Significant concentrations ($P< 0.05$), of IL-4 (M ±S.D 14.6±2.5, 10.6±1.9, 9.6±1.3 pg/ml) were observed between day 4 and 8 respectively but afterwards its levels remained low throughout the course of infection. IL-5 levels (M

±S.D 4.1±0.7, 3.4±1.6) had significant differences at day 11 and 20 pi. The study found IL-4 to be elevated between days 11 and 20 respectively with no significant differences ($P>0.05$) being reported. T-cell pathology was revealed by fragmentation of whole genomic DNA during the infection which coincided with elevated systemic pro-inflammatory (IFN-γ and TNF-α at day six) responses which further accelerated the severity of CM. The study demonstrated a parallel link between T-cell pathology and elevated levels of Th1 cytokines concentrations in the brain and the spleen. This study revealed that elevated levels of proinflammatory cytokines induce inflammation and cellular apoptosis inhibiting parasite clearance. Thus, interventions to regulate the Th1 cytokine responses may be beneficial in the prevention of severe CM. Further work is needed on IL-2 IL-10 and IL-12 cytokines that could be involved in the pathology

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background Information

The current World Health Organization estimate of malaria mortality is at 216 million cases and occurs mostly in children under the age of five (WHO 2012). Cerebral malaria (CM) is a major cause of malaria mortality.

Cerebral malaria is an immunophysiopathological process caused by *Plasmodium falciparum* in humans and *Plasmodium berghei* ANKA in rodents. C M seen in about 7% of *P. falciparum* malaria cases, is characterized by neurological features, especially impaired consciousness (Maitland and Newton, 2005). Several host and parasite factors have hindered the successful development of malaria vaccines. During acute stage of malaria large amounts of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interferon gamma (IFN-γ) are released into the circulation (Harpaz *et al*., 1992). This type 1-biased immune response contributes either directly or indirectly to kill parasites, but is also implicated in the development of clinical symptoms and complications of malaria. An adequate anti-inflammatory response, to limit the otherwise overwhelming inflammation, has also been shown to be important for beneficial outcome of malaria (Miller *et al.,* 2002). Therefore, a delicate regulation of cells producing pro- and/or anti-inflammatory cytokines appears to be essential for a successful anti-malarial response (Miller *et al.,* 2002).

The multi-stage life cycle of the parasite in the human host allows the parasite to present diverse antigens to the immune system (Miller *et al.,* 2002). Some of these antigens are highly polymorphic or undergo rapid antigenic variation to avoid immune recognition. The host immune responses during malaria are also varied, complex and incompletely understood (Riley *et al.,* 2006).

1.2 Life Cycle of *Plasmodium species*

The genus *Plasmodium* belongs to the apicomplexa, a large subphylum of parasitic protozoa that are characterized by the possession of an apical complex organelle that lends the phylum its name (Cox-Singh *et al.,* 2008). Four species of *Plasmodium; P. falciparum, P. vivax, P. ovale and P. malariae,* traditionaly cause malaria in the human host, although *P. knowlesi* has been established as the fifth causative agent of human malaria (Cox-Singh *et al.,* 2008). Of these, *P falciparum* causes the most life threatening clinical episodes and is associated with the highest case-fatality rate. The parasites are transmitted by the bite of female anopheline mosquitoes (Cox-Singh *et al.,* 2008).

Development in the human host begins when sporozoites are injected into the dermis by an infected anopheles mosquito as it takes a blood meal. The sporozoites enter the bloodstream and are carried into the liver within thirty minutes where they invade hepatocytes, multiply and differentiate into merozoites. *P. vivax* and *P. ovale* can differentiate into hypnozoites in the liver and persist for a long period of time resulting in relapses From the liver, merozoites are released into the bloodstream

and invade red blood cells to initiate the blood stage of infection. Following erythrocyte invasion, merozoites mature asexually into ring stages, trophozoites and finally schizont forms (Jones and Good, 2006). The schizonts rupture releasing between 16–20 merozoites (depending on species), which invade more erythrocytes. Some blood stage parasites differentiate into male or female gametocytes which are subsequently transmitted to the mosquito as it feeds. The male and female gametocytes undergo sexual reproduction in the mosquito mid-gut forming more sporozoites (Fig.1). The clinical symptoms of malaria are manifested during the erythrocytic stage of infection and include fever, chills, and headache. The more severe symptoms include, impaired consciousness, metabolic acidosis, coma, anemia, shock and organ failure (Jones and Good, 2006).

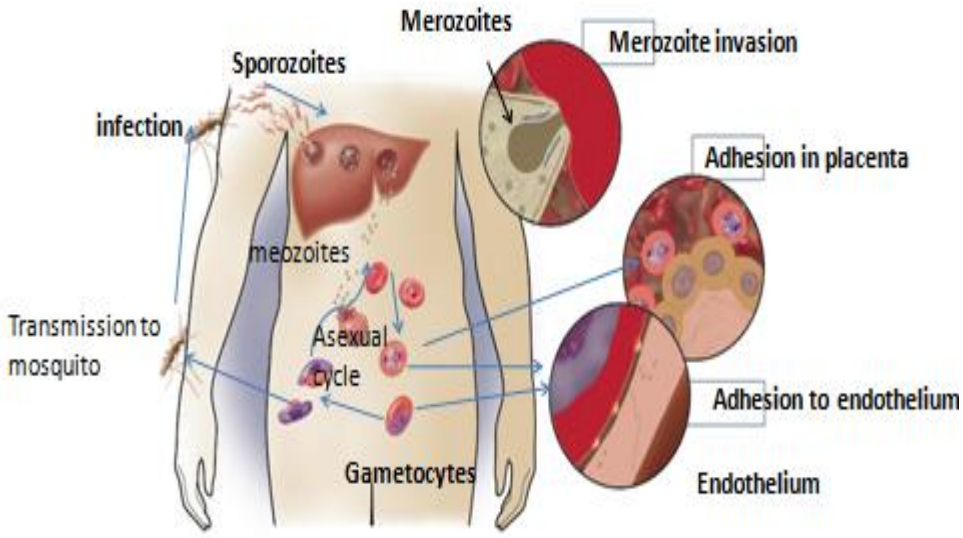

Figure 1 Life cycle of plasmodium species (Adapted from Jones and Good, 2006).

The phylogeny of human malaria parasites remains controversial, although there is a general consensus that the four species evolved separately and became parasitic on humans at different times in history (Cox-Singh *et al.*, 2008). It has been suggested that the malignancy associated with *P. falciparum* infections might reflect a relatively recent cross over by this species into humans (Ayala *et al.*, 1999). However, phylogenetic studies on the parasite's ribosomal RNA suggest that *P. falciparum* diverged from its closest relative, the apes parasite *P. reichenowi*, about 11 million years ago which is about the same time that man and apes are thought to have diverged. Thus, *P. falciparum* may have been infecting humans since the beginning of hominid evolution (Ayala *et al.*, 1999).

1.3 Clinical Features Of Malaria

Malaria presents as a spectrum of symptoms ranging from mild aches to a life threatening condition although only a minority of malaria case progress to become severe (WHO, 2010). General symptoms include raised body temperature in some cases above 39^{o}C, general body weakness with muscle pains, joint pains, occasional vomiting and headache (WHO, 2010). The severe form of malaria may lead to malaria-associated anaemia, cerebral malaria, respiratory complications and body bases imbalance leading to metabolic acidosis (WHO, 2010). Fever in malaria is thought to be mediated by tumour necrosis factor (TNF) and other pyogenic cytokines whose release by monocytes is triggered by toxins released during schizont rupture (WHO, 2010). Although high fever causes discomfort and could precipitate convulsions during an acute

malaria attack, it is not associated with risk of sequelae or death (Marsh *et al.*, 1995; Waller *et al.*, 1995).

The finding that fever can kill malaria parasites *in-vitro* while anti-pyretic drugs increases parasite clearance time *in-vivo* is an indirect evidence that fever may be important in regulating parasitaemia (Brandts *et al.*, 1997). Where cerebral malaria is seen, obstruction of the vasculature leading to the brain may precipitate seizures leading to malaria-associated coma in fatal cases (Miller *et al*., 2002). Splenomegaly is one of the common features of acute malaria infections in children (Mackintosh *et al*., 2004). There is no single symptom of malaria and the clinical picture is normally confirmed by confirmatory laboratory results to detect the presence of the malaria parasites or parasite products in patients (Dondorp *et al.*, 2000). The most severe complications in malaria infections are attributed to *P. falciparum* the most lethal of the four human infecting malaria parasites (Dondorp *et al.*, 2000).

1.3.1 Malaria-induced anaemia

Severe malarial anaemia was originally defined by a haemoglobin of below 50g/l or a haematocrit >15% in the presence of more than 10,000 malaria parasites/ul of blood but it is now recognised that there are other factors that might exacerbate anaemia even at lower parasite densities (Newton *et al.*, 1997). Increased immune haemolysis, phagocytosis and splenic clearance of both infected and uninfected erythrocytes following sensitisation with IgG and complement and changes in

deformability have been cited as potential mechanisms for anaemia (Waitumbi *et al.,* 2000; Dondorp *et al.,* 2000). Dyserythropoiesis induced by malaria toxins and cytokines (Miller *et al.,* 2002) has also been implicated.

1.4 Cerebral malaria

Cerebral malaria (CM) is one of the major clinical manifestations of severe malaria, leading to death or long-term neurological impairment if not treated. Patients often present with unarousable coma, impaired consciousness, respiratory distress and single or multiple organ failure (Marsh *et al.,* 1999). CM causes over one million deaths per year, mainly in young children primarily in sub-Saharan Africa (WHO, 2011). The clinical features of CM are well documented, but many aspects of its pathogenesis remain unclear. CM is a major cause of malaria mortality in endemic countries **(**Newbold, 1997).

1.4.1. Development of cerebral malaria

A number of hypotheses have been put forward to try and explain CM development. CM is an immunophysiopathological process caused by *P. falciparum* in humans and *P. berghei* ANKA in rodents (Milner *et al*., 2012). The current paradigm of CM pathogenesis suggests that parasite proliferation activates endothelial cells to produce adhesion molecules that enable sequestration of infected and uninfected red blood cells (RBCs) in brain capillaries which obstruct brain microvessels resulting to severe inflammatory processes that lead to CM syndrome (Milner *et al*., 2012). Recently it has been reported that regulatory inflammatory responses are associated

with CM but the mechanism by which they regulate its pathogenesis is unclear (Sarfo *et al.,* 2011). The mechanisms that lead to CM pathology are complex and often result from a contribution of several factors which are not well defined (Milner *et al*., 2012).

Histological studies from individuals who have died from CM have demonstrated the presence of mature parasites sequestered within the endothelium of brain blood vessels (Taylor *et al.,* 2004). This causes the blockage of normal blood flow leading to hypoxia, hypoglycemia and brain hemorrhage. Host adhesion molecules which include intercellular adhesion molecule–1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CD36 and E-selectin expressed in the brain endothelium mediate binding of parasitized erythrocytes to the endothelial lining of blood vessels (Schofield and Grau, 2005). In an autopsy study conducted in Malawi, 75% of children diagnosed with CM had parasites sequestered in the brain and demonstrable brain pathologies which included haemorrhages and accumulation of leukocytes (Taylor *et al.,* 2004*).* However, only 25% of the children diagnosed with CM harboured sequestered parasites in the brain with no other demonstrable pathology. These findings suggest that parasite cytoadherence is vital, but not sufficient to cause pathology in CM and therefore suggest a role of immune-mediated pathology (Milner *et al*., 2012).

The accumulation of monocytes, macrophages and platelets in the brain microvasculature and the release of inflammatory mediators such as, prostaglandins and microparticles obstruct blood flow within the brain and disrupt the blood brain

barrier, leading to brain damage (Patnaik *et al.,* 1994). The increased expression of the adhesion molecules, ICAM-1, E-selectin, VCAM-1 and inflammatory cytokines TNF-α and IL-1 have also been shown in autopsy brain tissues from fatal cases of CM (Brown *et al.,* 1999). Studies have also demonstrated that levels of inflammatory cytokines such as TNF-α, IL-1 and IL-6 in serum are elevated in CM patients (Kwiatkowski *et al.,* 1990). These studies suggest a link between leukocyte accumulation, inflammatory cytokines and the development of CM pathology.

1.4.2 Experimental cerebral malaria (ECM)

The host response to a primary infection with the erythrocytic stages of rodent malaria is accompanied by proinflammatory cytokines and a pronounced T-cell response (Li *et al.,* 2001). Experimental malaria models have greatly contributed to the understanding of immunological and pathological processes during CM (Jocelyn *et al.,* 2000). From the onset of infection, different cell types and organs can be examined in animals though ultimately, the relevance of these findings must be validated in humans (Jocelyn *et al.,* 2000). Susceptible mouse strains such as C57BL/6 infected with *P.berghei* ANKA (PbA) have been developed to investigate the pathogenesis of CM because they exhibit similar characteristics to human CM (Desruisseaux *et al.,* 2008), including hemorrhage, axonal and neuronal damage as well as behavioral and neurological impairments during active infection (Lackners *et al.,* 2006). Although a few *in vivo* studies have been performed in rhesus monkeys using *P. knowlesi* and *P. fragile,* most research have been done in mice using *P.*

berghei ANKA, which causes ECM in susceptible C57BL/6 and CBA mice (Lou *et al.,* 2001).

The clinical symptoms and pathological features observed during *P. berghei ANKA* infection are similar to those in humans (Kwiatkowski *et al.,* 1990**)**. Susceptible mice develop ataxia, convulsions and coma six to nine days following infection, with demonstrable brain hemorrhages, sequestered parasites and leukocytes in the brain (Fernanda *et al.,* 2010). The development of ECM is mainly mediated by lymphocytes (Renia *et al.,* 2001), initially shown by the resistance of nude mice, which lack T cells to ECM (Milner *et al*., 2012). Amongst the cells infiltrating the brain during ECM, cytotoxic $CD8^+$ T cells comprise the highest proportion and have been shown to cause brain damage by the production of perforin (Nitcheu, 2003).

The relative contribution of $CD4^+$ T cells to ECM pathogenesis however, is currently disputed due to contradictory findings. A study by Belnoue *et al.* (2002) demonstrated that $CD4^+$ T cells are only required during the onset of ECM as their depletion after establishment of clinical symptoms does not alter disease outcome in susceptible mice. Other studies have also shown that $CD4^+$ T cells are essential for the pathological manifestations of ECM (Renia *et al.,* 2001). The role of T regulatory cells has also been demonstrated in *P. berghei* ANKA infection (Fernanda *et al.,* 2010). Depletion of $CD4^+CD25^+$ (natural T regulatory cells) leads to a marked decrease in parasite sequestration in brain blood vessels and increased inflammatory responses in the spleen (Amante, 2007). Therefore, T regulatory cells play a role in inhibiting protective immune responses during *P. berghei* ANKA

infection. Other subsets such as Th17 cells have however not been described during *P. berghei* ANKA infection (Schmitz *et al.,* 2012).

The recruitment of cells to the sites of inflammation is mediated by chemokines (Rot and Andrian, 2007). During ECM, the expression levels of interferon-induced protein of 10kDa (IP-10), monokine induced by gamma interferon (MIG) and chemokine motif ligand 5 (CCL5) are upregulated in the brain during *P. berghei* ANKA infection (Nitcheu 2003). Their main effector function is trafficking T cells to the brain. In support of this, mice deficient in CXCR3, the receptor for IP-10 and MIG and CCR5 have been shown to harbor relatively few $CD8^+$ T cells in the brain compared to wild type mice which develops ECM (Belnoue *et al.,* 2002). Similar to human CM studies levels of TNF-α, LTα and IFN-γ increase during *P. berghei* ANKA infection (Amante, 2007). These cytokines may therefore influence the expression of chemokines and their receptors as well as contributing to other pathological features such as activation of endothelial adhesion molecules and cellular activation (Belnoue *et al*., 2002).

1.4.3 Murine Cerebral Malaria

Animal models of malaria have provided convincing evidence of the important role of inflammatory processes in the development of CM (de Souza and Riley, 2002). Monkey, rat, and mouse models for CM have been developed, although none of them completely duplicates the situation in humans. The most complete information has been obtained from experiments in mice. Lou *et al*. (2001) have

reviewed this issue, emphasizing the role of adhesion molecules and platelets in immune-mediated damage of vascular endothelium of the brain. A number of approaches have been used to understand CM pathogenesis in the murine model, including interventional approaches, such as the blockage or addition of a given mediator (de Souza *et al.,* 2009), comparisons between CM-susceptible and resistant mouse strains or between CM-inducing and non-inducing parasite strains (Beghdadi *et al.,* 2008).

In both human and mouse, tumor necrosis factor alpha (TNF)-induced upregulation of endothelial adhesion molecules has been invoked as a factor in pathology involving the sequestration of cells within the microvasculature of the brain and other major organs of the body (Grau *et al.,* 1993). However, other studies have suggested that there are differences between the two species, with a preference for leukocyte sequestration in the mouse Grau *et al.* (1993), rather than for parasitized red blood cell sequestration, as seen in humans (Aikawa *et al.,* 1990). de Souza *et al.* (2009) studied the role of cytokines during various blood-stage malaria infections and observed that *P. berghei* ANKA infections in "young" 8-week-old (BALB/c × C57BL/6) F_1 mice frequently resulted in neurological symptoms and early death. These characteristic CM-associated symptoms were less common in "older" (15 to 20 weeks) animals. These observations have now been extended further and show that this model of murine malaria does indeed bear a strong resemblance to human CM, including the characteristic feature of parasite sequestration within the microvasculature of the brain (Jocelyn *et al.,* 2000).

The current paradigm of CM pathogenesis suggests that parasite proliferation activates endothelial cells to produce adhesion molecules that enable sequestration of infected and uninfected red blood cells (RBCs) in brain capillaries which obstruct brain microvessels which results in severe inflammatory processes that lead to CM syndrome. Recently it has been reported that regulatory inflammatory responses are associated with CM but the mechanism by which they regulate CM pathogenesis is unclear. For example, mice lacking T-cells do not develop CM, and CM is attenuated when $CD4^+$ and $CD8^+$ T-cells are blocked. Thus, blocking activation of T cells appears to decrease the risk of CM severity (Hafalla *et al.,* 2011).

1.5 Immunology of Malaria

Innate and acquired immunity are important factors in determining the outcome of a malaria infection in an individual. Most episodes of infection with *P. falciparum* in malaria-endemic areas lead to mild clinical symptoms and after repeated exposure children eventually develop immune responses that protect against severe disease (Gupta *et al.,* 1999). This immunity does not fully prevent infection but reduces severity of the disease with low-grade infections continuing to occur with few or no clinical symptoms. Many targets for the humoral immune responses are polymorphic or clonally variant antigens exposed on the surface of merozoites or infected erythrocytes (Dodoo *et al*., 2001).

During acute infection, individuals develop specific antibody responses to antigens of the parasite variant they are exposed to. Maximum protection is generally achieved

by late childhood to early adulthood (Milner *et al.,* 2012). Over this time children in endemic areas develop an immunity that reduces the frequency and severity of disease, however immunity is not sterile. Immunity is a combination of variant-specific and non-variant responses (Bull *et al*., 2002).

In previous studies it has been shown that antibodies directed against the surface exposed protein PfEMP-1 agglutinate infected red blood cells (iRBC) in a variant-specific manner, associated with protection against disease (Newbold *et al*., 1992). As explained below, PfEMP1 are variant antigens, and cumulative exposure to a broad range of different *P. falciparum* serotypes leads to the development of a large number of anti-PfEMP-1 antibodies to cover most antigenic variants (Bull *et al.,* 2002). Mechanisms of cellular immunity thought to play a role in malaria includes direct activities of immune cells, such as phagocytosis and cytolysis, as well as soluble mediators, such as cytokines and nitric oxide, secreted or induced by these cells (Milner *et al.,* 2012). Such immunity can be specific or non-specific, and may require or be enhanced by antibody. Important immunologic cells thought to play a role in immunity to malaria includes; T cells, macrophages, B cells, Natural killer cells (NK) and dendritic cells (Milner *et al.*, 2012).

1.5.1 Humoral Response Important For Malaria Protection

Antibody responses are important in protection against malaria. Evidence for *in-vivo* protection against malaria by antibodies comes from passive transfer experiments both in animal models and humans (Groux *et al*., 1990).

Sera from malaria non-immune Europeans did not show the parasitocidal effect, indicating that antibodies in African sera were malaria specific (Cohen *et al*., 1961). In addition it was also shown that antibodies that protected against malaria could be obtained from cord blood thus demonstrating the maternal transfer of anti-malaria antibodies (Edozien *et al*., 1962). Sabchareon *et al.* (1991) repeated these experiments by treating malaria patients in Thailand with intravenous IgG from malaria immune African adults. A marked drop in parasitaemia within 24 hours of treatment was observed in the patients. *In-vitro,* antibodies from immune individuals have been shown to inhibit sporozoites invasion of hepatocytes, prevent merozoites invasion of red blood cells, depressing parasite growth, and promote parasite phagocytosis by macrophages (Pasquetto *et al*., 1997). In addition, immune serum can disrupt rosetting and the binding of infected erythrocytes to endothelial cell ligands, two processes that are implicated in the pathogenesis of severe malaria (Ricke *et al*., 2000). However it is not clear how the *in vitro* antibodies activates correlates with *in vivo* mechanisms. Despite the evidence cited, there is lack of a correlation between total antibody titres and malaria protection (Marsh *et al*., 1995).

Majority of malaria antibodies are probably directed against cellular debris released when schizonts burst and are of little consequence. However, even antibodies against antigens that are deemed to be important for parasite survival often do not correlate with protection (Hoffman *et al*., 1987). There are several reasons why this could happen. The immunodominant regions of many malaria antigens consist of tandem amino acid repeats, altering in number which generates polymorphisms that

may help the parasite escape immune recognition (Day *et al*., 1991). At the same time, polymeric antigens can cross-link B cell antigen receptors and induce T cell-independent antibody production that is characterised by IgM dominance and poor affinity, maturation and memory cells induction (Hoffman *et al*., 1987). Besides being short-lived and ineffective, T cell independent responses can also thwart protective responses to adjacent critical epitopes through epitope inhibition (Schofield 1991).

Under a variety of in vitro situations, malaria antibodies are often ineffective against parasites in absence of effector cells and may even promote parasite growth (Shi *et al.,* 1991). Despite exhibiting potent anti-parasitic activity in vivo, the antibodies used in transfer experiments showed no activity in vitro except in presence of monocytes (Sabchareon *et al*., 1991). Conversely antibodies that do not protect in vivo were unable to interact with monocytes in vitro (Groux *et al*., 1990). Thus the ability of antibodies to cooperate with effector cells may be more important than their quantity. It has been observed that humoral responses to malaria show pronounced tilting towards cytophilic antibodies IgG1 and IgG3, unlike responses to other antigens where IgG1 and IgG2 dominate (Ferrrante *et al*., 1997).

1.5.2 Role of Immune Cells in Malaria Infection

1.5.2.1 Role of B cells in Malaria Infection

B cells have ability to present antigen as well as activate production of antibody secreting plasma cells. The CIDR-1 domain of PfEMP1 has been implicated in

mediation of B cells activation *in vitro* and induction of hyper-gammaglobulinaemia, two distinct features of *P. falciparum* infections (Donati *et al*., 2004). It has been shown that iRBC of the laboratory isolate FCR3S1 attach to B cells and induce their activation from malaria non-exposed controls (Donati *et al*., 2004). This interaction is thought to be mediated by the *P. falciparum* erythrocyte membrane protein-1 and involve the extracellular portion of the cysteine-rich interdomain region-1 (CIDR-1). Stimulation with recombinant CIDR-1 derived from the *var* gene FCR3S1 induced multiplication and changes in B cell resulting in induction of activation molecules and production of cytokines accompanied by production of IgM. However the effect on B cells seems to be mediated by binding of this specific CIDR- 1 sequence to immunoglobulin molecules rather than binding to CD36 (Donati *et al*., 2004).

1.5.2.2 Role of Dendritic cells in Malaria infection

Dendritic cells (DCs) are a small population of white blood cells constituting less than 1% of the total white cell count in a normal person (Stevenson and Riley, 2004). DCs are unique antigen presenting cells with the ability to activate naïve immune responses and hence capacity to prepare subsequent memory immune responses. DCs participate in initiation of immune responses, and maintenance of peripheral tolerance (Banchereau *et al*., 2000). Studies on mouse models of malaria suggest activation and migration of DCs into the T-cell areas of the spleen upon malaria infection leading to induction of IFN-γ-producing T cells (Stevenson and Riley, 2004).

1.5.2.3 Role of Macrophages in malaria infection

Macrophage can activate T cells by presenting antigen in the context of MHC class II, and have the ability to ingest iRBC using opsonic or non-opsonic phagocytosis pathways (Ayi *et al*., 2005). Macrophage function is modulated in malaria infections to control DC function. Macrophages are essential effector cells of the host innate defence against malaria (Serghides *et al*., 2003). There is experimental evidence that non-opsonic phagocytosis contributes significantly to the uptake of iRBC by both rodent and human macrophages (Ayi *et al*., 2005). Wild-type macrophages displayed an enhanced phagocytic activity for non-opsonized iRBCs, compared with those for CD36 null mouse and rat macrophages (Patel *et al*., 2004). In addition, the uptake of ring-infected iRBCs by humans could be inhibited with anti-CD36 antibodies (Ayi *et al*., 2005). Haemozoin loading of macrophages, resulting from either the uptake of free malaria haemozoin or the phagocytosis of mature iRBCs, can lead to macrophage dysfunction (Schwarzer *et al*., 1996). Dysfunction is characterized by an inability to repeat phagocytosis, generate an oxidative burst upon stimulation and activate protein kinase C after exposure to haemozoin (Schwarzer *et al*., 1996). Macrophages are the only antigen presenting cells that can act as a vital connection in immune responses with the ability to link the innate and adaptive responses and are responsible for presenting antigen, activating naïve T-cells and enhancing antibody production (Liu *et al*., 2001).

1.5.2.4 Role of T cells in Malaria Infection

The role of T cell subsets in immunity to malaria have been described either by indirect observations in humans or in animal models. The limitation here is that many animals in which immunity experiments have been carried out are poor models of human malaria. The suppressive mechanism by which T- cells control CM pathogenesis is not fully elucidated. Regulatory T-cells (T-regs;$CD4^+CD25^+Foxp3^+$) are a class of T-cells which limit excessive activation of T-cells, and over-production of pro-inflammatory factors, and have been implicated in many infectious diseases but their role in CM immunopathogenesis is unclear (Stiles *et al.,* 2011).

1.5.2.5 Role of $CD4^+$ T cells in Malaria Infection

Mature $CD4^+$ T cells are placed in two groups that are associated with distinct cytokine profiles. Production of interferon alpha/gamma (IFN-α/γ), lymphotoxin-α (TNF-β), IL-12 defines type 1 helper cells (TH1) and is associated with a strong cell-mediated immunity, while production of IL-4, IL-6, IL-9, IL-10 and IL-13 defines type 2 (TH2) helper T cells associated with antibody production. However, because some T cells and non-T cells can produce both TH1 and TH2 cytokines, it may be more appropriate to distinguish them as type 1 (TR1) or type 2 response (TR2) (Clerici *et al*., 1994). In malaria, TR1/TR2 dichotomy is most clearly seen in the mouse *P. chabaudi* model. In this model, TR1 dominates the early response of mice to acute *P. chabaudi* infection and parasite killing is mediated by IFN-γ,

tumour necrosis factor (TNF-α) and nitric oxide (NO) secreted by activated TH1 $CD4^+$, macrophages and natural killer cells. TR1 cytokines- NO, INF-γ and TNF-α are also thought to mediate disease symptoms. On the other hand a shift toward TR2 leads to less symptomatic chronic infections. Along with inhibiting both INF-γ and TNF-α, type 2 cytokines also stimulate B cells to secrete antibodies (Fell *et al*., 1998). The dual anti-parasite/pathogenic nature of TR1 is also evident in *P. berghei* infections (Rudin *et al*., 1997). Other malaria-mouse models display variable tendencies towards either type of response during acute and chronic infections (Taylor-Robinson *et al*., 1999).

The distinction between type 1 and 2 responses is less clear in human malaria. Increased IFN-γ is associated with the resolution of parasitaemia in acute malaria episodes (Winkler *et al*., 1999) and a delay in re-infection (Liu *et al*., 2001), while reduced levels accompany hyper-parasitemia in children (Winkler *et al*., 1999). IFN-γ levels have been found to be higher in pregnant women with placental malaria than in those without malaria (Moore *et al*., 1999). These observations suggest a possible anti-parasite role of TR1 in human. On the other hand, IL-10 and IL-4 both type 2 cytokines have been associated with protection against malarial anaemia (Biemba *et al*., 2000).

1.5.2.6 Role of $CD8^+$ T cells (CTL) during Malarial infection

Hepatocytes unlike red blood cells, express class 1 HLA, the liver stage of malaria parasites is thought to be a target for induction of CTL responses ((Desruisseaux

et al., 2008). Classical adoptive transfer and depletion experiments have confirmed the protective role of CTL in animal models (Weiss *et al*., 1988). The fact that adoptively transferred CTL pre-primed with *P. berghei* failed to protect from infection by *P. yoelii* indicates that this protection is species-specific (Romero *et al*., 1989). Indirect evidence for CTL involvement in malaria immunity in man is borne in the association of some class I HLA alleles with protection against malaria (Hill *et al*., 1991). Over 40 epitopes on the sporozoites and liver stage antigens of malaria parasites have been identified as epitopes for human CTL (Aidoo *et al*., 2000). Some of the epitopes exhibit extensive polymorphism generated by non-synoymous mutations, an indication that they are under selection probably from host immunity (Hughes *et al*., 1995). CTL could kill parasites by perforin-mediated lysis, FAS-induced apoptosis of infected cells (Lowin *et al*., 1994), or via a cytokine pathway in which IFN-γ stimulates the host cell to kill the parasites through nitric oxide production.

1.5.2.7 Role of γδ T cells in Malaria Immunity

In healthy individuals, the majority of T cells receptors are made up of α and β chains, however a minority of T cells, whose restriction is uncertain, express receptors made of γ and δ chains. Infection with malaria causes a marked increase in the proportion of γδ T cells (Ho *et al*., 1990) but the significant of this phenomenon is yet to be established. Most of the work indicates a role for γδ T cells in resistance to malaria is based on mice that have immunological disruptions (Yanez *et al*., 1999) and may not necessarily reflect the situation in

immunologically intact mice. Nonetheless, there is evidence that γδ T cells from both malaria immune and non-immune individuals can produce IFN-γ, TNF-α in response to malaria infections (McKenna *et al*., 2000).

1.5.3 Pathological Consequences of Polarized Immune Response

Strong pro-inflammatory responses in rodent malarias and in human infections characterized by production of IL-12, TNF- α, IL-6, and IL-1 by DCs and macrophages, and IFN-γ by CD4β, CD8β, γδ T cells and NK cells (Seixas *et al*., 2002) can be associated with severe complications of malaria. However, the relationship between high TNF levels and pathology is not straightforward. Studies of human malaria disease showing lower IL-10 plasma concentration and IL-10/TNF-α ratios in children with malarial anaemia suggest that an imbalance in pro- inflammatory and anti-inflammatory cytokines, such as IL-10, may be related to the development of disease (Kurtzhals *et al*., 1999).

1.5.4 Cytokine Pathology During Cerebral Malaria

Studies using a murine model of experimental cerebral malaria (ECM) with *P. berghei* ANKA indicate that ECM is a T cell-mediated disease. In particular, $CD8^+$cytotoxic T cells are involved in the destruction of the blood-brain barrier (BBB) by perforin-dependent processes (Nitcheu *et al.*, 2003). Overall, parasite-triggered cerebral inflammation is considered to be a possible cause of death from CM (van der Heyde, 2006).

The harmful, dysregulated immune response leading to CM or ECM is mainly of the Th1 type, with overproduction of some cytokines, such as IFN-γ (initially produced by Th1 cells, and at a later stage also by $CD8^+$ cytotoxic T cells), combined with underproduction of others such as IL-10 (Hunt, 2003). Both sequestration and inflammation are needed for induction of cerebral pathogenesis (van der Heyde, 2006). Inflammatory cytokines play an important role in human immune responses to malaria although the balance between pro- and anti-inflammatory cytokines and the pathogenic effects that can result from dysregulation are poorly understood (van der Heyde, 2006).

Malaria disease manifestations differ and appear to be regulated by age and the acquisition of immunity, host and parasite genetic polymorphisms, and regional variation. Variations in human cytokine responses and their link to malaria disease manifestations are the subject of much debate (Sztein *et al.,* 2004). The pathogenic mechanisms underlying are not fully understood and remain a subject of debate (Wassmer et *al.,* 2003). CM has been associated with the sequestration of parasitized red blood cells (pRBC) in the brain microvasculature with the accumulation of mononuclear cells in brain tissue and with the increased expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF) both in the brain and systemically (Hunt and Grau, 2003**)**. Studies done both in humans and experimental murine models have demonstrated that IFN-γ and TNF play an important role in the development of CM mediated pathology **(**Sébastien *et al.,* 2004). IFN-γ can be secreted by a variety of cell populations such as NK cells, δγ T cells, $CD4^+$ T cells

or $CD8^+$ T cells. These cells contribute to ECM mediated pathology during *P. berghei* ANKA infection, nevertheless, the principal sources of IFN-γ during ECM are not well defined (Rest, 1982).

Accumulating leukocytes occlude brain blood vessels and cause damage by release of cytotoxic molecules such as perforin by $CD8^+$ T cells. Similarly, during malaria, IFN-γ can promote apoptosis of $CD4^+$ T cells which may contribute towards impaired parasite clearance and promote sequestration (Milner *et al.*, 2008). The mechanisms by which IFN-γ induces apoptosis during *P. berghei* ANKA infection are not known (Takahiko *et al.*, 2011).

IFN-γ is important in the effector phase of the immune response to activate macrophages and T cells for effective parasite clearance. It is however not well established whether IFN-γ is required for the activation and function of splenic macrophages, DCs and T cells during *P. berghei* ANKA infection (Takahiko *et al*., 2011). It is also unclear whether IFN-γ is required for the activation of brain-migrating T cells (Sébastien *et al*., 2004). Ultimately, IFN-γ production must be well regulated during *P. berghei* ANKA infection to achieve an anti-parasitic effect and also prevent its pathogenic effects (Xu *et al*., 2002). In a number of different studies and mouse strain/parasite combinations, it is clear that, in the absence of an IFN-γ response, mice are less able to control and clear their primary infection (Su and Stevenson, 2000). IFN-γ is produced by NK cells, γδ T cells, and CD4 T cells (Seixas *et al*., 2002), all of which may play some role in controlling parasitemia, but it is the CD4 T cells together with B cells that are crucial for the development of

protective immunity (Langhorne *et al*., 2002).

TNF-α has been postulated to have antiparasitic effects, but its role in protection *in vivo* is less easy to demonstrate. Neutralization of TNF by antibody treatment *in vivo* does not lead to a lethal infection, and mice are able to control a *P. chabaudi* infection with only minor increases in peak parasitemia (Li *et al.*, 2003). In addition to their contribution toward eliminating the parasite and to the development of protective immunity, IFN-γ and TNF-α are strongly associated with severe complications of malaria infections in humans and experimental models (Langhorne *et al.*, 2002). The most likely host cell to be responsible for the initiation of the pro-inflammatory response is the DC. DCs provide a critical link between the innate and adaptive immune response, and they are specialized for the uptake, processing, and presentation of pathogen-derived antigens to T cells (Langhorne *et al*., 2002). To initiate the adaptive immune response, parasites or parasite material is taken up by DCs, processed in the antigen-processing pathways, and presented to T cells (Langhorne *et al*., 2002). As malaria is a systemic infection, the spleen is the most likely organ to play a major role in antigen presentation (Langhorne *et al*., 2002).

1.5.5 The Immune Response to Malaria

During *Plasmodium* infection, the balance between host pro-inflammatory and anti-inflammatory immune responses play important roles in pathogenesis of CM. A weak pro-inflammatory response may lead to persistence and replication of parasites

while an excessive pro-inflammatory response may result in immunopathological consequences such as CM. Therefore, identifying regulatory mechanisms that control CM pathogenesis is important in developing interactions for CM (Stiles *et al.,* 2011).

Malaria infection induces innate, humoral, and cell-mediated responses, and the cross talk between these responses affect the outcome of the disease (Casares and Richie, 2009). Evidence from immune-epidemiological studies shows that immunity to malaria develops with exposure. Older children and adults experience fewer malaria episodes which are rarely life threatening This protected state is referred to as anti-disease immunity as sterilizing immunity is not achieved and individuals continue to become infected throughout their lives but remain asymptomatic (Trape *et al.,* 1994).

Several effector immune responses are induced during different stages of infection. During the liver stage, $CD8^+$ T cells and IFN-γ are the main effectors in the clearance of infected hepatocytes (Hafalla *et al.,* 2011). During blood-stage infection, the rapid clearance of parasites and their toxic products is both cell-mediated and humoral (Meding and Langhorne, 1991). These effector responses though crucial, ultimately require adequate regulation, since much of the pathology observed during malaria (and other infections) stems from the dysregulation of pro-inflammatory mediators (Riley *et al.,* 2006).

When merozoites exit from the liver, they are released into the bloodstream where they are exposed for seconds before invading erythrocytes. Merozoite antigens expressed on the surface or released from apical organelles prior to invasion are antibody targets. Antibodies can inhibit the invasion of erythrocytes (Pandey *et al.,* 2002*)* by binding to the merozoite surface and enhancing uptake and killing by macrophages (Bouharoun *et al.,* 1995). Parasites that evade antibodies at this stage mature within erythrocytes and encode proteins which are transported to the surface of the infected erythrocyte. These parasite proteins mediate binding to host receptors expressed on the microvasculature to evade splenic clearance, but are also targets of antibodies (Bull *et al.,* 1998). Recognition and uptake of the infected erythrocyte by macrophages and DCs can occur directly by binding of parasite ligands to the CD36 scavenger receptor (Serghides *et al.,* 2002). Other molecules released during schizont rupture such as glycosylphosphatidylinositol (GPI) and hemozoin (product of haem breakdown) interact with toll like receptors (TLR) on macrophages and DCs mediating release of the inflammatory mediators; TNF-α, IL-12 IL-18 and NO (Coban *et al.* 2005).

1.6 Statement of the problem

Cerebral malaria is a clinical manifestation of severe malaria, leading to death or long-term neurological impairment if not treated. Mechanisms leading to CM pathology are complex and often result from a contribution of factors which are not well defined. Cerebral malaria is associated with sequestration of parasitized red blood cells (pRBCs) in the brain microvasculature and secretion of toxic molecules

by parasites, as well as inflammatory components of the host immune response, including secretion of cytokines and recruitment of activated leukocytes to the brain (Amante *et al.,* 2010). During malaria, the successful resolution of infection is mediated by the early secretion of pro-inflammatory TH-1 cytokines that promote parasite killing by macrophages and thereafter to prevent immune-mediated damage (Desruisseaux *et al*., 2008). Cytokines imbalance contributes to pathological features such as activation of endothelial adhesion molecules and cellular activation (Takahiko *et al.,* 2011). The mechanisms by which the different TH-1 cytokines cause pathogenic effects during CM are diverse and have not been fully elucidated.

1.7 Justification of the study

Plasmodium berghei ANKA murine malaria has many features in common with human disease and is thus the best available model for certain important aspects of clinical malaria. Despite extensive research, the precise mechanisms leading to pathogenesis during cerebral malaria are not fully understood. Because of the difficulty in following up human cases with CM and the limited possibility to examine its pathological process, laboratory models are important to elucidate the immunological mechanisms involved in CM and the way of alleviating this serious condition. Experimental cerebral malaria (ECM), caused by infection of mice with *Plasmodium berghei* ANKA (PbA), is characterized by T cell-mediated neuropathology. Early immune events in the spleen during PbA infection are critical for ECM development. The development of ECM is mainly mediated by T cells. The recruitment of cells to sites of inflammation is mediated by cytokines. IFN-γ targets

various cell populations and influences their function during infection. It is however not well established whether TH1 cytokines are required for the activation and function of splenic macrophages, dendritic cells and T cells during *P. berghei* ANKA infection. It is also unclear whether they are required for the activation of brain-migrating T cells. Ultimately, TH1 cytokines production must be well regulated during *P. berghei* ANKA infection to achieve an anti-parasitic effect and also prevent its pathogenic effects. This study therefore seeks to elucidate the diverse mechanisms by which the different pro and anti-inflammatory cytokines cause pathogenic effects during CM.

1.7.1 Research questions

i. What is the role of T-helper 1 and T-helper 2 cytokines in the induction of CM pathology?
ii. Is there a relationship between elevated levels of Th1 cytokines and apoptosis of brain migrating T cells which thus interfere with parasite clearance in the spleen and brain?

1.8 HYPOTHESIS

Pathology during cerebral malaria infection do not concide with cytokine production

1.9 GENERAL OBJECTIVE

To determine cytokine levels and T cell apoptosis associated with cerebral malaria pathology during *Plasmodium berghei* anka infection in a mouse model

1.9.1 Specific Objectives

i. To determine parasitemia in Balb/c mice infected with *P. berghei* ANKA during ECM

ii. To determine production of T-helper 1 (TH1) cytokines by activated T cells in the spleen and brain-migrating T cells during ECM.

iii. To determine production of T-helper 2 (TH2) cytokines by activated T cells in the spleen and brain-migrating T cells during ECM.

iv. To assess apoptosis of splenic and brain migrating T cells during ECM.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Study site and Maintenance of Mice

The study was carried out at the Kenya Medical Research Institute's Centre for Biotechnology Research and Development (CBRD), Nairobi, Kenya.

BALB/c males of 7-9 weeks old were donated from Kenya Medical Research Institute's animal facility and used for experiments. They were weighed before the experiment to ascertain if they had acquired an adult weight of >20g. The animals were caged for a week to acclimatize to their new environment. They were allowed unlimited access to pellets (Laboratory chow, Unga Feed ® Co.) and water *ad libitum* on a twelve hour light–dark cycle with observation for vibrancy and any signs of disease.

2.2 Parasite acquisition and passage

Cryopreserved *P. berghei* ANKA parasites were obtained from KEMRI parasite bank, thawed and inoculated intraperitonealy (ip) into donor mice. Parasitaemia in mice used for passage was determined on the third day of infection and adjusted downwards to 5×10^4 parasites/ml using sterile physiological saline. Experimental mice were in turn infected (ip) by inoculations of 0.2 ml stock which translated to $1x10^5$ parasitized red blood cells (pRBC's) and randomized into 12 groups of 6 mice per cage. Control uninfected group received an equal dose of sterile normal saline.

2.2.1 Sample Size Determination

The experimental study compared two groups' means of continuous variables that is cytokine and apoptosis. The sample size was calculated using the formula $n=1+2C(s/d)^2$, where **s** is the standard deviation, **d** is the difference to be detected and **C** is a constant dependent on the value of α and β selected (Snedecor and Cochran 1989). C was determined from a table which gives values for C for two levels of α and β. The cytokine levels for the control group at day 0 of the experiment were used to compute the sample size required for the experiment, and in total 120 mice were used.

2.2.2 Sampling Design

Completely randomized sampling was applied, where 120 mice were assigned randomly into three groups namely;

i. 72 mice infected with 1 x 10^5 *P. berghei*
ii. 24 mice injected with a dose of normal saline
iii. Uninfected group (24 mice)

The infected mice were randomized into six groups of 6, each group corresponding to selected day post-infection when cytokine levels were determined. Each of the six groups received two control mice from each set of the control groups. Two parallel experiments (n=2) were modeled on this setup.

2.2.3 Determination of Parasitaemia in mice

Thin blood films were prepared on days 4, 6, 8, 11, 14 and 20 post infection by removing 1 mm section of the distal end of the tail and spotting 3µl of blood onto a microscope slide. Slides were air dried, fixed with 100% methanol and stained with a 10% Giemsa solution diluted in Phosphate Buffered Saline solution (PBS) for 10 min at room temperature (RT). Slides were observed at a 1000× magnification (oil-immersion) using an Olympus (CX31RBSF) microscope by reading 10 fields per slide (Homel and Gilles, 1998).

$$\text{Parasitaemia} = \frac{\text{Number of infected RBCs}}{\text{RBC per field} \times \text{fields counted}} \times 100$$

2.2.4. Serum Processing For Cytokine Analysis

Mice were sacrificed on days 4, 6, 8, 11, 14, and 20 post- infection by carbon dioxide asphyxiation and approximately one millimeter blood drawn into non-heparinised tubes via cardiac puncture using sterile 1cc syringe and 21 gauge needle. Serum was obtained by pooling blood from a set of six mice in the respective day post infection and processed by leaving the blood tubes to stand for 30 minutes at RT. The blood was then centrifuged at 1,000g for 10 minutes and serum collected was preserved at -80ºC until use.

2.3 Serum Cytokine Assays

Mouse cytokine kit bearing sensitivities for IFN-γ, IL-4, IL-5 and TNF-α representing the TH1 and TH2 cytokines was used (appendix 1-2). Cytokine profiles were analyzed from the serum samples by cytometric bead Array (CBA) according to the manufacturer's instruction (BD Pharmigen®, 2010).

2.3.1 Reconstituting the Standards for Cytokine Assays

One vial of lyophilized mouse Th1/Th2 standard was transferred in to a 15-ml conical polypropylene tube. The tube was labeled "Top Standard." The standards were reconstituted with 2.0 ml of assay diluent and allowed to equilibrate for 15 min at RT and mixed gently by pipetting. Nine (12 × 75-mm) polystyrene tubes were labeled and arranged as: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256 respectively.

Three hundred microliter of assay diluent was pipetted in each of the 12 × 75-mm tubes. Serial dilution was done by transferring 300 µL of the top standard to the 1:2 tube and continually diluted in a stepwise manner to 1:256 tube. One 12 × 75-mm tube containing assay diluent only was prepared to serve as the 0 pg/mL negative control (Hodge *et al.,* 2004).

2.3.2 Mixing Mouse Th1/Th2 Cytokine Capture Beads

Each capture bead suspension was vigorously vortexed for 5 seconds before mixing and 10-µL aliquot of each capture bead transferred into a single tube

labeled “Mixed Capture Beads” and the bead mixture vortexed thoroughly (Hodge *et al.*, 2004).

2.3.3 Mouse Th1/Th2 Cytokine Assay

The mixed capture beads were vortexed and 50 µL added to all assay tubes. Fifty microliters of the Mouse Th1/Th2 Cytokine Standard dilutions was added to the control tubes. Fifty microliters of each unknown serum sample was added to the appropriately labeled sample assay tubes followed by 50 µL of the Mouse Th1/Th2 PE detection reagent. The assay tubes were incubated for 2 hours at RT in the dark. One millimeter of Wash Buffer was added to each assay tube and centrifuged at 200g for 5 min. The supernatant was carefully aspirated and discarded from each assay tube. Three hundred microliters of wash buffer was added to each assay tube to resuspend the bead pellet. The samples were then acquired on a FACS calibur flow cytometer (BD Pharmigen®). Each sample was vortexed for 3-5 seconds immediately before acquiring it on the flow cytometer (Blank, 2004).

2.3.4 Analysis of Cytokine Levels

Analysis was done using CELLQUEST (3.3) software (BD Pharmigen®, 2010). The FACS Calibur flow cytometer (BD Pharmigen®) was calibrated with setup beads and the samples acquired. Cytokine concentration was indicated by its fluorescent intensities and then computed using the standard reference curve of

CELLQUEST software. Cytokine levels for IFN-γ, TNF-α, IL-4 and IL-5 were analyzed. FCAP array software (BD Pharmigen®, 2010) was used for analysis.

2.4 Isolation of T-cells from Mouse Brain and Spleen Tissues

Whole brain and spleen tissues from individual infected mice and obtained on day 4, 6, 8, 11, 14 and 20 post infection were perfused in 15ml of 2% Fetal Calf Serum-Hanks Buffered Saline Solution (FCS-HBSS) in sterile petri dish by teasing them until they turned pale in colour according to methods of Sébastien *et al.,* 2004 . Briefly, the pieces were aspirated using a syringe to break up the chunks. Each tissue suspension was transferred into a 50ml Falcon tube (BD) and centrifuged at 1500g for 5min at RT. The suspension was discarded and the supernatant resuspended in 10ml of collagenase diluted to 1µg/µl in 2% FCS-HBSS. The tissue suspensions were incubated for 45 min then centrifuged at 1500g for 5min at RT and the filtrate resuspended in 6ml of 2% FCS-HBSS. To separate leukocytes from tissue debris, the cell suspensions were layered onto 10ml of 30% (vol/vol) iso-osmotic percoll gradient solution (Pharmacia®) diluted in 2% FCS-HBSS in 15ml falcon tubes and centrifuged at 1500g for 10 min at room temperature without breaks. The upper layer cells were separated and stained with mouse anti-CD3 (Clone 145-2C 11) (BD, Biosciences) T-cell lineage marker then washed with 2% FCS-HBSS and finally washed with LymphoPrep™ and centrifuged at 1500g for 10 min. The white layer of leukocytes (plate 2) was carefully separated from the rest of the cell suspension and preserved at -80ºC for use in demonstrating DNA fragmentation.

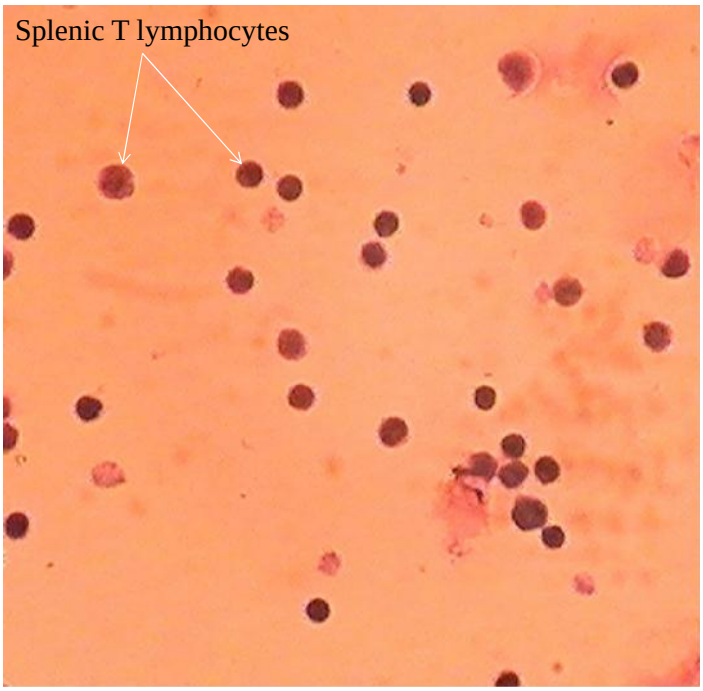

Plate 1 Spleen lymphocytes isolated from infected mice on day 6 post infection at a 1000× magnification.

2.5 DNA Extraction

Deoxyribonucleic acid(DNA) fragmentation was investigated using the whole genomic DNA extracted from spleen and brain lymphocytes since internucleosomal degradation of DNA is the biochemical hallmark of apoptosis (Muregi *et al.*, 2011). Genomic DNA was extracted from the isolated T-lymhocytes using a modifaction of the Chelex ® DNA extraction method (Abdel-muhsin, 2002). Two hundred microliter cell suspension of the T-lymhocytes was placed into sterile 1.5ml vial and 1ml of 0.5% saponin in 1×PBS was added. The vial was inverted several times and

incubated at 4ºC for 24 hr. The filtrate was resuspended in 1ml of ×1PBS and then incubated for further 30 min at 4ºC. The tube was centrifuged at 10,000g for 30 sec at R T. The filtrate was resuspended in 50µl of 20% chelex ® and 100µl of DNAse water added. The vial was heated for 10 min at 95ºC in a heating block while vortexing at intervals of 3 min. The vial was centrifuged at 10,000 g for 2 min at room temperature. The filtrate was collected into a sterile vial (Epperdorf®) and centrifuged at 10,000 g for 2 min at room temperature. The supernatant which had the eluted DNA sample was collected into a sterile 1.5ml vial for use to demonstrate apoptosis of T cells through DNA fragmentation (Abdel-muhsin, 2002).

2.5.1 Agarose Gel Electrophoresis

To demonstrate DNA cleavage, the eluted DNA samples were run on agarose gel and visualized on a UV light box. Agarose gel (1.0% w/v) was prepared by dissolving 1.0g of agarose powder into 100 ml of 1XTBE buffer. The gel solution was stirred, brought to boil in a microwave for 3 min to completely dissolve the powder. The gel solution was poured in a casting tray having combs and left for 10min to polymerise. Ethidium bromide (3µl) was incorporated before setting of the gel to facilitate visualisation of DNA under UV light. The DNA sample (5µl) was mixed with 3µl of loading dye (Bromophenol blue) and loaded into the wells and subjected to electrophoresis at 80V for 45 min. The gel was viewed on a UV light box to visualize the DNA ladder pattern (Sambrook *et al*., 1989). DNA samples (5µl) from non-infected healthy mice were run alongside as controls.

2.6 Ethical and Biosafety Considerations

Permission to carry out the study was obtained from Kenya Medical Research Institute (KEMRI) Scientific Committee and Ethical Review Committee (KEMRI SSC No 2217). The experiment was carried out in compliance with Animal Care and Use Committee (ACUC) Regulations of KEMRI. Standard operating procedures (SOPs) available at the malaria and immunology laboratories were followed in all experiments. These included injecting of BALB/c mice using a standard 21 gauge needle, anaesthetizing them using 6% sodium pentobarbitone and killing them using carbon ether asphyxiation. Dead animals were sterilized by dipping them in 70% ethanol and disposing in biohazard bags before transfer to the incinerator at KEMRI. Further precautionary measures included putting on protective gear and carrying out the experiments in a laminar flow hood (Nuair Biological Cabinets).

2.7 Data Analysis

Cytokine levels were measured in pg/ml from standard curves calibrated from reagent standards using the flow cytometer's software. Readings falling below the detectable threshold (≤0) were disregarded in the statistical analysis. The levels of TH1 and TH2 cytokines were expressed as means ± the standard deviation. The student t-test was used to examine the differences in cytokine concentrations using XLSTAT (2012) statistical software. Statistical significance levels were set at $p<0.05$.

CHAPTER THREE

3.0 RESULTS

3.1 Parasitemia Levels and Survival Rates

Cerebral symptoms such as convulsion, paralysis and coma were observed between day 6 and 14 post infection and the mice became moribund. All the experimental mice developed a patent parasitemia by day 4 post-inoculation with gradual increase in parasitemia between day 0 and day 4 and thereafter, the parasitemia rose rapidly reaching mean peak of 52.6 ± 0.8(mean±SD) at day 14 with only 6% of the mice surviving and dying at day 20 post infection due to overwhelming hyperparasitemia.

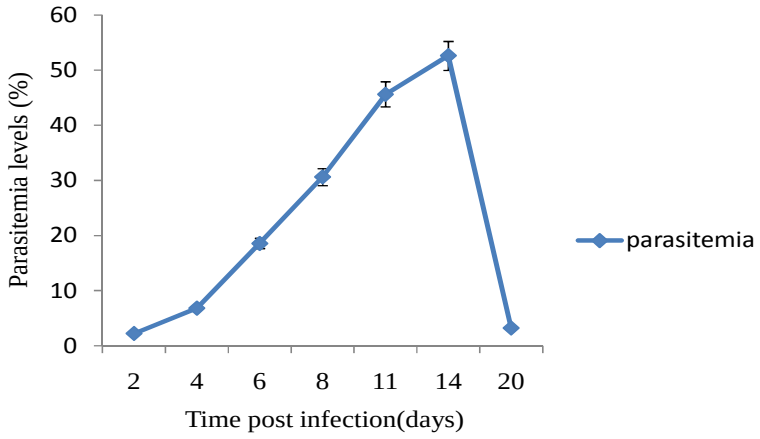

Figure 2 Parasitemia levels in BALB/c mice infected with *Plasmodium berghei* ANKA

Mice that developed CM were identified as ill by coat ruffling and general immobility prior to death. Mortality of the experimental mice was observed from day 6 post-inoculation, although some mice survived up to day 20 post infection as indicated in Fig. 3.2. On day 14, only 6% of the mice were still alive and all the surviving mice died at day 20 post infection respectively with a parasitemia of ≤3.2% ± 0.5 with few schizonts detectable on blood films. These mice died later at day 20 of severe anaemia and hyperparasitemia (Fig 3).

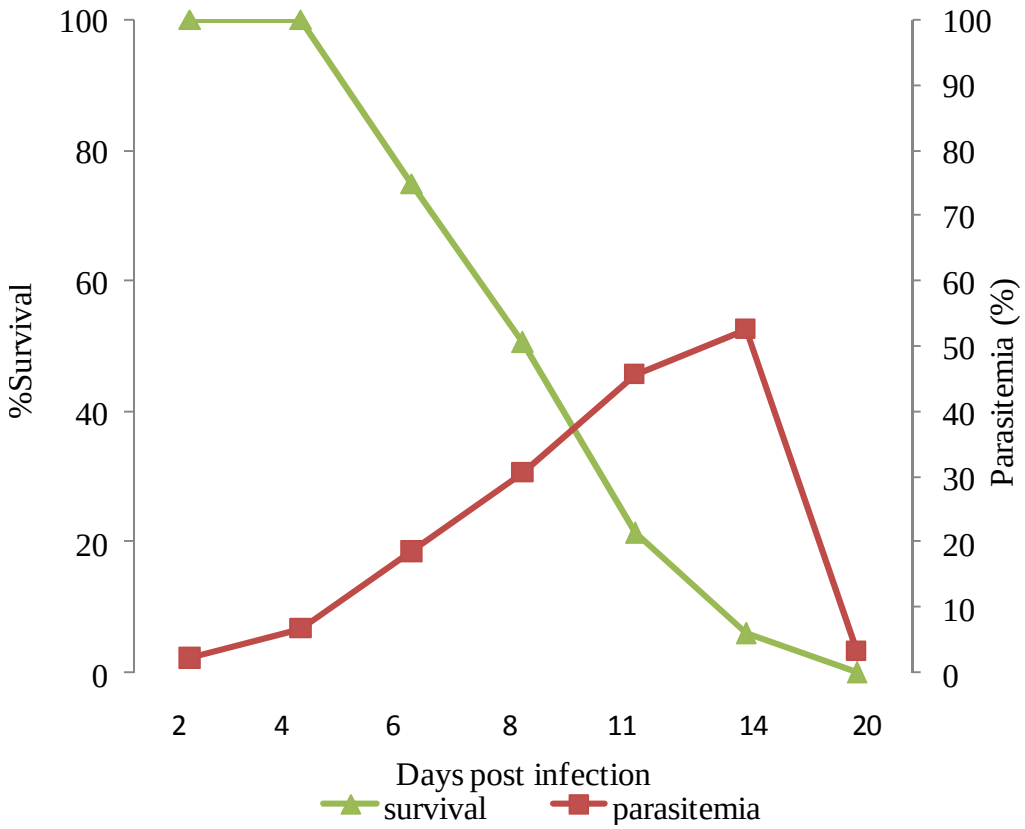

Figure 3 Comparison of parasitemia levels and survival incidence curve for BALB/c mice infected with *P. berghei* ANKA.

Serum was obtained from mice sacrificed at days 4, 6, 8, 11, 14 and 20 post infection. Four serum cytokine levels representative of both T-helper 1 and T-helper 2 cytokines were measured in BALB/c infected mice during the course of ECM (mean ± standard deviation of the mean). Serum was pooled from a set of six experimental mice per day.

3.2 T helper 1 cytokines

3.2.1 IFN-γ Levels

Analysis on IFN-γ showed no significant difference ($P>0.05$) in IFN-γ production at day 4 post infection between the test group (Mean ± S.D, 4.6±1.7), and that of control mice (2.3 ± 0.5) [$P=0.2$]). Similarly, no significant increase was registered at day 6 p.i (15.4 ± 9.5), compared to control mice (2.6 ± 0.3) Fig 4.

Peak IFN-γ levels were observed at day eight post infection (210.6±133.0) compared to control mice (2.2±0.5 [$P=0.05$]) followed by a drop in levels at day 11 post-infection (169.8±80.5) compared to control mice (2.3±0.3 [$P=0.02$] $P<0.05$). Conversely, there was a significant ($P<0.05$) upsurge at day 14 post-infection (203.6±91.6) while control mice (2.6± 0.2 [$P=0.02$]) registered an insignificant increase. There was a significant decline of the levels at day 20 post-infection (Fig 4) in the experimental group (22.0±3.5) compared to control mice (2.6±0.2 [$P=0.01$]).

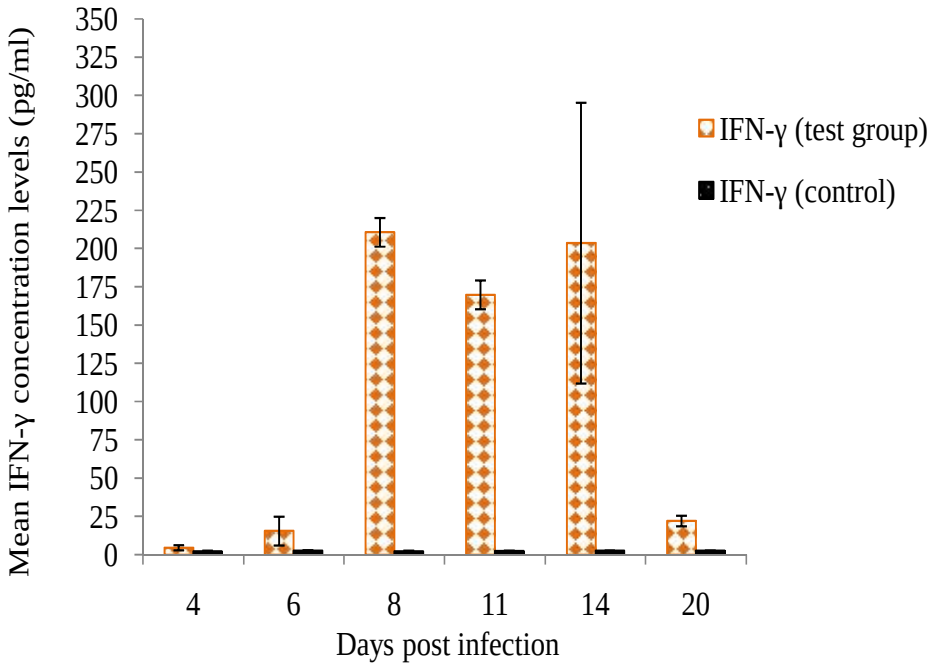

Figure 4 Mean IFN-γ levels for the experimental and the control groups during the course of ECM infection. Error bars represent standard deviation of the mean

3.2.2 TNF-α Levels

Tumour Necrosis Factor-α for the test group at day 4 post infection (2.9±0.2), was significantly different compared to that of control d mice (2.5±0.1 [P=0.04] P<0.05, Fig. 5). Although TNF-α levels rose exponentially (Fig. 5) for the test group at day 6 post infection (32.2±7.6) compared to control group (2.5±0.3 [P=0.4]), this change was not statistically significant (P>0.05). The levels for the test group were significantly higher (33.9±17.5) compared to control mice (2.5±0.1 [P=0.04]) at day 8 post-infection (P<0.05, Fig 5). The levels remained significantly higher (P<0.05)

for the test group (95.5 ±17.0) compared to controlgroup (2.2±0.2 [P=0.01]) at day 11 post-infection (Fig 5).

Conversely, although not statistically significant (P>0.05), the levels of the test group declined (31.6±19.1) while that of control group was 2.6±0.2 [P= 0.06] at day 14 post-infection (Fig. 5). At day 20 post-infection, the levels of the experimental group declined significantly (22.1±3.6, Fig 5) compared to control (2.6±0.2 [P= 0.01], P<0.05]).

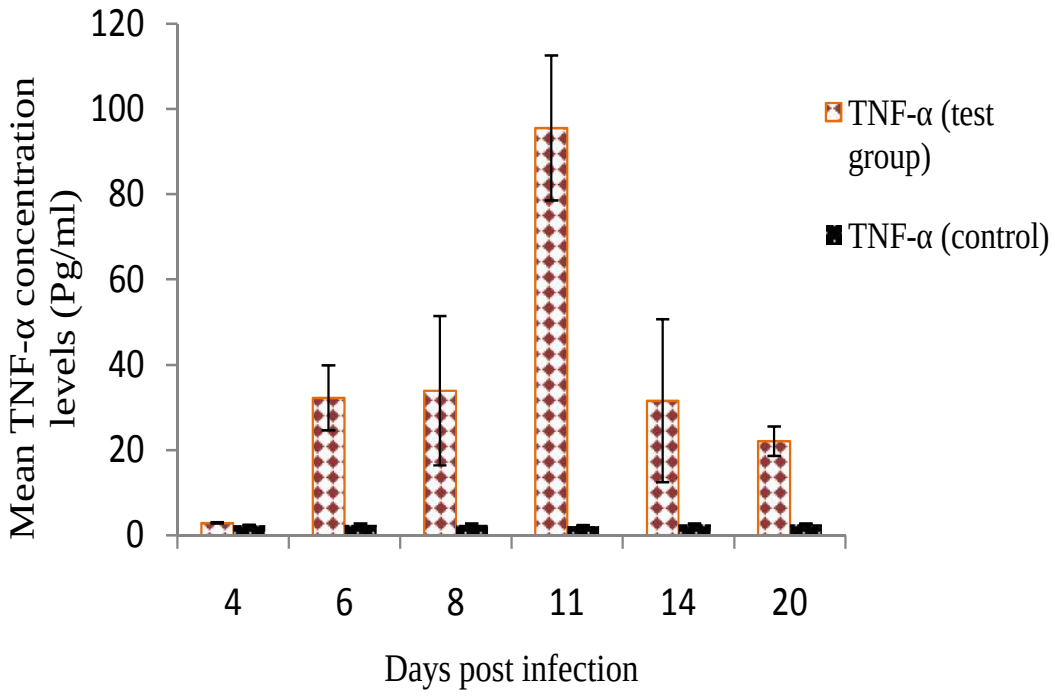

Figure 5 Mean IFN-γ levels for the experimental and the control groups during the course of ECM infection. Error bars represent standard deviation of the mean.

3.3 T helper 2 cytokines

3.3.1 Interleukin-4 Levels

Interleukin-4 (IL-4) for the test group was significantly higher (14.6±2.5) compared to that of control uninfected mice (3.1±0.6 [P=0.04], P<0.05]) at day 4 post-infection (Fig 6). The levels for the experimental group remained significantly elevated at day 6 p.i (10.6±1.9) compared to control mice (2.6±0.2, P<0.05, Fig 6).

There was a decline in the IL-4 levels for the test group (9.6±1.3) compared to control group (3.1±0.6, P<0.05) at day 8 post-infection (Fig. 3.5). There was an increase in the IL-4 levels in the test group at day 11 post-infection (10.6±2.8) compared to control mice (2.9±0.5 [P=0.5]) although no significant difference observed (P≥0.5) (Fig. 3.5). The levels then declined (4.9±4.3, Fig. 3.5) while control mice increased (2.5±0.2) while no significant differences were observed (P>0.05), whereas at day 20 post-infection, no significant increment was noted (5.1±3.9) compared to the control mice (2.7±0.3, P>0.05) Fig 6.

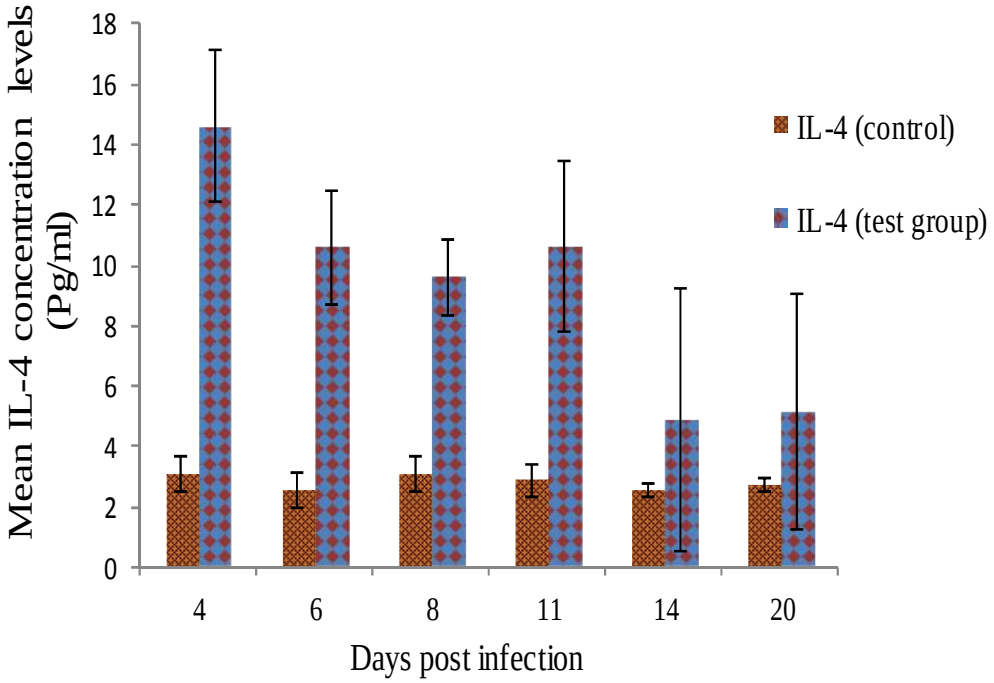

Figure 6 Mean IL-4 levels for the experimental and the control groups during the course of ECM infection. Error bars represent standard deviation of the mean

3.3.2 Interleukin-5 Levels

There was no significant increase in IL-5 levels for both the experimental group (4.5±0.7) compared to that of control mice (7.8±0.8, [P=0.06], P>0.05) at day 4 p.i. Correspondingly, the levels of the test group (4.9±1.1) and that of the control mice (5.8±1.1, [P=0.16]) were not significantly different (P>0.05) at day 6 p.i (Fig 7). Similarly, there was no significant difference for IL-5 levels (P>0.05), at day 8 p.i in the test group (4.3 ±0.8) compared to control group (5.9±0.9 [P=0.2])Fig 7..

The IL-5 levels in the test group was lower (4.1±0.7) than control group (5.8±1.4 [P=0.05], P<0.05) at day 11 post-infection. At day 14 post-infection, no significant production levels were observed in the test group (5.6±2.0) compared with control group (5.8±1.4 [P=0.9], P>0.05). However, the levels rose significantly in the test group (3.4±1.6) compared to control group (7.8±0.8 [P= 0.01], P< 0.05) at day 20 post-infection (Fig 7).

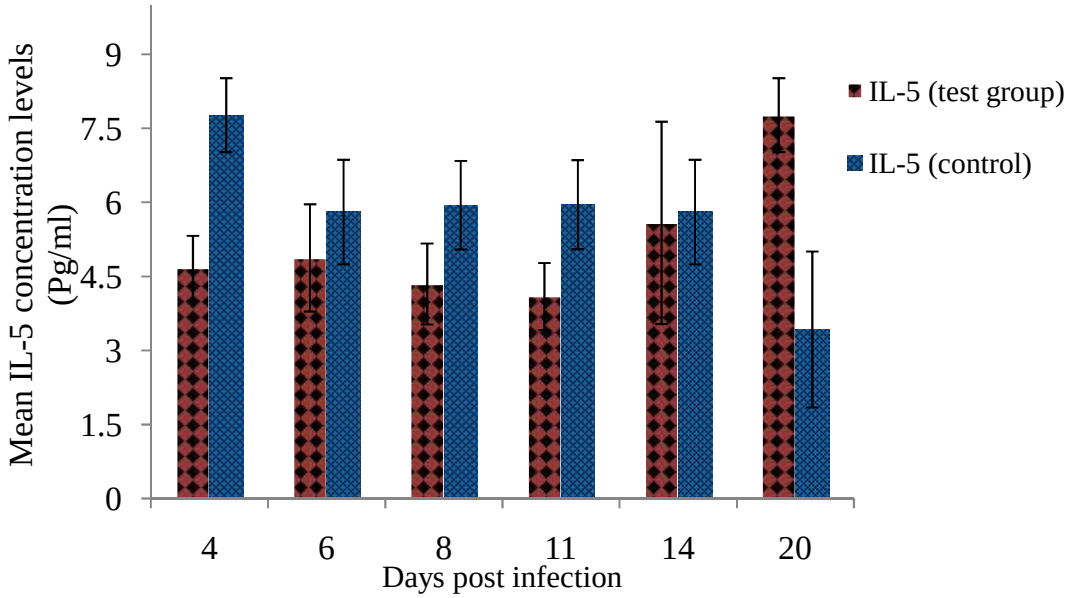

Figure 7 Mean IL-5 levels for the experimental and the control groups during the course of ECM infection. Error bars represent standard deviation of the mean

Interferon gamma-γ levels were the most dominant TH1 cytokine in circulation followed by TNF levels. An exponential upsurge in IFN-γ levels were detected between the 4th and 8th day and subsequently decreased on the 11th day, although the concentration rose again on the 14th day but decreased significantly (P<0.05) on the

20th day for the surviving mice. TNF-α levels were a distant second to IFN-γ levels. Between the 4th and 6th day, an exponential increment in TNF α levels was detected indicating its early involvement in protection against the parasite and constantly increased until day 11. However, TNF α production increased between the 6th and 8th day with peak levels being detected on the 11th day, and subsequently decreased between the 14th and 20th day (Fig 8).

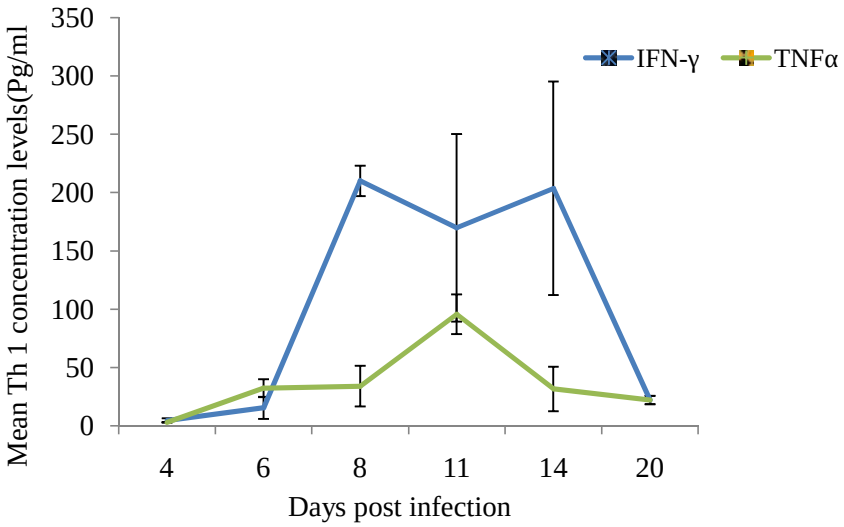

Figure 8 Basal T helper1 cytokine production trends during the course of infection with *Plasmodium berghei* ANKA. Error bars represent standard deviation of the mean

On the 4th day post infection significant levels (P<0.05) of IL-4 were detected while latter it tended to decrease, although on the 11th day the concentration rose again and subsequently decreased on the 14th day and thereafter it hit a bottom low till the 20th

day. Il-5 levels were the lowest across in all the intervals and there was a corresponding drop in IL-4 during the course of infection. IL-5 production was constant between the 4th and 11th day, although a rise was detected on the 6th day and subsequently increased between 11th to 20th day post infection (Fig 9).

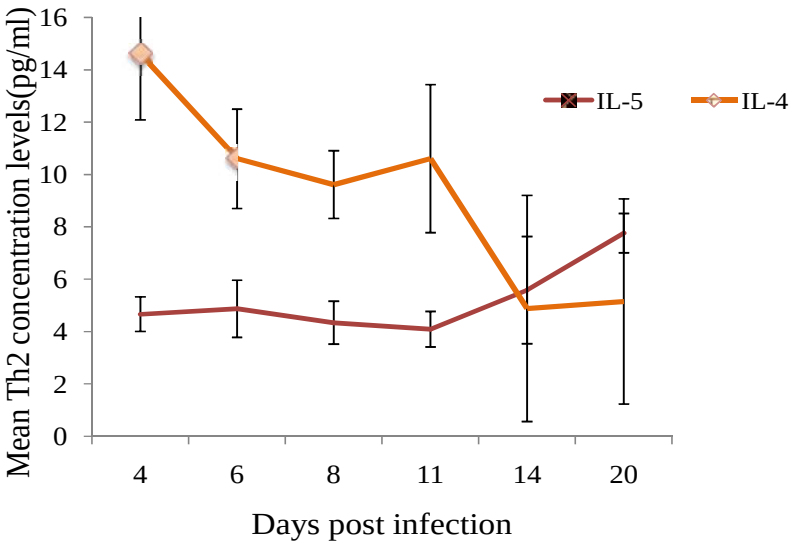

Figure 9 Basal T helper2 cytokine production trends during the course of infection with *Plasmodium berghei* ANKA. Error bars represent standard deviation of the mean

3.4 Cytokine Induced T-cell Apoptosis

In the experimental group, laddering patterns were observed showing that apoptosis was being induced by high levels of Th1 cytokines since internucleosomal cleavage of DNA is the biochemical hallmark of apoptosis (Plate.2A and B). Fragmentation of the DNA was shown to be extensively induced in T

cells from infected mice and was held to account for severity in pathology of malaria (Plate 2 A and B).

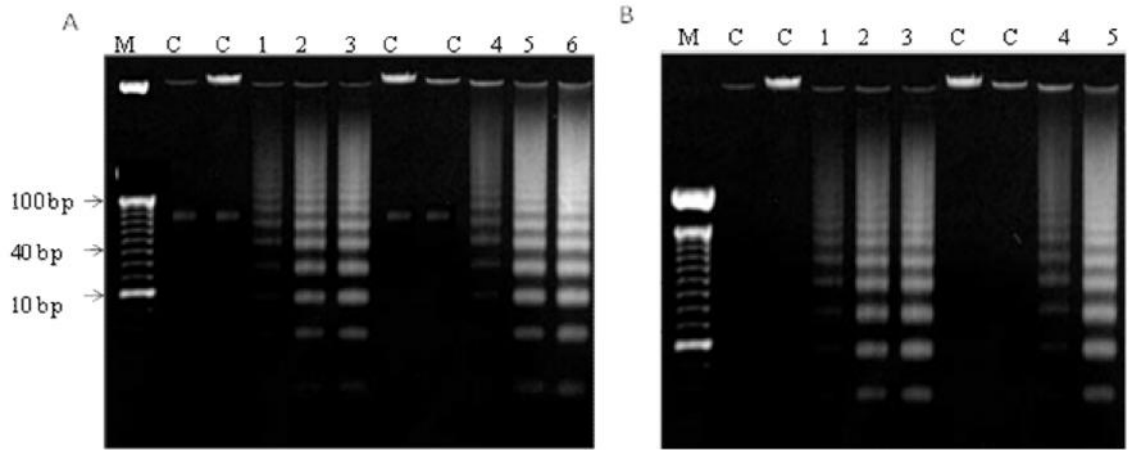

Plate 2 Cytokine induced DNA cleavage during infection with ECM

Key: DNA cleavage from infected mice observed Plate **2A** Lane M (100-bp DNA Molecular marker); Lane C, positive control (Cellular DNA from uninfected control mice); Lanes 1, 2, 3, 4, 5 and 6─Cellular DNA isolated on Day 6-20 post-infection). Initiation of DNA fragmentation from the spleen and brain lymphocytes was observed starting day 6 post-infection. Further DNA cleavage observed from brain Plate **2B**; Lane M(100-bp DNA Molecular marker); Lane C (negative control); Lanes 1, 2, 3, 4 & 5 (Cellular DNA cleavage as observed from day 6 p.i)

On day 8 post-infection when the levels of Th1 cytokines were at their optimum, concomitant DNA cleavage of T cells was detected with extensive apoptosis (lanes 1-6 in plate A and B). Apoptosis patterns of DNA cleavage were almost comparable at all intervals (Plate 2-A and B).

CHAPTER FOUR

4.0 DISCUSSION CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

4.2 Determination of Parasitemia in Balb/c Mice

In the present study, the development of cerebral complications was analyzed in BALB/c mice that are susceptible to ECM. Although several mechanisms have been proposed to explain cerebral malaria immunopathogenesis, the precise mechanisms is still unknown. Dysregulation of homeostasis of pro- and anti- inflammatory factors has been linked to the severity of CM (Stiles *et al.,* 2011). Upon infection with *P. berghei,* BALB/c mice exhibited varied parasitaemia responses to the infection which formed the basis for monitoring the cytokine concentrations depending on the survival rates.

This condition in *P. berghei* is well characterized and defined as the development of cerebral complications (ataxia, paralysis, convulsions, coma) at day 6–9 after infection of mice with 10^5 parasites (Engwerda *et al.,* 2005). In the current study, mice were injected intraperitonealy with 10^5 infected erythrocytes. The commencement of cerebral complications was determined by observing several clinical signs such as ruffled fur, hunching, limb paralysis and coma T helper 1 (Th1) cytokines (interferon-gamma, and tumour necrosis factor) have been implicated in driving the immunopathological process leading to CM, whereas some

Th2 cytokines such as interleukin-4 and Interleukin-5 appear to oppose this process (Hunt and Grau, 2006).

4.3 T helper 1 cytokines in ECM

The cytokine response is critical in determining the outcome of malarial infection (Maitland and Newton 2005). Cerebral Malaria is characterized by a Th1 response, with overproduction of some cytokines combined with underproduction of others (Hunt and Grau 2003). In this study T helper-1 set of cytokines showed significantly different patterns across the course of infection compared to their corresponding concentrations in control groups. The parasite load estimations correlated with high pro-inflammatory cytokines concentrations (IFN-γ and TNF-α) suggesting that the parasites were critical for inducing dysregulated pro-inflammatory disease pathogenesis among other factors. An exponential surge of IFN-γ and TNF-α for the test group was observed between day 6 and 14 respectively. IFN-γ levels in the test group were significantly different ($P<0.05$) between day 8 and day 14 post-infection respectively.

Nevertheless, unusual production of IFN-γ concentrations was observed all along in the progression of ECM infection which demonstrated that TH-1 pro-inflammatory cytokine dysregulation induced disease severity in ECM (Amante *et al.,* 2010). At start of the infection (day 4 post infection), the high levels of TNF-α production levels for the test group were significantly higher ($P<0.05$) suggestive of its early involvement in parasite clearance. This observation of high levels of TNF-α

cytokine in serum at the initial stages of the infection process (day 4 post infection) is consistent with previous studies in mice by Hunt and Grau (2003), equally, IFN-γ levels were exceedingly elevated which had higher impact on the pathology of T-lymphocytes this is because IFN-γ is absolutely critical for disease onset (Belnoue *et al.,* 2008), possibly by promoting tissue sequestration (Amante *et al.,* 2010). TNF-α is involved in the immune response to malaria as well as in the pathogenesis of severe disease (Rénia *et al.,* 2006). TNF-α is activated following the release of malaria antigens after schizont rapture and induces the local production of of IP-10. Subsequently IP-10 in concert with TNF-α may induce apoptosis of endothelial cells (Syarifah *et al.,* 2003). Conversely, with the surge in IFN-γ levels, TNF-α levels remained a distant second in circulation despite its earlier elevated production during the infection.

The mice displayed an increased trend in IFN-γ and TNF-α over the entire infection period which is initially beneficial by reducing the parasite load and later detrimental by decreasing the humoral response. A significant linear elevation for IFN-γ was observed between day 6 and day 8 post-infection when the parasitaemia levels were also on the upward trend.

Interferon -γ controls the activation and expansion of T cells by driving STAT1-dependent expression of T-bet in $CD4^+$ T cells, which is the initial step in the differentiation of T helper-1 cells and concurrently inhibiting both T helper-2 and T helper-17 lineages (Schulz *et al.,* 2009). In the present study, the cytokine seems to suppress T cell hyperactivity by limiting $CD4^+$ and $CD8^+$ T cell accumulation or

expansion through the induction of apoptosis as the high levels of the cytokine coincided with DNA cleavage as observed between the 6^{th} to the 14^{th} days post infection.

Tumour Necrosis Factor-α is the most potent pro-inflammatory cytokine during ECM that is induced by IFN-γ and is associated with severe malaria disease (Good *et al.,* 2005). IFN-γ and TNF-α cytokines initially mediate protection as elevated levels of TNF-α in serum have been demonstrated to correlate with protective immunity to *P. falciparum* malaria, but also it may be alternatively involved in disease severity (Syarifah *et al.,* 2003). The results presented in this study show that the TNF-α produced at day 4 post-infection correlates with protection from CM, as T-cell apoptosis was not observed converging with previous studies by Andrew *et al.,* (2005) showing that a later production of TNF-α is essential for the development of CM. Tumour Necrosis Factor-α up-regulates the expression of adhesion molecules, thereby exacerbating parasite sequestration and correlates with severe disease (Andrew *et al.,* 2005).

Moreover in murine models, IFN-γ and TNF-α cytokines initially mediates protection as elevated levels of IFN-γ in serum have been demonstrated to correlate with protective immunity to *P. falciparum* malaria (Syarifah *et al.,* 2003), but also they may be alternatively involved in disease severity. Similary, pro-inflammatory cytokines in murine models, seem to play an important role in protective immunity rather than in promoting CM by up-regulating expression of some adhesion molecules such as ICAM-1 (Syarifah *et al*., 2003). IFN-γ plays divergent roles at

different stages of *P. berghei* infection (Andrew *et al.,* 2005). These results confirm the observation that T helper-1 responses are initially involved in protection against infection which is consistent with the findings of Kashiwamura *et al.,* (2002) who found that IL-18 characterized as T helper-1 plays a protective role in murine malaria. These observations increase our understanding of the levels of T helper-1 cytokines in the pathogenesis of ECM and have relevance for understanding the role of cytokines in the pathogenesis of human CM. Although the study provides evidence that T helper-1 cytokines are detrimental in ECM it is reasonable to assume that optimal development and maintenance of severity relies on a complexity of several other host mechanisms rather than upon the levels of a single immune response.

4.4 T helper 2 cytokines in ECM

The kinetic pattern of T helper-2 cytokines during immune responses is to limit the consequences of an exaggerated proinflammatory response and to counterbalance the production of T helper-1 cytokines (Hunt *et al*., 2005). Significant transient increase in concentrations of IL-4 ($P<0.05$), was noted between day 4 and 8 post-infection but afterwards its levels remained low throughout the course of infection which is critical for biasing the immune reaction toward a T helper-2 phenotype (Waknine-Grinberg *et al.,* 2010). Interleukin-4 is an anti-inflammatory cytokine and thus increased production during initial parasite infection (days 4 to 8 post infection) was necessary to ameliorate the disease outcome as evidenced in the absence of CM symptoms in those mice (Hunt *et al.,* 2005). The current study show that *P. berghei*

infection caused an immediate significant shift in the IL-4 response of infected mice skewing towards a T helper 2 shift and later the mice displayed a prevalently T helper 1 response as it directs polarization of naïve T helper cells towards the Th2 phenotype variety of anti-inflammatory effects (Waknine-Grinberg *et al.,* 2010). This early regulation of pro-inflammatory response during murine malaria infection is mediated by regulatory T- cells in an IL-4 dependent manner (Chen, 2009). The regulation of TNF-α levels by IL-4 and IL-5 appears to contribute to the prevention of severe malarial anemia in humans (May *et al*., 2000) as observed in this study, however, the role that both IL-4 and -5 plays may depend on their levels, since very high levels of IL-4 have been associated with severe malaria in humans (Lyke *et al.,* 2004) and some animal models (Kobayashi *et al*., 2000).

The study reveals a relative deficiency in IL-5, albeit not statistically significant in days 6, 8, and 14 post-infection, whereas significant differences were observed in days 11 and 20 post-infection in mice that died of severe malaria suggesting a loss of down-regulatory function. IL-5 concentrations were remarkably low throughout the infection course but it was peculiarly significant ($P<0.05$) at day 11 and 20 post-infection because the transient increase was necessary to ameliorate the disease outcome in the late stages of the infection. Interleukin-4 and IL-5 levels in the test group were significantly higher ($P<0.05$) compared to control group at day 6 post-infection which demonstrates that T helper-2 cytokines were counter balancing the pro-inflammatory cytokines during the initial phase of infection. The inverse relationship between T helper 1 and T helper 2 cytokine milieu was striking as

revealed by a continued suppression of T helper--2 responses in the test group during the course of infection which in effect led to the overproduction of the T helper-1 immune responses. The early production of IL-4 together with IL-5 acts predominantly in an autocrine manner by down-regulating the activation of antigen-presenting cells, which then has the downstream effect of inhibition of T-cell activation and expansion (Andrew *et al*., 2005). The T helper-2 cytokines in the study were marginally up-regulated in and therefore they have played no role in CM pathology as this part of normal fluctuation.

4.5 Cytokine Induced Pathology on Splenic and Brain Migrating T Cells

In this study, evidence is provided indicating that various cytokines are altered in CM pathology. The elevated levels of IFN-γ and TNF-α is particularly remarkable since they are involved in parasite clearance and preserve erythropoiesis (Stevenson and Riley 2004) The possible involvement of cytokines production in disease severity was investigated by DNA fragmentation of T-lymphocytes in the spleen and brain tissues during malaria infection. The study demonstrates an elevated systemic significant increase in pro-inflammatory cytokines (IFN-γ and TNF-α) in the serum conciding with T-lymphocytes pathology in the spleen and brain. This is because while the Th1/Th2 dichotomy is highly stereotypic (Kelso, 1995) the activation of inappropriate effector responses conceivably could lead to the development of immune-mediated pathology.

The study found circulating elevated levels of TNF-α and IFN-γ to associate with severity of CM in mice because their concentrations are deleterious for the host lymphocytes although at low doses they promote the clearance of parasites (Peyron *et al.* 1994) since early secretion of T helper-1 cytokines promotes parasite killing by macrophages (Peyron *et al.* 1994). Although TNF-α concentrations were lower compared to IFN-γ, it is the most potent inducer of apoptosis. Thus, in the present study, death of T-cells in the course of infection was demonstrated to occur via apoptosis, confirmed by genomic DNA fragmentation, coinciding with elevated systemic pro-inflammatory (IFN-γ and TNF-α) responses. This inturn accelerated the severity of CM. This suggests that apoptosis results from paracrine signalling such as from one T cell to another rather than autocrine signaling. Although the study did not determine the concentrations of cytokines in the brain and spleen, it can be deduced that up-regulation of TNF-α and IFN-γ in these tissues was higher as indicated by the fragmentation of the T-lymphocytes DNA from this tissues.

This study established that high levels of parasitemia and cytokines correlate with T-cell apoptosis. This concurs with previous studies by Tewari (2007) which demonstrated that IFN-γ induces apoptosis of activated $CD4^+$ T cells as apoptosis was largely observed in the brain lymphocytes. T lymphocytes become activated in response to adequate stimulation to undergo proliferation or to be effector cells; this is accompanied by the expression of a variety of genes, including those for various cytokines or effector molecules (Ucker *et al.*, 1994).

Through a comparison of the patterns of cytokine responses in the mice, the present study was able to exhibit different courses and outcomes of infection that can be used to deduce instances of cytokine excess or deficiency. Elevated cytokine levels for IFN-γ and TNF-α coincided with the development of CM, implying their dual roles in the pathogenesis of malaria. Therefore, the complications seen in ECM may be related to cytokine-mediated injury required for parasite clearance. A study by Ramnath *et al.* (2006) on inflammatory mediators in microbial sepsis found that detectable plasma Thelper1 cytokines were indeed the cause of pathophysiology of T-cells by inducing DNA fragmentation. An elevated systemic significant increase in pro-inflammatory (IFN-γ and TNF-α) in the serum resulted to T-lymphocytes pathology in the spleen and brain. Under several circumstances, however, T lymphocytes respond to the stimulation by a programmed cell death response or apoptosis rather than activation (Helmby *et al.*, 2000). Apoptotic cell death is induced with several kinds of reagents, cytokines, and stimulations. Parasite or cytokines induced apoptosis in the host may also mediate the severity of malaria.

The study was able to compare the patterns of cytokine responses in the mice exhibiting different courses and outcomes of infection used to deduce instances of cytokine excess or deficiency. The cytokine levels for IFN-γ and TNF-α appears to coincide with the development of CM implying their dual roles in pathogenesis of malaria thus implying that complications seen in ECM may be related to cytokine-mediated injury required for parasite clearance.

4.6 Conclusions

i. Parasitemia induces cytokine production

ii. Elevated Levels of T helper 1 cytokines (IFN-γ and TNF-α) induces inflammation inhibiting parasite clearance

iii. TNF-α and IFN-γ appear to coincide with disease severity and suppression of IL-4 and IL-5 since elevated levels were noted in mice that had severe malaria

iv. The study demonstrated a parallel link between T-cell DNA cleavage and elevated levels of serum TH1 cytokines in respect to the findings above, the null hypothesis is rejected.

4.7 Limitations

These results suggest that cytokine production is a dynamic process. The limitations inherent in examining cytokine production at a single time point and in circulation rather than in the local microenvironments complicate the interpretation of these results. Nevertheless, insights could be gained from the cytokine differences observed because cytokines appear to maintain a delicate balance between the control of infection and contribution to disease in ECM infection. The mouse CBA kit used for the analysis handles four cytokines only (IL-4, IL-5, TNFα and IFN-γ) and therefore does not allow the profiling of other major cytokines such as IL-10, IL-12 and IL-1 that are important during ECM. Due to limited resources it was not possible to test for other key cytokines during ECM.

4.8 Recommendations

i. TNF-α and IFN-γ for use in cytokine therapy and vaccine development

ii. Further work on other cytokines such as IL-2 IL-10 and IL-12 that could be involved in the pathology

iii. Interventions to regulate T helper 1 cytokine response may be beneficial in prevention of cerebral malaria

REFERENCES

Abdel-Muhsin, A. M., Ranford-Cartwright, L. C., Medani, A. R., Ahmed, S., Suleiman, S., Khan, B. (**2002**). Detection of mutations in the *Plasmodium falciparum* dihydrofolate reductase (dhfr) gene by dot-blot hybridization. *American Journal of Tropical Medicine and Hygiene* **67**(1):24-27

Aidoo, I. and Udhayakumar, I. (**2000**). Field studies of cytotoxic T lymphocytes in malaria infections: implications for malaria vaccine development. *Parasitology Today* **16**(2): 50-56

Aikawa, M., Iseki, M., Barnwell, J. W., Taylor, D., Oo, M. M. and Howard, R. J. (1990). The pathology of human cerebral malaria American *Journal of Tropical Medicine and Hygiene* 43:30–37

Amante, F. H. (**2007**). A role for natural regulatory T cells in the pathogenesis of experimental cerebral malaria. *American Journal of Pathology* **171**(2): 548-59

Amante, F.H., Haque, A,. Stanley, A. C., Rivera, F. L. and Randall, L. M. (2010). Immune-mediated mechanisms of parasite tissue sequestration during experimental cerebral malaria. *Journal of Immunology* **185**:3632–3642.

Andrew, J., Mitchell, A. M., Hansen, L. H., Helen, J. B., Sarah, M., and Nicholas, H. H. (2005). Early Cytokine Production Is Associated with Protection from Murine Cerebral Malaria *Infection and Immunity* **73**(9): 5645–5653

Ayala, F. J., Escalante, A. A. and Rich, S. M. (**1999**). Evolution of *Plasmodium* and the recent origin of the world populations of *Plasmodium falciparum*. *Parasitologia* **41***(*1-3***)**:55-68

Ayi K, Patel, S. N., Serghides, L., Smith, T. G. and Kain, K. C. (**2005**). Nonopsonic phagocytosis of erythrocytes infected with ring-stage *Plasmodium falciparum*. *Infection and Immunity* **73**: 2559–2563

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebeque, S., Liu, Y.-J. (**2000**). Immunobiology of dendritic cells.*Annual Review of Immunology* **18**:767-811.

Beghdadi, W., Porcherie, A., Schneider, B.S., Dubayle, D., Peronet, R., Huerre, M. (**2008**). Inhibition of histamine-mediated signaling confers significant protection against severe malaria in mouse models of disease. *Journal of Experimental Medicine* **205**:395–408

Belnoue, E., Kayibanda, M., Vigario, A. M., Deschemin, J. C., van Rooijen, N., Viguier, M. (**2002**) CCR5 deficiency decreases susceptibility to experimental cerebral malaria. *Blood* **101** (11): 4253-4259

Belnoue, E., Potter, S. M., Rosa, D. S., Mauduit, M. and Gruner, A. C. (**2008**). Control of pathogenic CD8+ T cell migration to the brain by IFN-gamma during experimental cerebral malaria. *Parasite Immunology* **30**:544–553

Biemba, G., Gordeuk, V. R., Thuma, P. and Weiss, G. (**2000**). Markers of inflammation in children with severe malarial anaemia. *Tropical Medicine & International Health* **5**(4): 256-262

Blank, C., Sabri, M., and Di Lorenzo, C. (**2004**). Pediatric gastric and duodenal disorders. *Current Opinion in Gastroenterology* **20**(6):551-555

Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T. and Druilhe, P. (1995). Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro* but act in cooperation with monocytes. *Journal of Experimental Medicine* **172** (6):1633–1641

Brandts, C. H., Mordmüller, B. G., Lehman, L. G. and Kremsner, P. G. (1997).The dual role of tumor necrosis factor (TNF) during a malarial attack *Sante* **7**(4):271-274

Brian de Souza, J. and Riley, E. M. (2002). Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis. *Microbes and Infection* **4:**291–300

Brown, H., Turner, S., Rogerson, M., Tembo, J., Mwenechanya, M., Molyneux, K. *et al.*, (**1999).** Cytokine expression in the brain in human cerebral malaria. *Journal of Infectious Diseases* **180** (5): 1742-1746

Bull, P. C., Lowe, B. S., Kortok, M., Molyneux, C. S., Newbold, C. I. and Marsh, K. (2002). Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nature Medicine* **4**:358 - 360

Casares, S. and Richie, T. L. (2009). Immune evasion by malaria parasites: a challenge for vaccine development. *Current Opinion in Immunology* **21**:321-330

Chen, G., Liu, J., Wang, Q.H., Wu, Y., Feng, H., Zheng, W., Guo, S.Y., Li, D.M., Wang, J.C. and Cao, Y. M. (2009). Effects of CD4+CD25+Foxp3+ regulatory T cells on early *Plasmodium yoelii* 17XL infection in BALB/c mice. *International Journal for Parasitology* **2**:1–14

Clerici, M. and Shearer, G. M. (**1994**). The Th1-Th2 hypothesis of HIV infection: new insights. *Immunology Today* **15**(12): 575-581

Coban, C., Ishii, K. J., Kawai, T., Hemmi, H., Sato, S., Uematsu, S. (**2005**). Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *Journal of Experimental Medicine* **201**(1): 19-25

Cohen, S., Mac, G. I. and Carrington, S. (**1961**). Gamma-globulin and acquired immunity to human malaria. *Nauchni Tr Vissh Med Inst Sofiia* **192**:733-756

Cox-Singh, J., Davis, T. M., Lee, K. S., Shamsul, S. S., Matusop, A., Ratnam, S., *et al.*, (**2008**). *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life-threatening. *Clinical Infectious Diseases* **46**:165–171

Day, K., Karamalis, F., Thompson, J., Barnes, D. and Peterson, C. (**1991**). Genes necessary for expression of a virulence determinant and for transmission of *Plasmodium falciparum* are located on a 0.3-megabase region of chromosome 9. *Proceedings of the National Academy of Sciences* **90**(17): 8292-8296

de Souza, J. B. and Riley, E. M. (**2002**). Cerebral malaria: the contribution of studies in animal models to our understanding of Immunopathogenesis. *Microbes and Infection* **4:**291–300

de Souza, E. T., de Lira, D. P., de Queiroz, A. C., da Silva, D. J., de Aquino, A. B., Mella, E. A., (**2009**). The antinociceptive and anti-inflammatory activities of caulerpin, a bisindole alkaloid isolated from seaweeds of the genus Caulerpa. *Marine Drugs* **7**(4):689-704

Desruisseaux, M. S., Gulinello, M., Smith, D. N., Lee, S. C., Tsuji, M., Weiss, L. M., Spray, D. C. and Tanowitz, H. B. (2008). Cognitive dysfunction in mice infected with *Plasmodium berghei* strain ANKA. *Journal Of Infectious Diseases* **197**:1621–1627

Dodoo, D., Omer, F. M., Todd, J., Akanmori, B. D., Koram, K. A. and Riley, E. M. (**2001**). Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *Plasmodium falciparum* malaria, *Journal of Infectious Diseases* **185**: 971-979

Donati, D. Todd, J., Akanmori, B. D., and Zhang, P L. (**2004**). Identification of a polyclonal B-cell activator in *Plasmodium falciparum*. *Infection and Immunology* **72**(9): 5412-5418

Dondorp, A. M., Kager, P. A., Vreeken, J. and White, N. J. (**2000**). Abnormal blood flow and red blood cell deformability in severe malaria. *Parasitology Today* **16**(6): 228-232

Edozien, J. C. and Gilles, **H. M.** (**1962**). Adult and cord-blood gamma-globulin and immunity to malaria in Nigerians. *Lancet* **2**: 9511-9555

Engwerda, C., Belnoue, E., Gruner, A. C. and Renia, L. (**2005**). Experimental models of cerebral malaria. *Current Topics in Microbiology and Immunology* **297**:103–143

Fell, A. and Smith, N. (**1998**). Immunity to asexual blood stages of *Plasmodium*: Is resistance to acute malaria adaptive or innate. *Parasitology Today* **14**(9): 364-368

Fernanda, G., Baptista, A., Pamplona, A. C., Pena, M. M., Mota, S. P. and Ana, M. V. (2010). Accumulation of *Plasmodium berghei*-Infected Red Blood Cells in the Brain Is Crucial for the Development of Cerebral Malaria in Mice *Infection and Immunity* **78**:4033-4039

Ferrante, A. and Rzepczyk, C. (**1997**). Atypical IgG subclass antibody responses to *Plasmodium falciparum* asexual stage antignes. *Parasitology Today* ***13***(4): 145-148

Foulds, K. E., Rotte, M. J., Paley, M. A., Cox-Singh, B., Douek, D.C. and Hill, B. J., (2008) IFN-gamma mediates the death of Th1 cells in a paracrine manner. *Journal of Immunology* **180**:842–849

Good, M. F., Xu, H., Wykes, M., and Engwerda, C. R. (2005). Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. *Annual Review of Immunology* **23:69**–99

Grau, G. E., Heremans, H., Piguet, P. F., Pointaire, P., Lambert P. H., Billiau A. and Vassalli, P. (**2003**). Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *Journal of Infectious Diseases* **187**(3):461-466

Grau, G. E., Tacchini-Cottier, F., Vesin, C., Milon, G., Lou, J., Piguet, P. F., and Juillard, P. (1993). TNF-induced microvascular pathology: active role for platelets and importance of the LFA-1/ICAM-1 interaction. *European Cytokine Network* **4**:415–419

Grau, G. E., Bieler, G., Pointaire P., DeKossodo, S., Tacchini-Cotier, F., Vassalli, P. (**1990**). Significance of cytokine production and adhesion molecules in malarial immunopathology. *Immunology Lett*ers **25**:189–194

Groux, H. Davies, J. R and Gysin, J. (1990). Opsonization as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum*: functional role of IgG subclasses. *Research in Immunology* **141***:529*-531

Gupta, S., Trenholme, K., Anderson, R. M. and Day, K. P. (**1999**). Antigenic diversity and the transmission dynamics of *Plasmodium falciparum*. *Science* **263**(5149): 961-963

Hafalla, J. C., Cockburn, I. A. and Zavala, F. (**2011**). Protective and pathogenic roles of CD8+ T cells during malaria infection. *Parasite Immunology* **28** (1-2): 15-24

Harpaz, R., Edelman, R., Wasserman, S. S., Levine, M. M., Davies, J. R. and Sztein, M. B. (**1992**). Serum cytokine profiles in experimental human malaria. *Journal of Clinical Investigations* **90**:515–523

Helmby, H., Jonsson, G. and Troye-Blomberg, M. (**2000**). Cellular changes and apoptosis in the spleens and peripheral blood of mice infected with blood-stage *Plasmodium chabaudi* AS. *Infection and Immunity* **68**:1485–1490

Hill, A. V., Allsopp, C. E., Kwiatkowski, D., Anstey, N. M. and Twumasi, P. (**1991**).Common West African HLA antigens are associated with protection from severe malaria. *Nature* **352**(6336)*:*595-600

Ho, M., Webster, H. K., Tongtawe, P., Pattanapanyasat, K. and Weidanz, W. P. (**1990a**). Increased gamma delta T cells in acute *Plasmodium falciparum* malaria. *Immunology. Letters* **25**(1-3): 139-141

Hoffman, S., Oster, C., Plowe, C., Woollett, G. and Beier, J. (**1987**). Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications. *Science* **237** (4815): 639-642

Homel, M., and Gilles, H. M. (**1998**). Malaria in F.E.G. Cox, J.P. Krier, and D. Wakelin (ed.), Topley & Wilson's. *Microbiology and Microbial Infections* **9**:361-409

Hughes, M., and Hughes, A. (**1995**). Natural selection on *Plasmodium* surface proteins. *Molecular and Biochemical Parasitology* **71**(1): 99-113

Hunt, N. H. and Grau, G. E. (**2003)**. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends in Immunololgy* **24:**491-523

Hunt, N. H., Mitchell, A. J., Hansen, A. M., Hee, L., Ball, H. J., Potter, S. M., Walker, J. C., (2005) Early cytokine production is associated with protection from murine cerebral malaria. *Infection and Immunity* **73**:5645-5653

Hunt, N. H,, and Grau, G. E., (2006) Current perspectives on the mechanism of action of artemisinins. *International Journal of Parasitology* **36**(14):1427-1441

Jocelyn, H., Neil, R., David, N. L., David, R. K. and Brian de Souz, J. (2000). Immunopathology of Cerebral Malaria: Morphological Evidence of Parasite Sequestration in Murine Brain Microvasculature *Infection and. Immunity* **68**:5364-5376

Jones, M. K. and Good, **M. F.** (**2006).** Malaria parasites up close. *Nature Medicine* **12** (2):170-181

Kashiwamura, S., Ueda, H. and Okamura, H. (2002). Roles of interleukin-18 in tissue destruction and compensatory reactions. *Journal of Immunotherapy* **1**: 4-11

Kelso, A. (1995). Th1 and Th2 subsets: paradigms lost? *Immunology Today* **16:**374-379

Kern, P., Hemmer, C. J., Van Damme, J., Gruss, H. J. and Dietrich, M. (**2000**). Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria. *American. Journal of Medicine* **87:**139-143

Kobayashi, S., Akiyama, T., Nata, K., Abe, M., Tajima, M. and Shervani, N. J. (2000). Identification of a receptor for reg (regenerating gene) protein, a pancreatic beta-cell regeneration factor. *Journal of Biological Chemistry* **275**:10723-107266

Kojima, S., Syarifah, H. P. and Masashi, H. (2003). Cytokine and chemokine responses in a cerebral malaria susceptible or resistant strain of mice to *Plasmodium berghei* ANKA infection: early chemokine expression in the brain. *International Immunology* **15** (5): 633-640

Kurtzhals, J. A., Akanmori, B, D., Goka, B.Q., Adabayeri, V., Nkrumah, F.K., Behr, C. and Hviid, L. (1999). The cytokine balance in severe malarial anemia. *Journal of Infectious Diseases* **180** (5):1753-1755

Kwiatkowski, D., Hill, A. V., Sambou, I., Twumasi, P., Castracane, J., Manogue, K., Cerami, A. (**1990).** TNF concentration in fatal cerebral, non-fatal cerebral and uncomplicated *Plasmodium falciparum* malaria *Lancet* **336** (8725):1201-1204

Lackner, P., Beer, R., Heussler, V., Goebel, G., Rudzki, D., Helbok, R. (**2006**). Behavioural and histopathological alterations in mice with cerebral malaria. *Neuropathology and Applied Neurobiology* 32:177–188.

Langhorne, J., Quin, S. and Sanni, L. (**2002**). Mouse models of blood stage malaria infections: immune responses and cytokines involved in protection and pathology. *Chemical Immunology* **80**:204–228

Li, C., Sanni, L. A., Omer, F., Riley, E. and Langhorne, J. (2003). Pathology and mortality of *Plasmodium chabaudi chabaudi* infection in IL-10-deficient mice is ameliorated by anti-TNF-a and exacerbated by anti-TGF-b antibodies. *Infection and Immun*ity **71**:4850–4856

Li, C., Seixas, E. and Langhorne, J. (2001). Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. *Medical Microbiology and Immunology* **189**:115–126

Liu, M. Y., Xu, S. M., Song, G. H., Zhongguo, J. S., Chong, X. Y., . and Za, Z. (**2001**). Dendritic cells and protozoan infection. *Immunity to Protozoan Parasites* **19**(**6**):367-370

Lou, J., Lucas, R. and Grau, G. E. (**2001**). Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. *Clinical. Microbiology* **14**:810–820

Lyke, K. E., Burges, R., Cissoko, Y., Sangare, L., Dao, M., Diarra, I., (**2004**). Serum levels of the proinflammatory cytokines interleukin-1 Beta (IL-1ß), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12 (p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. *Infection and Immunity* **72**:5630–5637

Mackintosh, C. L., Beeson, J. G. and Marsh, K. (**2004).** Clinical features and pathogenesis of severe malaria. *Trends in Parasitology* **12**:597-603

Maitland, K. and Newton, C. R. (**2005**). Acidosis of severe falciparum malaria: heading for a shock *Trends in Parasitology* **2:**11–16

Marsh, K,. Forster, D., Waruiru, C., Mwangi, I., Winstanley, M. and Marsh, V. (**1999**). Indicators of life-threatening malaria in African children. *New England Journal of Medicine* **332** (21): 1399-1404

May, J., Lell, B. A., Luty, J. C., Meyer, G. and Kremsner, P. G. (**2000**). Plasma interleukin-10: tumor necrosis factor (TNF)-alpha ratio is associated with TNF

promoter variants and predicts malarial complications. *Journal Of Infectious Diseases* **182**:1570-1573

McKenna, K. C., Tsuji, M., Sarzotti, M., Sacci, J.B. and Witney A. A. (**2000**). Gamma delta T cells are a component of early immunity against pre-erythrocytic malaria parasites. *Infection and Immunity* **68**(4): 2224-2230

Meding, S. J., and Langhorne, J. (**1991)**. CD4+ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi. European Journal of Immunology* **21** (6):1433-1444

Miller, L. H., Baruch, D. I., Marsh, K. and Doumbo, O. K. (**2002**). The pathogenic basis of malaria. *Nature* **415** (6872):673-679

Milner, A. M. D., Jimmy, C. V., Jacqui, M., Rachel, F. D., Sarah, K. V., Daniel, E. N. (2012) Human cerebral malaria and *Plasmodium falciparum* genotypes in Malawi. *Malaria Journal* **11**:35 1475-2875

Moore, J. M., Nahlen, B. L., Misore, A., Lal, A. A. and Udhayakumar, V. (**1999**). Elevated production of interferon- gamma by placental blood mononuclear cells is associated with protection in an area with high transmission of malaria. *Journal of Infectious Diseases* 179(**5**): 1218-1225

Muregi, F. W., Isao, O., Uchijima, M.,Hideto K. and Akira, I. (2011). Resistance of a Rodent Malaria Parasite to a Thymidylate Synthase Inhibitor Induces an

Apoptotic Parasite Death and Imposes a Huge Cost of Fitness *PLoS One* **6(**6): 21251-21255

Newbold, C. I., Craig A. G., Kyes, S., Berendt, A. R., Snow, R. W., and Marsh, K. (**1992**). PfEMP1, polymorphism and pathogenesis. *Tropical Medical Parasitology* **1**:551-557

Newton, C. R., Crawley, J. and Sowumni, A. (**1997**). Intracranial hypertension in Africans with cerebral malaria. *Archives of Disease in Childhood* **76** (3): 219-226

Nitcheu, J., Bonduelle, O., Combadiere, C., Tefit, M., Seilhean, D., Mazier, D. (**2003**). Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. *Journal of Immunology* **170**:2221–2228

Pandey, C., Sanjay, S., Priyabrata, P., Pillai, R,. Usha, P., Andrew, L. (**2002**). Bacterially expressed and refolded receptor binding domain of Plasmodium falciparum elicits invasion inhibitory antibodies EBA-175.*Molecular and Biochemical Parasitology* **123** (1): 23-33

Pasquetto, V., Fidock, D. A., Gras, H., Badell, E. and Eling, W. (**1997**). *Plasmodium falciparum* sporozoite invasion is inhibited by naturally acquired or experimentally induced polyclonal antibodies to the STARP antigen. *European Journal of. Immunology* **27 (**10): 2502-2513

Patel, S N., Serghides, L. and Smith, T. G. (**2004**). CD36 mediates the phagocytosis of Plasmodium falciparum-infected erythrocytes by rodent macrophages. *Journal of Infectious Diseases* 189: 204–213

Patnaik, J. K., Das, B. S., Mishra, S. K., Mohanty, S., Satpathy, S. K. and Mohanty, D. (1994). Vascular clogging, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. *American Journal of Tropical Medicine and Hygiene* **51**:642–647

Peyron, F., Caux-Menetrier, C., Roux-Lombard, P., Niyongabo, T., Aubry, P. and Deloron, P. (**1994**). Soluble intercellular adhesion molecule-1 and E-selectin levels in plasma of falciparum malaria patients and their lack of correlation with levels of tumor necrosis factor alpha, interleukin 1 alpha (IL-1 alpha), and IL-10. *Clinical and Diagnostic Laboratory Immunology* **1**(6):741-753

Ramnath, R. D., Welch, K., Siddiqui, J., and Remick, D. G. (2006) Inflammatory mediators in sepsis: Cytokines, chemokines, adhesion molecules and gases. *Journal. Of Organ Dysfunction* 2:80-92

Renia, L., Gr¨uner, A. C. and Snounou, G. (**2001**). Pathogenic T cells in cerebral malaria. *International Journal of Parasitology* **36** (5):547-554

Rénia, L., Potter, S. M., Mauduit, M., Rosa, D. S., Kayibanda, M., Deschemin, J.C. (2006). Pathogenic T cells in cerebral malaria. *International Journal of Parasitology* **36**:547–554

Rest, J. R. (**1982).** Cerebral malaria in inbred mice. I. A new model and its pathology *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76** (3): 410-415

Ricke, C. H., Staalsoe, T., Koram, K., Akanmori, B. D., and Riley, E. M. (**2000**). Plasma antibodies from malaria exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. *Journal of Immunology* **165**(6):3309-3316

Riley, E. M., Couper, K. N., Helmby, H., Hafalla, J. C., de Souza, J. B. and Langhorne, J. (2006). Regulating immunity to malaria. *Parasite Immunology* **28**(1-2):35-49

Romero, P., Maryanski, J., Corradin, G., Nussenzweig, R. and Nussenzweig, V., (**1989**). Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature* **341**(6240): 323-326

Rot, A. and von Andrian, U. H. (**2007**). Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annual Reviews in Immunology* **22**:891-928

Rudin, W., Favre, N., Bordmann, G. and Ryffel, B. (**1997**). Interferon-gamma is essential for the development of cerebral malaria. *European Journal of Immunology* **27**(4): 810-815

Sabchareon, A., Burnouf, T., Ouattara, D., Attanath, P., Bouharoun-Tayoun, H., Chantavanich, P. (1991). Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. *American Journal of Tropical Medicine Hygeine* **45**(3):297-308

Sambrook, J. and Gething, M. J. (1989). Protein structure. Chaperones, paperones. *Nature* **342**(6247):224-225

Sarfo, B. Y., Wilson, N. O., Bond, V. C. and Stiles, J. K. (**2011).** *Plasmodium berghei* ANKA infection increases Foxp3, IL-10 and IL-2 in CXCL-10 deficient C57BL/6 mice. *Malaria Journal* **28**:10-69

Schmitz, S., Garden, O. A., Werling, D. and Allenspach, K. (2012). Gene expression of selected signature cytokines of T cell subsets in duodenal tissues of dogs with and without inflammatory bowel disease. *Veterinary Immunology and Immunopathology* **146** (1):87-91

Schofield, L. (**1991**). On the function of repetitive domain in protein antigen of *Plasmodium* and eukaryotic parasites. *Parasitology Today* **7**(5): 99-105

Schofield, L. and Grau, G. E. (**2005).** Immunological processes in malaria pathogenesis. *Nature Reviews Immunology* **5**(9): 722-35

Schulz, E.G., Mariani, L., Radbruch, A. and Hofer, T. (**2009**). Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Infection and Immunity.* **30**:673–683

Schwarzer, E. and Arese, P. (**1996**). Phagocytosis of malarial pigment hemozoin inhibits NADPH-oxidase activity in human monocyte-derived macrophages. *Biochim Biophys Acta* **1316**: 169–175

Sébastien, B., Fatima, N., Alexis, C., Virgilio do Rosario, F. L., Pierre-André, C. and Sylviane P. (**2004**). Comparative study of brain $cd8^{+}$ t cells induced by sporozoites and those induced by blood-stage *plasmodium berghei* anka involved in the development of cerebral *Malaria Infection and Immunity* **72**:2817-2826

Seixas, E., Fonseca, L. and Langhorne, J. (2002**).** The influence of gammadelta T cells on the CD4Þ T cell and antibody response during a primary *Plasmodium chabaudi* infection in mice. *Parasite Immunology* **24**:131–140

Serghides, L. Kain, K. C and Patel, S. N. (**2002).** CD36 and malaria: friends or foes *Trends in Parasitology* **19**(10):461-469

Serghides, L., Smith, T. G., Patel, S. N. and Kain, K. C. (**2003**). CD36 and malaria: friends or foes . *Trends in Parasitology* **19**: 461–469

Sherry, A.D.,Peng, M., Emig, F.A., Mao, A., Lu, W., Kirby, E. P., Niewiarowski, S., and Kowalska, M. A. (1995) Interaction of echicetin with a high affinity thrombin binding site on platelet glycoprotein GPIb. *Journal of Thrombosis and Haemostasis* **74**(3):954-957

Shi, Y. P., Udhayakumar, V., Oloo, A. J., Nahlen, B. L. and Lal, A. A. (**1991**). Differential effect and interaction of monocytes, hyperimmune sera, and

immunoglobulin G on the growth of asexual stage *Plasmodium falciparum* parasites. *American Journal Of Tropical Medicine and Hygine* **60** (1): 135-41

Snedecor, G. W., and Cochran W. G., (1989) statistical methods 8th edition Ames: Iowa state press 67: 456-461

Stevenson, M. M. and Riley, E. M. (**2004).** Innate immunity to malaria. *Nature Reviews Immunology* **4**(3):169-180

Stiles, J. K., Sarfo, B., Y, Nana, O. W. and Vincent, C. B. (**2011**). *Plasmodium berghei* ANKA infection increases Foxp3, IL-10 and IL-2 in CXCL-10 deficient C57BL/6 mice. *Malaria Journal* **1269**:10-69

Su, Z. and Stevenson, M. (2000). Central role of endogenous gamma interferon in protective immunity against blood-stage *Plasmodium chabaudi* AS infection. *Infection and Immunity* **68** :4399–4406

Syarifah N. A., Munchar, M. J, , Jamal, R., and Looi, L. M., (2003) CD44s expression correlated with the International Neuroblastoma Pathology Classification (Shimada system) for neuroblastic tumours. *Pathology* **35** (2):125-129

Sztein, M. B. and Levine, M. M. (2004). Vaccine development strategies for improving immunization: the role of modern immunology *Nature Immunology* **5** (5):460-464.

Takahiko, T., Kazumi, K., Masao, Y. and Katsuyuki, Y. (2011). Host Response and Inflammation Prevention of Experimental Cerebral Malaria by Flt3 Ligand during Infection with *Plasmodium berghei* ANKA. *Infection and*. *Immuntiy* **79**:3947-3957

Taylor, T. E., Fu, W. J., Carr, R. A., Whitten, R. O., Mueller, J. G., Fosiko, N. G. (2004**).** Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nature Medicine* **10**:143–145

Taylor-Robinson, A. and Smith, E. (**1999**). A role for cytokines in potentiation of malaria vaccines through immunological modulation of blood stage infections. *Immunology*. *Reviews* 17105-17124

Tewari, K., Nakayama, Y. and Suresh, M. (2007). Role of direct effects of IFN-gamma on T cells in the regulation of CD8 T cell homeostasis. *Journal of Immunoogy* **179**(4):2115-2125

Trape, J.F., Rogier, C., Konate, L., Diagne, N., Bouganali, H., Canque, B. (**1994**). The Dielmo project: A longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. *American Journal of Tropical Medicine and Hygiene* **51**(2):123-137

Ucker, D. S., Hebshi, L. D., Blomquist, J. F. and Torbett. B. E. (1994). Physiological T-cell death: susceptibility is modulated by activation, aging, and

transformation, but the mechanism is constant. *Immunolology Review* **142**:273–299

van der Heyde, H. C., Nolan, J., Combes, V., Gramaglia, I,. and Grau, G. E. (**2006**). A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. *Trends in Parasitology* **22**:503–508.

Waller, D. K., Pujazon, M. A., Canfield, M. A., Scheuerle, A. E., and Byrne, J. L. (1995) Frequency of prenatal diagnosis of birth defects in Houston. *Fetal Diagnosis Therapy* **15** (**6**):348-354

Waitumbi, J. N., Opollo, M. O., Muga, R. O., Misore, A. O., and Stoute, J. A. (2000) Red cell surface changes and erythrophagocytosis in children with severe *Plasmodium falciparum* anemia. *Blood* **95**:1481–1486

Waknine-Grinberg, J. H., Daniel, G., Ariel O., Eliezer, F., Alina H., Michael, J. D. (**2010)**. Schistosoma mansoni infection reduces the incidence of murine cerebral malaria. *Malaria Journal* **10**:1185-1185

Wassmer, S. C., Coltel, N., Combes, V. and Grau, G, E. (**2003**) Pathogenesis of cerebral malaria: facts and hypotheses *The American Journal of Tropical Medicine and Hygiene*. **63**(3):254-257

Weiss, W., Sedegah, M., Beaudoin, R., Miller, L. and Good, M. (**1998**). CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proceedings of National. Academy of Science.* **85**(2): 573-576

Winkler, S., Willheim, M., Baier, K., Schmid, D. and Aichelburg, A. (**1999**). Frequency of cytokine-producing T cells in patients of different age groups with *Plasmodium falciparum* malaria. *Journal Of Infectious Diseases* **179**(1): 209-216

World Health Organization, (**2002**) World Malaria Report

World Health Organization, (**2012**) World Malaria Report

World Health Organization, (**2011**) World Malaria Report

Xu, H., Wipasa, J., Yan, H., Zeng, M., Makobongo, M.O., Finkelman, F.D., Kelso, A. (2002). The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. *Journal of Experimental Medicine* **195**(7): 881-889

Yañez, D. M., Batchelder, J., van der Heyde, H. C., Manning, D. D. and Weidanz, W. P. (1999). Gamma delta T-cell function in pathogenesis of cerebral malaria in mice infected with *Plasmodium berghei* ANKA. *Infection and Immunity* **67**(1):446-448.

APPENDICES

Appendix 1; **Cytometric Bead Array (CBA) Mouse Th1/Th2 Cytokine Kit**

The BD™ CBA Mouse Th1/Th2/Cytokine Kit can be used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interferon-γ (IFN-γ), and Tumor Necrosis Factor (TNF), protein levels in a single sample. The kit performance has been optimized for analysis of physiologically relevant concentrations (pg/mL levels) of specific cytokine proteins in tissue culture supernatants and serum samples. The kit provides sufficient reagents for 80 tests.

Principle of CBA Assays

BD CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead in a BD CBA kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector. The Kit uses bead array technology to simultaneously detect

multiple cytokine proteins in research samples. Five bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL- 2, IL-4, IL-5, IFN-γ, and TNF proteins. The five bead populations are mixed together to form the bead array, which is resolved in a red channel (FL3 or FL4) of a flow cytometer.

Table 1 Theoretical limit of detection for serum cytokine levels using the Mouse CBA kit

Cytokine	Limit of detection(pg/mL)
IL-2	0.1
IL-4	0.03
IL-5	1.4
IFN-γ	0.5
TNF-α	0.9

Appendix 2; Bd CBA mouse kit contents

Bead reagents

Mouse Cytokine Capture Beads (A1–A7): An 80-test vial of each specific capture bead (A1–A7). The specific capture beads, having discrete fluorescence intensity characteristics, are distributed from brightest (A1) to dimmest (A7).

Cytometer Setup Beads (D): A 30-test vial of setup beads for setting the initial

instrument PMT voltages and compensation settings is sufficient for 10 instrument setup procedures. The Cytometer Setup Beads are formulated for use at 50 µL/test.

Antibody and standard reagents

Mouse Th1/Th2/Th17 PE Detection Reagent (B): An 80-test vial of PE-conjugated anti-mouse IL-2, IL-4, IL-5, IFN-γ and TNF α antibodies that is formulated for use at 50 µL/test.

Mouse Th1/Th2/Th17 Cytokine Standards (C): Two vials containing lyophilized recombinant mouse cytokine proteins. Each vial should be reconstituted in 2.0 mL of Assay Diluent to prepare the top standard.

PE Positive Control Detector (E1): A 10-test vial of PE- conjugated antibody control that is formulated for use at 50 µL/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings.

FITC Positive Control Detector (E2): A 10-test vial of FITC-conjugated antibody control that is formulated for use at 50 µL/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings.

Buffer reagents **Wash Buffer (F):** A 130-mL bottle of phosphate buffered saline (PBS) solution (1X), containing protein and detergent, used for wash steps and to resuspend the washed beads for analysis.

Assay Diluent (G): A 30-mL bottle of a buffered protein solution (1X) used to reconstitute and dilute the Mouse Th1/Th2 Cytokine Standards and to dilute unknown samples.

Advantages over ELISA

1) The required sample volume is approximately one-seventh the quantity necessary for conventional ELISA assays due to the detection of seven analytes in a single sample.

2) A single set of diluted standards is used to generate a standard curve for each analyte.

3) A BD CBA experiment takes less time than a single ELISA and provides results that would normally require seven conventional ELISAs.

Limitations

The theoretical limit of detection of the BD CBA Mouse Th1/Th2 Cytokine Kit is comparable to conventional ELISA, but due to the complexity and kinetics of this multi-analyte assay, the actual limit of detection on a given experiment may vary.

The BD CBA Kit is not recommended for use on stream- in-air instruments for which signal intensities may be reduced, adversely affecting assay sensitivity.

.

Appendix 3 Cytokine levels analysis for day 4 post infection with P berghei anka

Cytokines	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
				Lower	Upper			
IFN-γ vs IFN-γ(control)	2.33333	2.19393	1.26667	-3.1167	7.78336	1.842	2	0.207
TNF-α vs TNF-α (control)	0.62333	0.33262	0.19204	-0.2029	1.4496	3.246	2	0.043
IL4 vs IL4 (control)	-0.175	0.16039	0.0926	-0.5734	0.22343	-1.89	2	0.04
IL5 vs IL5(control)	-3.1033	1.41737	0.81832	-6.6243	0.41761	-3.792	2	0.063

Appendix 4; Cytokine levels analysis for day 6 post infection with *P berghei* anka

Cytokines	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
				Lower	Upper			
IFN-γ vs IFN- (control)	1.12E+01	9.84133	5.68189	-13.251	35.6439	1.971	2	0.188
TNF-α vsTNF-α (control)	2.98E+01	47.8547	27.6289	-89.094	148.661	1.078	2	0.404
IL-4 vs IL-4 (control)	8.04167	1.88595	1.08885	3.35671	12.7266	7.385	2	0.018
IL-5 vs IL-5 (control)	-0.92333	0.73112	0.42211	-2.7395	0.89286	-2.19	2	0.16

Appendix 5; Cytokine analysis levels for day 8 post infection with *P berghei* anka

Cytokines	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper	t	df	Sig. (2-tailed)
IFN-γ vs IFN-γ (control)	2.08E+02	133.0989	76.84471	-122.246	539.0261	2.712	2	0.05
TNF-α vs TNF-α (control)	3.15E+01	17.41427	10.05414	-11.7861	74.73279	3.13	2	0.04
IL-4 vs IL-4 (control)	6.50333	1.64233	0.9482	2.42357	10.5831	6.859	2	0.021
IL-5 vs IL-5 (control)	-1.60333	1.62608	0.93882	-5.64274	2.43607	-1.708	2	0.23

Appendix 6; Cytokine analysis levels for day 11 post infection with *P berghei* anka

Cytokine	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper	t	df	Sig. (2-tailed)
IFN-γ vs IFN-γ (control)	1.67E+02	80.13438	46.26561	-31.6182	366.5115	3.619	2	0.029
TNF-α vs TNF-α (control)	9.33E+01	17.17336	9.91504	50.62235	135.9443	9.408	2	0.011
IL-4 vs IL-4 (control)	7.71333	3.31219	1.9123	-0.51461	15.94128	4.034	2	0.056
IL-5 vs IL-5 (control)	-1.70333	0.70671	0.40802	-3.45889	0.05222	4.175	2	0.053

Appendix 7; Cytokine analysis levels for day 14 post infection with *P berghei* anka

Cytokine	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
				Lower	Upper			
IFN-γ vs IFN-γ (control)	2.00E+02	91.72386	52.95679	-28.0347	427.6747	3.773	2	0.024
TNF-α vs TNF-α (control)	2.90E+01	18.98759	10.96249	-18.2078	76.1278	2.642	2	0.063
IL-4 vs IL-4 (control)	2.32333	4.40519	2.54334	-8.61977	13.26644	0.913	2	0.457
IL-5 vs IL-5 (control)	-0.22	2.39357	1.38193	-6.16597	5.72597	-0.159	2	0.888