A study of the immune response in murine experimental malaria, with special reference to the effects of South African medicinal plants, artesunate and chloroquine

Bonginkosi Gumede

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Supervisors: Prof. P. I. Folb, Prof. B. Ryffel and Prof. D. Kwiatkowski (Oxford)

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DECLARATION

I, Bonginkosi Gumede hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part of this work has been, is being, or is to be submitted for another degree in this or any other University.

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Publications and Presentations

The following publications and presentations were based on the studies that are described in this thesis:

Publications

Bonginkosi Gumede, Peter Folb and Bernhard Ryffel.

Oral artesunate prevents *Plasmodium berghei* ANKA infection in mice

Parasitology International, 52 (2003) 53-59.

Manuscripts in preparation

Gumede B, Leisewitz A, Jones M, Turner G, Kwiatkowski B and Rockett K
Early events in the spleen of mice infected with *Plasmodium chabaudi chabaudi* AS infections.

Gumede B, Madikane E, Cailean C and Folb P.
In vivo antiplasmodial activity of plants that are traditionally used to treat malaria in South Africa

Gumede B, Folb P and Ryffel B.
Effect of artesunate and chloroquine on the production of IFN-γ, TNF-α, IL-10 and IL-12 by mitogen treated splenocytes.

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The role of pro-inflammatory cytokines in the pathogenesis of murine cerebral malaria.
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Abbreviations

A. annua	*Artemisia annua*
APC	Antigen-presenting cell
B6X129	C57BL/6 strain X 129Sv strain of mice
CD	Cluster of differentiation
CM	Cerebral malaria
CNS	Central nervous system
Con A	Concanavalin A
DAB	3,3 diaminobenzidene tetrahydrochloride
DC	Dendritic cells
DHA	Dihydroartemisinin
ELISA	Enzyme linked immunosorbent assay
FP	Ferriprotoporphyrin IX
GC	Germinal centre
GM-CSF	Granulocyte macrophage colony stimulating factor
H&E	Haematoxylin and Eosin
H. procumbens	*Harpagophytum procumbens*
ICAM	Intercellular adhesion molecule
IFN	Interferon
$IFN\gamma^{-/-}$	Interferon-gamma knockout mice
IL	Interleukin
KZN	KwaZulu Natal
LFA	Lymphocyte function associated molecule
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MZ	Marginal zone
Na^+/H^+	Sodium/hydrogen
$NaHCO_3$	Sodium bicarbonate
NK	Natural killer

NO	Nitric oxide
P. falciparum	*Plasmodium falciparum*
PbA	*Plamsodium berghei* ANKA
PCAM	Platelet cell adhesion molecule
Pcc	*Plasmodium chabaudi chabaudi* AS
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RP	Red pulp
TACE	TNF-converting enzyme
TCR	T-cell receptor
$TFN^{-/-}$	Tumour necrosis factor knockout mice
Th	T-helper
TNF	Tumour necrosis factor
TNF-R	Tumour necrosis factor receptor
VCAM	Vascular cell adhesion molecule
W. salutaris	*Warburgia salutaris*
WHO	World Health Organization
WP	White pulp
WP	Western Province
WPS	White pulp score
WT	Wild type
µg	micrograms
µM	micro-Molar

Abstract

The role of pro-inflammatory cytokines (TNF and IFN-γ) in a murine experimental malaria model for cerebral malaria is reported in this thesis. Wild type and receptor knockout mice (IFN-γ deficient mice (IFN-$\gamma^{-/-}$) and TNF-$\alpha/\beta^{-/-}$ double deficient) were infected with *Plasmodium berghei* ANKA (PbA). PbA induced fatal cerebral malaria in wild type mice, which died within 5 to 8 days. In contrast, IFN-$\gamma^{-/-}$ and TNF-$\alpha/\beta^{-/-}$ were completely resistant to PbA-induced cerebral malaria. Both wild type and mutant mice developed a similar degree of parasitaemia in the initial phase, and anaemia and leukocytosis were not different, showing that both anaemia and mobilisation of leukocytes occur in the absence of TNF and IFN-γ. The results show that TNF$^{-/-}$ and IFN-$\gamma^{-/-}$ mice are resistant to PbA-induced cerebral malaria, and confirm the role played by Th1 cytokines in its pathogenesis.

Plasmodium chabaudi chabaudi AS infection results in splenomegaly, and activation of the immune system. Resistant C57BL/6 mice, which eliminate the parasites and survive the infection, developed marked splenomegaly. Susceptible A/J mice develop minimal splenomegaly. In this work it has been shown that there is a rapid deterioration in splenic architecture, although immunohistochemistry confirmed preservation of a high level of structure and organisation. CD11c (dendritic) cells moved from the marginal zone into the $CD4^+$ T cell area (where their antigen presenting function would be maximal). The juxtaposition of CD11c and T cells might be associated with immune complex formation in the spleen during the infection. The findings were similar for C57BL/6 and A/J mice.

A 14-day course of artesunate 100 mg/kg prevented completely the development of parasitaemia and cerebral malaria in *Plasmodium berghei* ANKA infected mice, with survival of more than 60d. Chloroquine enhanced production of IL-10. Artesunate displayed enhanced IL-10 activity but no effect on production of pro-inflammatory cytokines. Extracts of W. *salutaris*, a South African medicinal plant, reduced parasitaemia by >50% at doses of 100 and 500mg/kg, and of *A. annua* reduced parasitaemia by 64% at a dose of 200 mg/kg. These extracts, and extracts of *H. procumbens,* had immunomodulatory activity on TNF-α, IFN-γ, IL-12 and IL-10 production by Con A- and LPS-induced splenocytes.

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CHAPTER 1

Literature Review

Introduction

Malaria is one of the major infectious diseases and causes severe morbidity and mortality in tropical and sub-tropical regions of the world. Nearly half of the world's population lives in malaria-endemic areas in Africa, Asia and South America (Figure 1). There are 300-500 million cases of malaria annually resulting in an estimated one million deaths in Africa alone (WHO, 1998). Pregnant women and children under 5 years old constitute the two most important risk groups. Malaria not only causes considerable problems to human health and an economic burden in developing countries but, with increasing global travel, it also poses a threat to human health worldwide. The parasite is resistant to older anti-malarial drugs such as chloroquine, and there is the spread of multidrug resistance to newer classes of antimalarial drugs (Newton P *et al.*, 1999).

Malaria is caused by the infection of hemaprotozoan parasite belonging to the genus *Plasmodium*. There are four parasite species; *Plasmodium falciparum (P. falciparum), P. vivax*, *P. ovale*, and *P. malariae*, responsible for human malaria infections. *Plasmodium falciparum* and *P. vivax* are the most common causative agents of severe malaria; *P. falciparum* causes the most severe disease and is responsible for more than 95% of malaria deaths worldwide (Stevenson M *et al.*, 2001).

The mechanisms leading to the morbidity observed during malaria, eventually leading to death are largely unknown. A better understanding of these mechanisms would allow the development of new drugs and therapies. In this thesis, the development of immunity and the nature of discrete pathologies during malaria infection were investigated, and the effects of intervention with herbal medicine and known antimalarial drugs on the immune response, using murine experimental models have been described.

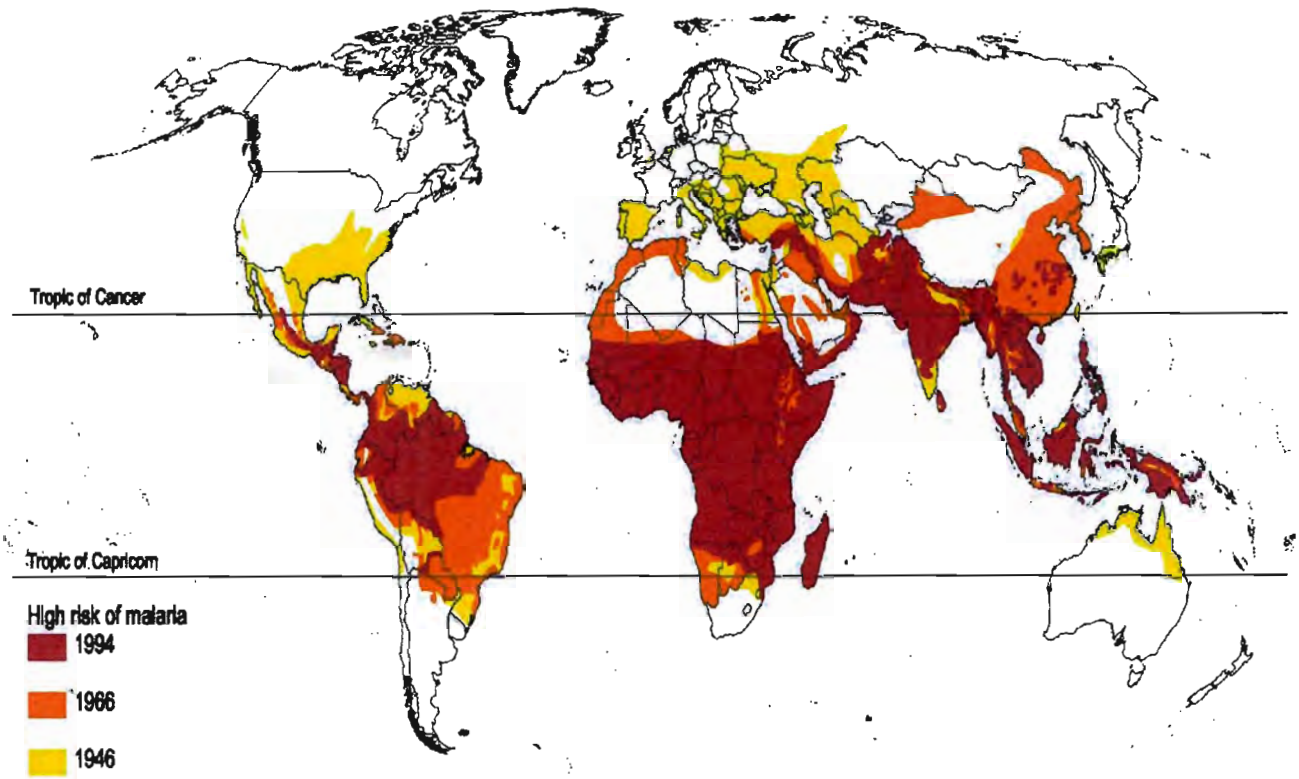


Figure 1 Global distribution of malaria (Sachs J et al., (Feb. 2002); Nature. www.nature.com

1.1 Malaria

1.1.1 Transmission and exposure

Malaria parasites are transmitted by the female of various species of *Anopheles* mosquitoes. Infection is initiated during blood feeding by infected mosquitoes, which inoculate sporozoites into the mammalian host *(Figure* 1.1.1). Sporozoites migrate through the lymphatics and bloodstream to the liver and invade hepatocytes, where the

parasite undergoes a series of asexual replications, the liver-stage of infection. Infected hepatocytes rupture and release merozoites which invade host erythrocytes. In the case of *P. falciparum*, literally thousands of merozoites are released and the ensuing blood-stage infection is considered to be the most serious and deadly form of malaria. Merozoites then undergo a series of asexual replications within the host erythrocyte. Following rupture of the infected erythrocytes, merozoites are released and infect new erythrocytes. Within the erythrocyte some merozoites develop into either male or female gametocytes which are ingested by the mosquito vector during a blood meal, released from the host erythrocyte, and fuse to form a zygote in the mosquito mid-gut. A series of sexual replications occur within the mosquito, resulting in production of infectious sporozoites that migrate to the salivary glands and initiate infection in a new host during the next blood feeding by the mosquito.

There are many factors regulating the transmission of malaria. The combination of these factors is different for every geographical region. They may be natural, such as the presence of enough for breeding mosquitoes, or temperature. Other factors regulating the transmission of malaria are social and economical, such as the behavioral and social situation of the human populations living in a particular environment. Moreover, these combinations are not constant and are likely to change, with the general weather conditions or modernization. Therefore, effective regional methods of control of the infection might be useless in another region, and the efficiency of the given methods might change with time. Control of the infection at the individual level, with drugs and vaccines, might be of advantage in combination with intervention at community level.

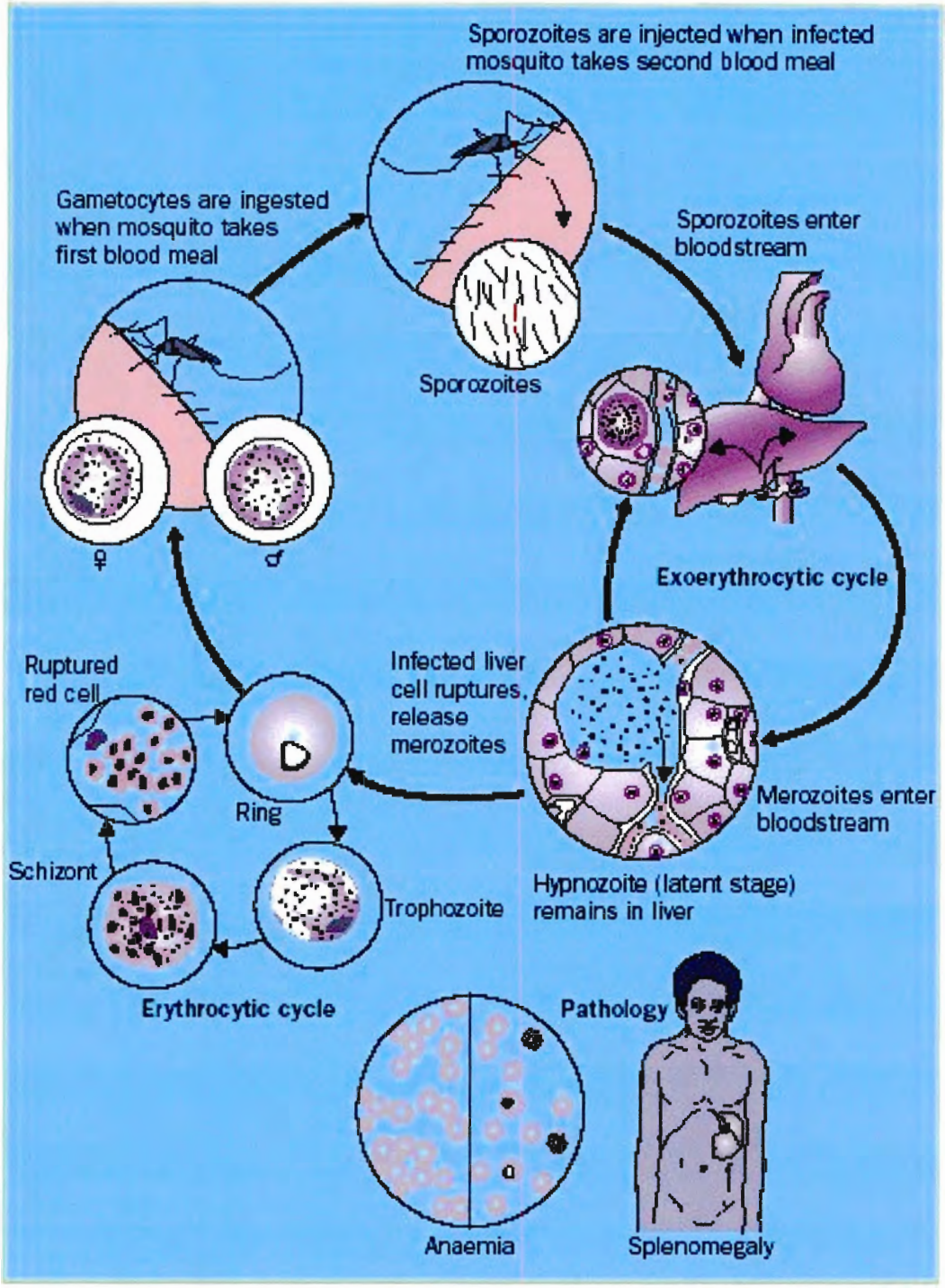


Figure 1.1.1 Life cycle of the malaria parasite in man and mosquito ***(Reproduced with permission from Dr Lucia Malaguarnera, Department of Biomedical Sciences, University of Catania, Via Androne 83, Catania, Italy)***. Malaguarnera L *et al.*, 2002, *The Lancet.*

1.1.2 Infection and disease

The clinical symptoms and pathological consequences of human malaria are associated with the asexual blood-stage of the parasite life cycle [Good M *et al.*, 1998]. The clinical symptoms range from mild headache or mild fever to severe, life-threatening complications such as cerebral malaria, anemia, renal failure, and pulmonary edema. Of these, cerebral malaria and severe anemia are commonly observed in children, and pulmonary and renal disorders often occur in adults [Kwiatkowski D *et al.*, 1990]. Both parasite and host factors determine the severity and outcome of malaria infection [Kwiatkowski D *et al.*, 2000]. Development of acquired immunity to malaria, which is only partially protective, requires persistent, sub-clinical infection over several years. This may account for the higher prevalence and more severe outcome of malaria infection in children than in adults, and indicates that the immune status of the host influences the severity of disease due to malaria. The active multiplication of parasites and the massive destruction of both infected and non-infected erythrocytes are the primary pathogenic mechanisms for severe malaria. However, not all infected children develop severe malaria, suggesting that genetically determined host factors also play a role in the pathogenesis of malaria-associated sequelae (McGuire W *et al.*, 1999). Most of the clinical manifestations associated with malaria are due to host response to parasitized erythrocytes and metabolites released by the parasites. Increased production of inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α, and expression of adhesion molecules by the host, has been suggested to mediate the pathogenesis of malaria (Medana IM *et al.*, 2001; Grau GE *et al.*, 1989; Aikawa M *et al.*, 1990).

1.2 The immune system

The immune system is a surveillance mechanism vertebrates use to protect themselves against parasites and pathogenic microorganisms such as bacteria and viruses. It is also able to recognize and destroy transformed cells such as cancer cells.

1.2.1 Humoral immunity

B-lymphocytes (B-cells) are the lymphocytes generally associated with the humoral immune response. They carry clonally distributed membrane-bound immunoglobulins. When appropriately activated they can proliferate and differentiate into B memory cells, or into antibody forming cells which secrete antibodies into the surrounding medium. Memory cells can be reactivated by subsequent contact with the same antigen, and will rapidly produce a new wave of antibody forming cells. B-cells are produced in the bone marrow of adult mammals. In fetal mice and humans, the first site of B-lymphopoiesis is the liver. B-cells recognize antigens in their native conformation either free in solution, bound to the membranes, or on the surface of the cells. They are able to process soluble antigens and are involved in antigen presentation to the T-cells.

1.2.2 Cellular immunity

T-lymphocytes (T cells) are the lymphocytes associated with the cellular immune response. Most T-cells can only recognize antigens, or more specifically fragments of processed antigens, if they are presented to them physically bound to the major histocompatibility complex (MHC) molecules of antigen-presenting cells. The receptor involved in this recognition is called the T-cell antigen receptor (TCR). The T-cell receptor is composed of two glycoprotein chains, α and β, linked by disulphide bonds (Male D *et al.*, 1995). It possesses two extracellular domains, one variable and the other constant, and a cytoplasmic tail. Although the predominant part (90%) of the T-cell population bears α/β TCR, a minority (10%) possesses a TCR composed of two other glycoprotein chains γ and δ.

The acquired cellular immune response is composed of $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ T-cells recognize proteins commonly referred to as antigens after they have been processed by a specific group of cells termed antigen-presenting cells (APC). $CD4^+$ T-cells recognize antigens that have been processed through the exogenous pathway by antigen-presenting cells (such as dendritic cells, macrophages and B-cells), expressing

major histocompatibility complex (MHC) class II molecules (Roitt *et al.* 1997). After the recognition of antigen in association with MHC class II molecules, $CD4^+$ T-cells become activated, and differentiation can occur into functional subsets termed T helper 1 (Th1)-type and T helper 2 (Th2)-type cells. The distinction of these subsets is based on their production of a variety of proteins called cytokines. The signature cytokines for Th1 and Th2 cells are interferon-gamma (IFN-γ) and interleukin 4 (IL-4), respectively. Th1 cells, through their production of IFN-γ, mediate the killing of organisms responsible for a variety of intracellular infections. For many intracellular infections the induction of a functional Th1 response is crucially dependent on another cytokine, IL-12, which is produced by antigen-presenting cells such as macrophages and dendritic cells after exposure to the pathogen at the initiation of the immune response. Thus, in response to many intracellular infections, IL-12 is the inducer of Th1 cells, and IFN-γ is the effector cytokine that mediates protection. $CD8^+$ T-cells also mediate their effector function through the production of cytokines such as IFN-γ and tumour necrosis factor-alpha (TNF-α) and/or through a direct cytolytic mechanism. The mechanism of cytolytic killing is mediated by release of granule contents such as perforin and granzyme from $CD8^+$ T-cells. In addition, $CD8^+$ T-cells can kill cells by process of Fas-mediated lysis (Roitt I *et al.* 1997).

1.3 Processes of immune response in malaria

Malarial infections stimulate each component of the immune response. $CD4^+$ and $CD8^+$ T-cells, αβ T-cell receptor (TCR), γδ TCR, B-cells and macrophages are all rapidly activated during malaria infection. $CD4^+$ T-cells, however, play the major role in protective immunity against asexual blood stages, with minor contributions from $CD8^+$ T-cells and γδ T-cells. This has been demonstrated in murine experimental malaria by depletion of different populations of T-cells *in vivo*, adoptive transfer of purified T-cells and T-cell lines and clones, and more recently, by disruption of all appropriate genes by homologous recombination (Taylor-Robinson W *et al.* 1995, Yap G *et al.* 1994, von der Weid *et al.* 1993). Immunization with irradiated sporozoites induced $CD8^+$ T-cells specific for the epitopes SYIPSAEKI and SYVPSAEQI, located in the circumsporozoite

protein of *P. berghei* and *P. yoelii*, respectively. The identification of these epitopes permitted the generation of epitope-specific $CD8^+$ T-cell clones that were used to study the in vivo antiparasite activity of these T cells (Romero P *et al.*, 1989, Rodrigues M *et al.*, 1991, Weiss W *et al.*, 1992). Cloned $CD8^+$ T cells transferred into mice subsequently challenged with viable sporozoites inhibited the development of liver stages and thereby prevented the infection of red blood cells. $CD4^+$ T-cells are divided into two major subsets, Th1 and Th2, based upon the repertoire of cytokines secreted following stimulation, with distinct cytokine profiles that they display indicating their function (Mosmann T *et al.* 1987). Th1 cells produce interleukin 2 (IL-2), IFN-γ and TNF-α, and through these mediate macrophage activation and delayed type hypersensitivity responses. Th2 cells produce IL-4, IL-5, IL-6, IL-13 and IL-10, and provide help for maturation of B-cells to plasma cells and for the production of antibodies. This implies that Th1 and Th2 cells mediate qualitatively distinct immune responses, with largely non-overlapping cell-mediated and humoral elements. Central to an understanding of the processes leading to resolution of parasitaemia or pathology is elucidation of the interplay of the major $CD4^+$ T-cells, and their associated cytokines and other effector molecules (macrophages and dendritic cells) involved in the immune response to the malaria parasite.

1.3.1 Role of macrophages and dendritic cells in malaria

An antigen-presenting cell (APC) is defined as any cell that expresses MHC or related molecules (e.g. CD1) that bind antigenic components such as peptides, and is recognized by one class of T-cell or another (Austyn J., 2000). Thus, cytotoxic T-cells and helper T-cells generally recognize peptide-class I and class II MHC complexes, respectively, on the surface of the APC. Dendritic cells (DCs) are antigen-presenting cells with a unique ability to induce primary immune responses (Banchereau J *et al.*, 1998, Bell D *et al.*, 1999). DCs capture the antigen and transfer information from the outside to cells of the adaptive immune system. DCs are critical not only for the induction of the primary immune responses, but also in the induction of immunological tolerance, as well as in regulation of the type of T-cell-mediated immune response (Banchereau J *et al.*, 2000,

Hart D *et al.*, 1997, Steinman R *et al.*, 1991). DCs reside in tissues as immature cells with high phagocytic capacity. Following antigen capture and activation, they migrate to the lymphoid organs where they prime antigen-specific $CD4^+$ and $CD8^+$ T-cells (Bruna-Romero O *et al.*, 2001). DCs play a pivotal role in initiating immune responses to malaria. Urban and colleagues have shown that falciparum-infected erythrocytes bind to the surface of myeloid DCs *in vitro* and profoundly modulate the maturation and function of DCs (Urban B *et al.*, 1999). Infected erythrocytes inhibit the normal upregulation of MHC class II molecules, adhesion molecules, and co-stimulatory molecules (CD83 and CD86) on DCs after stimulation with lipopolysaccharide (LPS). The ability to stimulate not only the allogenic but also antigen-specific primary and secondary T-cell responses is reduced in malaria (Urban B *et al.*, 1999). It has recently been shown that dendritic cells presenting *Plasmodium yoelii* sporozoite antigens are able to activate specific $CD4^+$ and $CD8^+$ T-cells and initiate protective immune responses against malaria in mice (Bruna-Romero *et al.*, 2001).

Macrophages are large phagocytic cells, derived from blood monocytes that also function as antigen-presenting cell. Animal models and indirect evidence from clinical observations suggest that phagocytosis of infected red cells by splenic macrophages is a critical component of host defence mechanisms against blood-stage parasites. Macrophages phagocytose malaria-infected erythrocytes. Macrophages not only ingest intact erythrocytes but also extract parasites from recently infected erythrocytes, leaving the erythrocytes to continue to circulate (Urban B *et al.*, 2002, Angus B *et al.*, 1997, Chotivanich K *et al.*, 2002). Macrophages participate in the control of the infection through both antibody-dependent and independent phagocytosis, and secretion of soluble factors directly or indirectly toxic to the parasite, such as IL-1, TNF-α, granulocyte-macrophage colony stimulating factor (GM-CSF), reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Pichyangkul S *et al.*, 1994, Yamada-Tanaka M. S. *et al.*, 1995, Prada J *et al.*, 1996). Haemozoin is a key factor in malaria-associated immunosuppression, affecting both the antigen-processing and immunomodulatory functions of macrophages (Metzeger W *et al.*, 1995, Taramelli D *et al.*, 2000). The accumulation of pigment inside the macrophages has been shown to impair macrophages

activation and function. The percentage of heavily haemozoin-laiden leukocytes and macrophages correlate with disease severity (Metzeger W *et al.*, 1995, Taramelli D *et al.*, 2000).

1.3.2 Role of cytokines in malaria

Cytokines are low molecular weight proteins that stimulate or inhibit differentiation, proliferation or function of immune cells. An appropriate immune response is critical in determining the outcome of malaria infection. It has been suggested that IFN-γ contributes to the immunity against blood-stage infection, as it is a potent activator of effector cells, such as macrophages, in which it induces phagocytosis and killing (Ockenhouse C *et al.* 1984, Gyan *et al* 1994). Increased serum levels of IFN-γ have been reported for some *P. falciparum* infected individuals (Kwiatkowski *et al.* 1989, Wenisch *et al.* 1995). Cytokines may be beneficial for the host but they are also involved in the pathogenesis of malaria. This dual role is most apparent for the inflammatory cytokine TNF. Morbidity and mortality of severe *P. falciparum* malaria are related to the blood concentrations of TNF (Grau G *et al.* 1989, Kwiatkowski D *et al.* 1990) which, in addition to other cytokines, also plays a critical role as a mediator of malaria fever (Kwiatkowski D *et al.* 1993, Kwiatkowski D *et al.* 1995). A critical role of IL-4 for parasite clearance has been suggested by its elevated serum levels in parasitaemic individuals living under perennial and holoendemic *P. falciparum* transmission (Mshana *et al.* 1991). IL-10 has been shown to increase during the acute attack and then return to normal levels when clinical symptoms have disappeared (Mshana *et al.* 1991). Other cytokines believed to play a role in malaria immunity include GM-CSF and IL-8. GM-CSF acts synergistically with TNF in neutrophil-mediated phagocytosis and killing of asexual blood stages (Kumaratilake *et al.* 1996).

1.3.2.1 Pro-inflammatory cytokines and malaria

A minimal pro-inflammatory response is beneficial in limiting parasitaemia, but an exacerbated host response invariably leads to tissue damage. In human malaria, altered

immune reactivity appears late in the acute phase of the disease and can last a long time after the clearance of parasites from circulation. An explanation for the poor acquisition of malaria immunity in naturally exposed populations is that the parasite actively modulates the immune system of the host, preventing the development of specific immune responses (Plebanski M *et al.* 2000). The inflammatory response that is needed to remove parasites leads to considerable tissue damage, and activation of phagocytes to kill intracellular or extracellular parasites requires the production of inflammatory cytokines, which can cause or exacerbate systemic effects such as severe anaemia and cerebral malaria (McGuire W *et al.*, 1994, Luty A *et al.*, 2000). The outcome of the malaria infection then depends on a delicate balance between appropriate and inappropriate induction of these mediators.

1.3.2.1.1 Tumour Necrosis Factor (TNF)

TNF and its receptors are members of the extended super gene families (Thomson *et al.*, 1998). Although several different cell types synthesize TNF under *in vitro* conditions (Vassalli *et al.*, 1992), its production is mainly attributed to macrophages and T-cells. TNF and related proteins such as lymphotoxin are structurally homologous and encoded by genes clustered on human chromosome 6 and mouse chromosome 17 within the MHC locus (Locksley R *et al.*, 2001). TNF can be expressed in two biologically active forms namely as a precursor membrane bound protein (26 kD) which can be cleaved to a soluble mature protein (17 kD) by TNF converting enzyme (TACE). TNF and membrane bound TNF can bind to TNF-receptor (TNFR)-1 and TNFR-2. TNFR1 and TNFR2 are expressed on a broad spectrum of cell types and can exist in both a soluble form or can be attached to the membrane (Locksley R *et al.*, 2001).

The role of TNF in malaria has been extensively studied in both humans and mice. The first characterized parasite-induced cytokine is TNF-α, induced in macrophages by erythrocytes infected with plasmodium, malarial pigment (Pichyangkul *et al.*, 1994). The amount of TNF-α produced by malaria parasites varies between people in the same endemic area exposed to similar parasites and inoculation rates (Peyron F *et al.*, 1990). It has also been shown that the variation of concentrations and appearance of TNF-α in

people with severe malaria and subclinical malaria could be modulated by other factors such as reactive nitrogen, oxygen radicals, leukotrienes, and cytokines such as IFN-γ, IL-4 and IL-10 (Fiorentino D *et al.*, 1991, Essner R *et al.*, 1989). Luty *et al.*, 2000 showed a close association between the presence of severe anaemia, high TNF-α concentrations, and a large number of circulating haemozoin-containing monocytes, suggesting that TNF-α-production plays a part in either initiation or exacerbation of anaemia as a clinical outcome of chronic, uncontrolled parasitaemia. Several clinical studies have attempted to determine the association between serum TNF-α levels and cerebral malaria (Grau G *et al.*, 1989, Kwiatkowski D *et al.*, 1990, Shaffer N *et al.*, 1991). In a Malawian study, the mean serum level of TNF-α was higher in patients with *P. falciparum* infection who died than those who survived. In another study involving children from Gambia, a similar correlation between serum TNF-α levels and the severity of cerebral malaria was reported (Grau G *et al.*, 1989, Kwiatkowski D *et al* 1990). In this study it was further reported that TNF-α was a better indicator of fatal outcome in childhood cerebral malaria than other parameters such as parasite density and blood glucose levels. The role of TNF-α in cerebral malaria has been studied extensively using rodent malaria models. Infection of susceptible CBA/Ca mice with *Plasmodium berghei* Anka (PbA) induces a fatal cerebral disease (Grau G *et al.*, 1987). In these mice, anti-TNF antibody treatment has been found to abrogate the symptoms of CM (Grau G *et al.*, 1987). However, it is difficult to extrapolate these findings to human cerebral malaria because this rodent model does not reproduce the pathological features observed in human cerebral malaria. There are at least two theories that explain the role of TNF-α in the pathogenesis of cerebral malaria. Ian Clark and Cowden *et al.*, 1992, proposed that TNF-α maybe directly involved in cerebral malaria by inducing toxic compounds, such as nitric oxide, which may cause brain dysfunction. An alternative hypothesis takes into account the role of TNF-α inducing the expression of receptors for the cytoadherence of *P. falciparum* on microvascular endothelial cells (de Kossodo *et al.*, 1993). TNF-α activates expression of cell adhesion molecules such as ICAM-1, VCAM-1, and ELAM-1 on microvascular endothelial cells (Mantovani A *et al.*, 1992). These molecules on the cerebral microvascular endothelial cells serve as receptors for the binding of parasitized

erythrocytes. In contrast to the pathogenic role of TNF-α during malaria infection, TNF-α can increase the phagocytic capacity due to an increased expression of Fc-receptors on monocytes, or to the modulation of Fc-receptor signaling pathways by signals originating from the binding of TNF-α to its receptors. TNF-α also acts on lymphocytes and monocytes by increasing the inhibition of *P. falciparum* via a mechanism unrelated to phagocytosis. This then suggests that TNF-α has a pleiotropic antimalarial effect and that this protective effect depends on the interplay of different factors, such as monocyte/macrophages, lymphocytes, and antibodies, in addition to the other cells and molecules (Muniz-Janqueira M *et al.*, 2001).

1.3.2.1.2 Interferon-gamma (IFN-γ)

Interferon-γ is a macrophage-activating factor involved in the innate immune response to malaria. It is mainly produced by $CD4^+$ and $CD8^+$ T lymphocytes in a specific immune response and by NK cells in a non-specific response (Weiss W *et al.*, 1993). Studies of experimental murine models as well as human models suggest an important role for interferon-γ in protective immune responses to blood stage malaria. Luty A *et al.*, 1999, demonstrated that IFN-γ production by $CD4^+$ T-cells to specific erythrocytic antigens is associated with protection against malaria reinfection in Africa. IFN-γ, chiefly produced by T-cells, may also help to induce IgG blood-stage specific antibodies and assist in antibody-dependent cellular inhibitory mechanisms (Bouharoun-Tayoun H *et al.*, 1995). There is evidence which suggests that nitric oxide (NO) has an important role in the destruction of intrahepatic malaria parasites in response to IFN-γ and other cytokines released by T-cells and NK-cells. IFN-γ, via signal transducers associated with transcription, activates inducible nitric oxide synthase and induces the L-arginine-dependent NO pathway, subsequently eliminating the infected hepatocytes or the hepatic schizonts within the cells (Malaguarnera L *et al.*, 2002). The *in-vitro* treatment of plasmodium-infected hepatocytes with IFN-γ eliminated *P. falciparum* parasites from culture, and *in-vivo* administration of IFN-γ partly protected against sporozoite challenge with *Plasmodium berghei* in mice. Winkler S *et al.*, 1999, demonstrated that children

with *P. falciparum* hyperparasitaemia have lower concentration of $CD4^+$ T-cells secreting IFN-γ than children with uncomplicated malaria. IFN-γ is essential for the resolution of primary infection by limiting the initial phase of parasite replication, but also contributes to the acute symptoms of malaria infection such as fever, nausea, and headache through the induction of TNF-α and IL-1 (Riley E *et al.*, 1999). IFN-γ plasma concentrations are higher in clinical cases of malaria than in symptomless cases and a temporal association between IFN-γ or TNF-α predisposes to severe pathology (Riley E *et al.*, 1999). Treatment of mice with exogenous IFN-γ delays the onset of parasitaemia and decreases the number of infected erythrocytes during *P. chabaudi adami* infection (Clark I *et al.*, 1987). Shear *et al.*, 1989 demonstrated that daily treatment with recombinant IFN-γ resulted in a less severe course of infection and increased survival in mice infected with the lethal strain of *P. yoelii* 17X. They further found a correlation between timing and level of IFN-γ production *in vitro* by spleen cells and outcome of infection with lethal versus nonlethal strains of *P. yoelii* 17X.

1.3.2.1.3 Interleukin-12 (IL-12).

IL-12 is a 70-kDa-heterodimeric cytokine composed of two disulphide-linked subunits of 35 and 40 kDa (Thomson A *et al.*, 1998, Gately M *et al.*, 1998). In both man and mouse, the two genes for these proteins are located on separate chromosomes. The two subunits together form the p70 protein, which is the biological active molecule of IL-12. For release of IL-12p70, the two genes must be expressed in the same cell. Earlier evidence suggests that while the expression of p40 mRNA is highly upregulated following activation of IL-12-producing cells, p35 mRNA expression is constitutive. Evidence now suggests that the induction of both genes can be upregulated, but compared to p35, the p40 gene appears to be much more highly regulated. IL-12 is produced primarily by antigen presenting cells, macrophages and dendritic cells, in response to bacteria and bacterial products such as LPS, intracellular parasites and viruses, and through engagement of CD40 on APC's by its ligand, CD40L, on activated T-cells (Trinchieri G *et al.*, 1998). It has also been described in other cell types, including neutrophils and microgial cells. Multiple mechanisms appear to regulate IL-12 production, both

positively and negatively. The cytokines IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce IL-12 production while other cytokines, including IL-4, IL-10, IL-13 and TGF-b are inhibitory (Trinchieri G *et al.*, 1998). This cytokine is a potent immunomodulatory cytokine that has been proven to be effective in conferring protection against viral, bacterial, and intracellular parasitic infections. It does not only increase cell-mediated immune response but also affects humoral immunity by inducing isotype switching through both IFN-γ -dependent and independent mechanisms (Trinchieri G *et al.*, 1995). IL-12 seems to stimulate antibody production in B-cells and it has been shown that IL-12 is effective in inducing protective immunity against blood-stage infection in the murine model (Crutcher J *et al.*, 1995). The major immunological role of IL-12, and of relevance to protective immunity to blood-stage malaria, is its ability to induce IFN-γ production by both NK-cells and T-cells. It also induces T-cell proliferation and is a potent stimulus for Th1 cell development (Trinchieri G *et al.*, 1998). A Th1 response has been implicated in host protective immunity against a number of protozoan parasitic diseases, including the acute phase of blood-stage *P. chabaudi* AS (Yap G *et al.*, 1994). The early events in the cell-mediated immune response needed for the defence against malaria, initiated by the release of IL-12 from monocytes, macrophages, B-cells and other cell types and consequently the concentration of IL-12, reveal a prognostic significance in malaria infection. In combination with an anti-malarial drug chloroquine, low dose IL-12 was found to induce protective immunity to primary *P. chabaudi* AS infection in mice, to alleviate anaemia associated with this infection, and to induce immunity to re-infection (Mohan K *et al.*, 1999). There is convincing evidence for a protective role for IL-12 in immunity to blood-stage malaria, but this cytokine has also been implicated in the pathological response to malaria. The pathogenic effect of IL-12 is related to the dose of exogenous IL-12 administered *in-vivo* or the level of endogenous IL-12 produced during infection.

1.3.2.2 Anti-inflammatory cytokines and malaria

During malaria infection early pro-inflammatory cytokine responses seem to mediate protective immunity, whereas late responses contribute to pathology. This suggests that a

crucial balance might exist during the inflammatory response to malaria infection. An unbalanced response between Th1 and Th2 type of cytokine response leads to severe disease in malaria. In mild malaria, the inflammatory response might be down-regulated by anti-inflammatory cytokines, including IL-4 and IL-10.

1.3.2.2.1 Interleukin-4 (IL-4)

IL-4 was identified in 1982 for its ability to induce activated mouse B-lymphocytes to proliferate and to secrete immunoglobulin IgG_1. cDNAs coding for both human and murine molecules were isolated in 1986, followed in 1989-90 by those coding for IL-4 high-affinity glycoprotein receptors. The human IL-4 gene, composed of four exons and three introns, is localized on the long arm of chromosome 5 on band q23-31 together with genes of other related cytokines including IL-3, IL-5, IL-9, IL-13 and GM-CSF (Thomson *et al.*, 1998). The wide range of cells on which IL-4 induces specific biologic functions highlights the pleiotropic nature of this molecule. IL-4 is produced by Th2 and activated basophil and mast cells, and it has been seen to be involved in the activation of CTL, NK-cells, and macrophages. IL-4 is an important component of the immune response stimulating growth of Th2 and inhibiting Th1 response by depressing the production of IFN-γ (Luty A *et al.*, 2000). IL-4 and Th2 cells are important in antibody response to plasmodia. $CD4^+$ T-cells are crucial to the development of $CD8^+$ T-cell responses to hepatocytes infected with malaria parasite (Carvalho *et al.*, 2002). In the absence of $CD4^+$ T-cells, $CD8^+$ T-cells initiate seemingly normal differentiation and proliferation during the first few days after immunization, suggesting that IL-4 is a mediator of CD4/CD8 cross-talk, leading to the development of immunity against malaria (Kumaratilake *et al.*, 1992). Production of IL-4 by T-cells stimulated by malaria antigens *in vitro* is associated with increased concentrations of serum antibodies to the same activating malaria antigens *in vivo*. IL-4 helps to induce the antibody response directed against the malaria parasite (Carvalho *et al.*, 2002).

1.3.2.2.2 Interleukin 10 (IL-10)

IL-10 is an immunosuppressive and anti-inflammatory cytokine involved in regulation of the immune response during infection. IL-10 is produced by monocytes, Th2 cells, mast cells and B-cells. Human IL-10 is synthesized as an 18kDa protein whereas differential glycosylation yields three proteins of 17kDa, 19kDa and 21kDa in mice (Moore *et al.*, 1990). IL-10 signaling is mediated through the IL-10 receptor (IL-10R), which is composed of two subunits namely IL-10Rα and CRF2-4 (Liu *et al.*, 1994). Although several cell types can transcribe IL-10 mRNA, not all cells can translate it into functional protein. IL-10 has been reported in the plasma of patients with acute malaria (Wenish C *et al.*, 1995). It inhibits cytokine production in Th1 and $CD8^+$ T-cells, but not Th2 cells. IL-10 induces B cell proliferation, resulting in immunoglobulin production, a mechanism that is important for the development and maturation of antimalarial antibodies. IL-10 seems to have an important role in defining the T helper cell response to malaria. It downregulates MHC class II molecules on macrophages, leading to decreased antigen presentation, inhibits oxygen radical and reactive nitrogen production, prevents T-cell priming and proliferation, and suppresses the production of IFN-γ, IL-6, TNF-α and GM-CSF by T-cells (Akdis C *et al.*, 1999).

In 1999 Winkler S *et al.* showed that malaria-infected children and adults in Gabon recorded many IL-10-producing $CD4^+$ and $CD8^+$ T-cells co-expressing IFN-γ. These cells may provide a fertile ground for parasite-driven immune modulation. It was shown that the increase of IL-10 is more pronounced and more specific than IL-6 and IL-8 in patients with malaria parasitaemia, compared with other infections (Jason J *et al.*, 2001). It is not clear whether the increased concentration of IL-10 during the malaria infection has a beneficial role by reducing the parasite-induced inflammatory response, or a detrimental one by decreasing the cellular immune responses. A strong indication that IL-10 plays a protective role in cerebral malaria was provided by a study in which IL-10 was shown to mediate the murine acquired immune deficiency syndrome (MAIDS)-induced protection against cerebral malaria (Eckwalanga M *et al.*, 1994). Kossodo S *et al.*, 1997, evaluated whether resistance to cerebral malaria correlated with IL-10

expression. In this study it was demonstrated that IL-10 inhibited IFN-γ and TNF-α secretion, and this has been reported to be important to counteract the pathological role of macrophages in cerebral malaria. It has been shown that severe anaemia is associated with reduced concentrations of circulating IL-10, and an increased ratio between TNF-α and IL-10 contributes to the reversible bone marrow suppression seen in malaria patients (Kurtzhals J *et al.*, 1998; Othoro C *et al.*, 1999).

1.4 Malaria pathologies

Malaria is associated with several pathologies, including fever, cerebral malaria, hyperparasitaemia, haemoglobinuria, hypoglycemia, pulmonary eodema and jaundice. Although some of these are found in almost every *Plasmodia* infection, others are only found in a minority of patients. Fever is found in most plasmodium infections; cerebral malaria, severe anaemia and renal failure are only found in the minority of patients. The factors determining the appearance and the underlying mechanisms of these symptoms are still unknown.

1.4.1 Cerebral malaria

Cerebral malaria (CM) is one of the most severe complications of *P. falciparum* infection. It most commonly occurs in infants, pregnant women and non-immune visitors to areas endemic for malaria, and carries a mortality of 30-50% (Brewster D *et al.*, 1990; Kwiatkowski D *et al.*, 1991, Roman G. *et al.*, 1992). Most often, patients present with fever, headache, delirium progressing to an acute febrile stupor, followed by coma (Phillips R *et al.*, 1990). Cerebral complications may be the predominant organ pathology evident and either present abruptly, as is typical in African children living in malaria-endemic areas, or develop as a late complication with multisystem involvement, which is typical of non-immune adults from South-East Asia (Eling W *et al.*, 1995; Ho M *et al.*, 1990). Given the different patterns of neurological involvement in African children and South-East Asian adults, there can be problems drawing parallels with neuropathological pathways. Some authors have tried to explain the pathophysiology of

CM by proposing that cytokines or other soluble mediators could cause the symptoms of coma directly, independently of sequestration (Clark I *et al.*, 1997). Sequestration results from parasitised red blood cells to receptors on the brain endothelium (Turner G *et al.*, 1997, Berendt A *et al.*, 1994). Cytokines such as TNF-α and IL-1, or neuro-active mediators such as nitric oxide can be toxic to the central nervous system when overproduced. Such short-lived mediators might explain the reversible symptoms of coma in CM. It has been observed that children with severe malaria and CM cases have significantly higher plasma levels of TNF-α than those with mild disease (Kwiatkowski D *et al.*, 1990). Another potential soluble toxic mediator is nitric oxide, which is produced by a group of enzymes called nitric oxide synthase. Recent studies found widespread expression of one of these enzymes, inducible nitric oxide synthase, in endothelium and many other central nervous system cells during cerebral malaria (Maneerat *et al.*, 2000). Cytokine-induced nitric oxide might damage neurons or disrupt nerve function indirectly by disturbing axonal transport or synaptic transmission. Medana I *et al.*, 2001 showed in human and mouse studies morphological and functional changes in the resident glial cells of the central nervous system (CNS). The degree of immune activation and degeneration of glial cells was shown to reflect the extent of neurological complications in murine cerebral malaria. From these results they highlighted the need to consider the potentially important contribution within the CNS of glia and their secreted products, such as cytokines, in the development of human cerebral malaria. However, the results from studies that measure derivatives of nitric oxide in plasma and cerebrospinal fluid during human CM are inconclusive, and some data suggest that nitric oxide might play a protective rather than a pathogenic role (Taylor A *et al.*, 1998; Dondorp A *et al.*, 1998; Chiwakata C *et al.*, 2000). It is difficult to envisage how a single mediator could be responsible for all the symptoms of CM because these mediators could be over-produced in other neurological conditions. The systemic and local production of a variety of mediators, including cytokines, chemokines and nitric oxide, form a complex network. This may be disturbed by features of the disease, such as changes in parasite load, host inflammatory and immune responses, or by treatment. It is not possible to follow the course of CM within the human brain, and hence theories of its pathogenesis are often based on data from animal models, particularly mice. Several

theories have been proposed to explain the appearance of different symptoms observed during CM and are further considered in Chapter 3.

1.4.2 Anaemia

Severe anaemia is one of the most serious complications of falciparum malaria. All children with clinically significant malaria have some degree of anaemia. The pathogenesis of malarial anaemia is complex. Parasitized red blood cells are destroyed within 72 hours, either because they must burst during schizogony or are removed by the spleen. However, there is increasing evidence to suggest that non-parasitized erythrocytes have shortened life span. Studies of severe malaria in children in the Gambia and Kenya show that anaemia occurs with a high frequency in children between 1 and 4 years of age in the absence of neurological symptoms associated with cerebral malaria (Bojang K et al., 1997, Greenwood B *et al.*, 1987). The peak of severe anaemia in children antedates the peak of cerebral malaria, and that severity is not consistently associated with the degree of parasitaemia. Moreover, in most malaria endemic areas there are also other causes of anaemia, particularly iron deficiency, and the contribution of each cause is difficult to determine in any individual (Newton C *et al.*, 1997). This has led to a certain amount of confusion in the definition of severe malarial anaemia. Similar to other pathophysiological events associated with malaria, anaemia is dependent on factors connected with the status of the host, the genome of the host and the parasite, as well as the environment. In human and animal models, the development of anaemia is the result of the loss of the haemopoietic steady state due to accelerated destruction of parasitised and non-parasitised red cells, and to an impairment of the red cell genesis in parallel.

1.4.3 Hypoglycaemia

Hypoglycaemia has been reported to complicate severe malaria in children in 13-32% of cases (Marsh K *et al.*, 1995; Waller D *et al.*, 1995) and is associated with a higher risk of poor outcome, either by the development of neurological sequelae or death (Molyneux M *et al.*, 1989). In a few cases hypoglycaemia has been shown to be due to

hyperinsulinaemia following quinine therapy (Holloway P *et al.*, 1991). Hypoglycaemia is not a specific complication of severe malaria but rather the result of many severe childhood illnesses in the tropics, possibly being related to prolonged fasting due to the weakness of the patient (Kawo N *et al.*, 1990). Studies in which some of the gluconeogenic precursors have been directly measured indicate that the levels of alanine and lactate are normal or high while levels of the counter-regulatory hormones (glucagons, cortisol and growth hormone) are also elevated (Taylor T *et al.*, 1988, White N *et al.*, 1987). There is no agreed common mechanism that explains the development of these symptoms. Different hypotheses have been proposed, such as reduced levels of liver glycogen, impaired gluconeogenesis, and increased glucose consumption by both host and parasite. A correlation with raised TNF-α serum levels has been proposed. It has been shown that some of malaria toxin preparations had the ability to induce hypoglycaemia. This ability was, however, independent from their capacity to induce TNF-α production. It has been shown recently that the induction of hypoglycaemia during malaria might be due to a direct interaction of glycosylphosphatidylinositol toxin of malaria parasites in the signal transduction pathway of the insulin receptor (Schofield *et al.*, 1993).

1.5 Animal models in experimental malaria

There are several different animal models that are currently being used for experimental malaria. These include primates (chimpanzees and monkeys), birds, lizards and rodents. Chimpanzees have been shown to react in a similar fashion as humans react to *P. falciparum* infections. Among other monkey models that have been proposed, Aotus monkeys (*Aotus nancymae*) seems to be promising as experimental models for *P. falciparum* infections. However, the animals that are widely used in research today are mice. The mouse has evolved as the choice animal of researchers for the investigation of several diseases. Besides the relative low maintenance cost compared to other animals, the mouse is genetically similar to man as most human genes have murine counterpart (Malakoff *et al.*, 2000). There are several well-characterized inbred strains of mice that are available, and rapid advances in molecular technology have assisted in specific gene

manipulation of these mouse strains. Currently, targeting specific genes for alteration is confined to mice only. Today the use of gene-deficient or knockout mice is common and the emphasis has shifted more to the use of conditional gene-targeted mice, which can be defined as a genetic modification that is restricted to specific cell types, or developmental stages of the mouse.

In order to gain a better insight into pathogenesis of CM and into protective immunity *in vivo*, various murine model systems have been developed with parasites isolated from African wild rodents. These offer an advantage over other models in that they are easy and cheap to maintain, and that the mouse immune system is well characterized. These also offer advantage in that the large scale dissective or intervention studies of a nature not ethically permissible to humans and non-human primates may be performed in these models. There are four rodent malaria species which provide, in their different host association, ample opportunity to investigate the most diverse aspects of the immune response to malaria. These are *Plasmodium berghei, Plasmodium chabaudi, Plasmodium vinckei* and *Plasmodium yoelii*. There is no single model that reflects exactly infections in humans, but taken together, different systems provide valuable information on the antigens necessary to induce the mechanisms of immunity and immunopathogenesis. *Plasmodium berghei* ANKA and *Plasmodium vinckei vinckei* are uniformly lethal to mice and they are recognized experimental model for studies on the development of cerebral malaria (Taylor-Robinson A *et al.*, 1998). *Plasmodium chabaudi, Plasmodium vinckei petteri* and other isolates of *P. yoelii* cause infections in most mouse strains, that resolve after an initial parasitaemia and are then either eliminated completely or cause recrudescences over several months (Taylor-Robinson A *et al.*, 1998). Lethal infections are better suited for testing putative vaccine candidate molecules and also examining the efficacy and safety of remedies and antimalarial drugs. Non-lethal infections have been used as best models to investigate the mechanisms of acquired immunity. In this thesis, experimental models based on two strains of rodent malaria, *Plasmodium chabaudi chabaudi* AS and *Plasmodium berghei* ANKA have been used. *Plasmodium berghei* ANKA infections in mice induce cerebral malaria, and serves as experimental models for studies of pathogenesis of cerebral malaria. *Plasmodium chabaudi chabaudi* AS

infections in mice have been shown to have strong analogies at immunological level to *P. falciparum* infections in humans (Taylor-Robinson A *et al.*, 1998). It is therefore a broadly used experimental model to study development of immunity during malaria infections.

1.6 Drugs used in the treatment of malaria

Although malaria is a curable disease if promptly diagnosed and adequately treated, a limited number of drugs for its treatment are available. The antimalarial drugs can be classified by their selective actions on different phases of the parasite's life cycle. The majority of the antimalarial drugs target the intra-erythrocytic stage of the parasite life cycle and this results in the prevention of the development of disease in humans. The drug of choice for the treatment of severe and complicated malaria in most parts of the world is quinine. Quinine is one of the four main alkaloids isolated from the bark of the *Cinchona* tree. Mefloquine, an antimalarial drug that is structurally related to quinine, was introduced for therapy in 1971. It has been found to be effective against all malarial species, including multidrug resistant *P. falciparum*. But it has since been reported in various parts of the tropics in the naturally resistant parasite populations (Milijaona R *et al.*, 2000; Calvosa *et al.*, 2001; Caniato R *et al.*, 2003). Chloroquine is the 4-aminoquinoline most widely used for the treatment of all malaria species. This drug was synthesized as a less toxic analogue of quinine and has been used extensively since then. Chloroquine is still widely used to treat malaria in areas where notable drug resistance has not yet appeared. Despite the parasite's resistance, chloroquine shows low toxicity, low costs and very high tolerance. Artemisinin, extracted from the Chinese herbal remedy *Artemisia annua*, and its derivatives (artesunate, artemether, arteether and dihydroartemisinin) have proven highly effective in clinical practice in adults and children with severe malaria and they are well tolerated. Malarone is a new drug combination of proguanil and atovaquone, which has a synergistic effect (Looareesuwan *et al.*, 1999). The combination is a very effective antimalarial treatment. The other drug combination used to treat malaria is Fansidar, which is a combination of pyrimethamine and sulfadoxine.

1.6.1 Mechanism of chloroquine action

Chloroquine is a member of quinoline-containing antimalarials. The quinolines include quinine and its derivatives mefloquine, amadiaquine and chloroquine (Figure 1.6.1.). The exact mechanism of antimalarial action has not been determined. There are three possible ways in which chloroquine might act once it is inside the parasite. The lysosomal theory states that chloroquine, being a basic compound, is protonated in the lysosome thus raising lysosomal pH (Meshnick S *et al.*, 1990). This effect may raise the intralysosomal pH above a critical level and bring about a loss of lysosomal function. This would reduce the parasite digestion of hemoglobin, and thus prevent its growth (Homewood C *et al.*, 1972). Chloroquine may act by blocking the enzymatic synthesis of DNA and RNA in both mammalian and protozoal cells or by forming a complex with DNA that prevents replication or transcription to RNA. This intercalation theory suggest that chloroquine may be bound with increased affinity by certain parts of the genome and be toxic to the malaria parasite by selective accumulation in specific genes, inhibiting their expression (Meshnick S et al., 1990). The ferriprotoporphyrin IX (FP) interaction hypothesis states that instead of interacting with protons, chloroquine forms a complex with FP, which inhibits sequestration of FP into malaria pigment. This could impair hemoglobin degradation and permit damage to the food vacuole sufficient to discharge its pH gradient (Fitch C *et al.*, 1986).

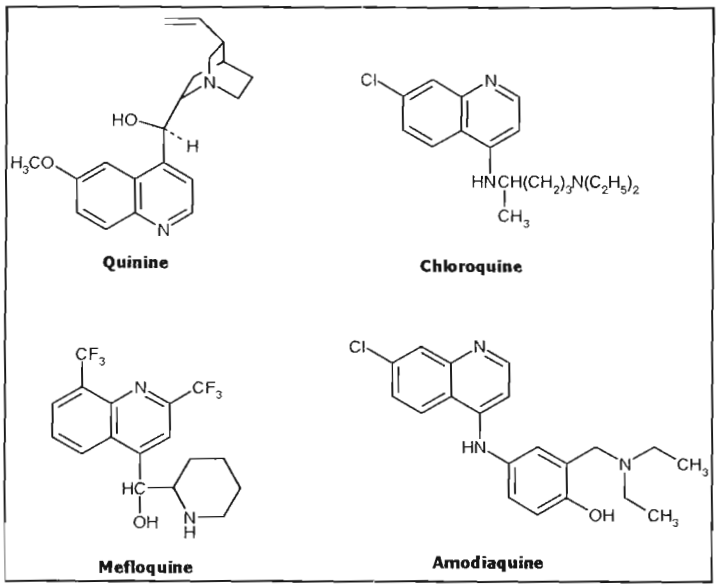


Figure 1.6.1 The structure of quinine and its derivatives

1.6.2 Mechanism of chloroquine resistance

The need to understand the mechanisms of action of the quinoline antimalarials is becoming more urgent as levels of resistance to these drugs increase. Resistance to chloroquine was slow to develop, taking almost 30 years, despite the extensive use of the drug, suggesting that mutations in several genes were required to produce the resistance phenotype. Chloroquine-resistant parasites accumulate chloroquine much less efficiently in their acidic vacuoles than chloroquine-sensitive strains, suggesting that drug resistance results mainly from exclusion of the drug from site of action rather than an alteration in the chloroquine target, although a decreased sensitivity of the target may also be involved (Yayon *et al.*, 1984; Geary *et al.*, 1990). Other studies indicate that chloroquine resistance is associated with an elevated level of drug efflux. Drug-resistant parasites are reported to release pre-accumulated chloroquine almost 50 times faster than sensitive isolates (Verdier *et al.*, 1985; Krogstad *et al.*, 1987). The third possibility is that the

decreased steady-state chloroquine concentration could result from the loss or alteration of a protein involved in chloroquine uptake or the loss of an intracellular receptor (Warhurst *et al.*, 1986; Hawley *et al.*, 1996). It has recently been shown that a plasmodial Na^+/H^+ exchanger is implicated in the facilated import of chloroquine. This work showed that both chloroquine-resistant and chloroquine-sensitive *P. falciparum* possesses a chloroquine importer with a single binding site for chloroquine. However, the kinetics of uptake differed between the resistant and sensitive strains (Sanchez *et al.*, 1997).

1.6.3 Artemisinin and its derivatives

Artemisia annua, a plant belonging to Asteraceae family, is an annual herb native to China and occurs naturally as a part of steppe vegetation in northern parts of Chahar and Suiyan province (Dhingra *et al.*, 2000). Artemisinin or qinghaosu, an endoperoxide sesquiterpene lactone produced by aerial parts of *Artemisia annua* leaves, is effective even against multi-drug resistant strains of the malaria parasite. The isolation and characterization of artemisinin from *Artemisia annua* is considered as one of the most novel discoveries in recent medicinal plant research. It was isolated from the leaves of this plant in 1972 and its structure elucidated in 1979 (Klayman D, 1985). It has an empirical formula of $C_{15}H_{22}O_5$ (Figure 1.6.3). Artemisinin has a peroxide bridge to which its antimalarial properties are attributed. It has a unique structure and lacks a nitrogen-containing heterocyclic ring, which is found in most antimalarial compounds. Artemisinin is an odourless, colourless compound and forms crystals with a melting point of 156-157^{0}C (Jansen F, 2002). This drug can be easily reduced with sodium borohydride, resulting in the formation of dihydroartemisinin, which has even more antimalarial activity *in vitro* than artemisinin itself (van Agtmael *et al.*, 1999). Many derivatives have been synthesized from dihydroartemisinin, and out of these, artemether, arteether and artesunate are either currently in use or being evaluated for use. Oral formulations of artemisinin and its derivatives are absorbed rapidly, but incompletely, with considerable inter-individual variability. The peak plasma concentrations are reached in 1-2 hours, and most compounds have a short elimination half-life (Balint *et*

al., 2001). Artemisinin is converted primarily into inactive metabolites such as deoxyartemisinin and dihydroxydeoxyartemisinin. Both artemether and arteether are converted into a number of different metabolites with retained antimalarial activity (Meshnick *et al.*, 1996). Artesunate acts like a prodrug, with fast transformation into dihydroartemisinin, which accounts for its antimalarial effect (Lee *et al.*, 1990). In healthy persons, orally administered artemisinin is metabolized primarily in the liver, with a considerable first pass effect. The drugs are likely to behave differently in various patients, given the rapid uptake of the drug by infected erythrocyte. Moreover, the artemisinins are highly protein bound, mainly to a-1-acid glycoprotein, an acute-phase protein whose level is significantly elevated in acute malarial illness (Li *et al.*, 1982).

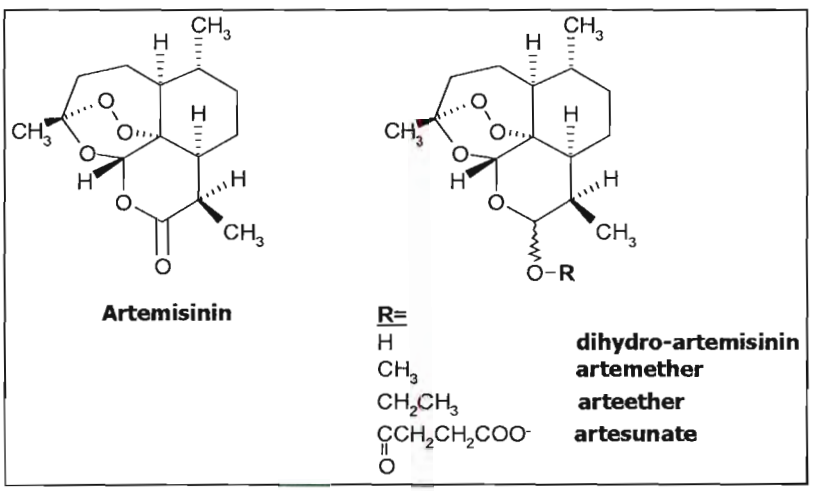


Figure 1.6.3 The structure of artemisinin and its derivatives

1.6.4 Mechanism of action of artemisinin and its derivatives

The artemisinins kill parasites more rapidly than any other antimalarial agent due to its unique pharmacodynamic action. They are toxic to malarial parasite at nanomolar concentrations. These drugs cause the structural changes in erythrocyte stage of the parasite by affecting the membranes surrounding the vacuole, the nucleus, the mitochondria, endoplasmic reticulum and nucleoplasm. Artemisinin localizes in parasite

specific membranes and such changes lead to the formation of autophagus vacuoles and loss of cytoplasm, which then kills the parasite (Maeno Y *et al.*, 1993). The peroxide function in artemisinin and related compounds is vital for antimalarial activity and the results of *in vitro* studies indicate that the carboxyl function is not necessary for antimalarial action (Jung M *et al.*, 1990). It is postulated that the killing of the malarial parasite by artemisinin and its derivatives is mediated by the production of cytotoxic compounds such as free radicals and reactive aldehydes (Zhang F *et al.*, 1992). Artemisinin derivatives lacking the endoperoxide bridge, a known source of oxygen free radicals, are devoid of antimalarial activity (Klayman D, 1985). Most free radical generating drugs cause oxidant damage by producing oxygen free radicals. However, artemisinin derivatives affect the malarial parasite very differently from other oxidant drugs. Instead of reacting with oxygen and producing large quantities of oxygen-containing free radicals, artemisinin itself becomes a free radical in reaction catalyzed by iron (Meshnick S, 1994). The biochemical action of artemisinin depends on two sequential steps: activation and alkylation (Meshnick S *et al.*, 1996). Activation step comprises an iron-mediated cleavage of the endoperoxide bridge generating an unstable organic free radical and other electrophilic species. Iron activates artemisinin into a free radical. The second step, alkylation, involves the formation of covalent adducts between the drug and the malarial proteins. Thus, artemisinin derivatives are free radical generators but are different from oxidant free radical generating drugs in that the resulting free radicals are carbon centred and there are specific target proteins (Meshnick *et al.*, 1996).

1.7 Traditional medicines

The term "traditional medicine" refers to ways of protecting and restoring health that existed before the arrival of modern medicine (WHO, 2000). As the term implies, these approaches to health belong to the traditions of each country, and have been handed down from generation to generation. Traditional systems have had to meet the needs of the local population for many centuries, dating back in South Africa to the times of King

Shaka and his forefathers. According to the World Health Organization (WHO), traditional medicine refers to the following components: oriental therapies, acupuncture, Ayuverdic medicine, traditional birth attendant, mental healers and herbal medicine (WHO, 2000). More than 80% of the world's population use plants as their primary source of medicinal agents (WHO, 1995). It is therefore not surprising to find that in many countries there is a well-established system of traditional medicine. Therefore, plants continue to be the major source of biologically active natural products that may serve as commercially significant entities themselves or provide lead structures for the development of modified derivatives possessing enhanced activity and/or reduced toxicity. It has since been suggested by the WHO that because of the importance of medicinal plants, their use should be encouraged not only under an empirical value, but also under scientific approach. Traditional systems of health are often more holistic in that they treat the whole person, including mental, social and spiritual factors rather than just disease symptoms. In 1993, the Global Initiative For Traditional Systems (GIFTS) of health, based at the University of Oxford was established to bring policy and funding attention to the areas of traditional medicine. The goal of the initiative is to promote and strengthen the role of traditional health systems in providing affordable, effective and sustainable health care in developing countries. GIFTS emphasizes that international agencies should focus on strengthening existing health traditions rather than replacing them with western biomedical services which many countries find impossible to sustain.

There are many reasons for strengthening the role of traditional health system and traditional healers. One of the most important reasons is that in most cases, modern medicines are often too expensive, uncertain and irregular in supply, and pathogens are increasingly becoming resistant to many of them (e.g. MDR strains of TB and malaria). Furthermore, traditional healers know the sociocultural background of the people and are very highly respected and experienced. Traditional healing is deeply embedded in a wider belief system and is an integral part of the lives of the locals. For this reason, the WHO assessed the best strategy for future studies on traditional medicines, mainly to establish guidelines for a better use of traditional medicine in developing countries like

South Africa, where it has a fundamental importance (WHO, 2000). This thesis focuses largely on medicinal plants used in traditional medicine practice in South Africa.

1.7.1 Traditional medicines in South Africa

Western ideology largely underestimates the inordinate belief in traditional medicines by many people, particularly those in developing countries. The reliance of South Africans on traditional remedies is no exception. Despite the westernization of a large proportion of South Africans through urbanization and education, the belief in traditional remedies and healers remains firm. There are an estimated 200 000 indigenous traditional healers in South Africa, and up to 80% of South Africans consult these healers, usually in addition to using modern biomedical services. Traditional healers in South Africa are most commonly known as "*izinyanga*" and "*izangoma*" (Zulu), "*amaxhwele*" and "*amagqira*" (Xhosa) as well as "*dingaka*" (Sotho). They are also referred to as "*bossiedokter*" and "*kruiedokter*" in the Western and Northern Cape. The terms *izinyanga* and *izangoma* refer exclusively to herbalists and diviners respectively, but in modern times the distinction has become blurred since some healers tend to practise both arts. In addition to the herbalists and diviners who are believed to be spiritually empowered, there are traditional birth attendants (Zulu: *abazalisi*), prophets, spiritual healers (Zulu: *abathandazi*), spiritual mediums, and dreamers. Most elderly people in rural areas have knowledge of herbal medicines, and function as first-aid healers within a family repertoire of herbal remedies (van Wyk *et al.*, 1997).

Ethnobotany, the study of the use of plants by South African indigenous people is still a relatively underdeveloped discipline in South Africa, and knowledge of indigenous plant use in the country needs urgent scientific documentation before it is irretrievably lost to future generations. There is, however, ongoing scientific documentation of indigenous knowledge for future generations, and most importantly, the application and beneficiation of this knowledge as instruments for sustainable development in South Africa. There is a well-established system of traditional medicine research within the Department of

Pharmacology of the University of Cape Town, where medicinal plant information research is compiled in the form of Phytochemical and Ethnobotanical database. The South African government has embarked on integrating traditional medicinal practice into the national health care system since it recognizes traditional healers as an important resource in primary health care. Unique collaborations are being forged between government departments, science research councils, universities, local communities, traditional healers, farmers and entrepreneurs for recognition of traditional healing in South Africa.

1.7.2 Medicinal plants used in South Africa

Medicinal plants are an important aspect of the daily lives of most South Africans and they also form an important part of cultural heritage. Southern Africa has over 30 000 species of plants. With South Africa's remarkable biodiversity, it is not surprising to find that approximately 3000 taxa are used as medicines (van Wyk *et al.*, 1997). South Africa has the most diverse and temperate flora on earth, rivaling the tropical rainforests in terms of species richness. Medicinal plants used to be the primary source of all medicines in the world and they still continue to provide mankind with new remedies. Natural products and their derivatives represent more than 50% of all drugs in clinical use in the world (van Wyk *et al.*, 1997). Higher plants contribute no less than 25% to the total. In South Africa, a large part of day-to-day medicine is still derived from plants. South Africa's botanical contribution to world medicines includes Cape aloes (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*), but local equivalents exist for many of the famous remedies used elsewhere (van Wyk *et al.*, 1997).

1.7.3 Plant-derived antimalarial drugs

Traditional remedies have always been an important source of antimalarial drugs and continue to provide novel and effective treatments. In 1972, Professor Zenxhen Wei made an extract of the leaves of the plant *Artemisia annua* (sweet wormwood) and observed that it acted against the malaria parasite (Jansen F *et al.*, 2002). Further investigation yielded the isolation of the compound artemisinin (*qinghaosu*) and the crystalline structure was elucidated in 1979 with an empirical formula of $C_{15}H_{22}O_5$. A number of derivatives have been synthesized from artemisinin resulting in artesunate, arteether, artemether and dihydroartemisinin. The first antimalarial drug systematically extracted from medicinal plants was quinine from the Peruvian *Cinchona* tree. The Peruvians used to chew the bark of this tree to treat malaria-related symptoms. In 1820, further work was done on the bark of the tree and alkaloid quinine was isolated. Many other compounds used as antimalarial drugs have been developed synthetically by using quinine as the template. Chloroquine, mefloquine, amadiaquine are derivatives synthesized from quinine. Several compounds have been isolated from medicinal plants. In two tropical liana families (*Ancistrocladaceae* and *Dioncophyllaceae*) known in the traditional medicine of several African and Asian countries for the treatment of malaria, naphthylisoquinoline alkaloids are present (Francois *et al.*, 1997). These compounds have a considerable *in vitro* antiplasmodial potential against forms of both *P falciparum* and *P berghei* (Francois *et al.*, 1997). Some furanonaphthoquinones isolated from species of Bignoniaceae, traditionally used in Colombian Amazon, demonstrated a significant inhibition on the erythrocytic stages of *in vitro P. falciparum* and against *P berghei* (Perez *et al.*, 1997). In the Asteraceae species, new sesquiterpenes lactones of the germacranolide type were shown to be active against *P. falciparum in vitro*. The compound *Neurolaena lobata* showed an interesting activity (Francois *et al.*, 1996). Among naphthoquinones isolated from higher plants, plumbagin from Droseraceae possess the strongest antimalarial activity against *P. falciparum* (Likhitwitayawuid *et al.*, 1998). A number of South African medicinal plants have been evaluated for their *in vitro* antiplasmodial activity and their active constituents are still being investigated (Clarkson, 2002 and Matsabisa, 2001).

1.8 Aims

This research aimed to establish in our laboratories the murine experimental malaria model for the purpose of studying the role of cytokines and immune cells in experimental malaria, and to study the effects on experimental malaria of artesunate and of traditional medicines used to treat malaria in South Africa, with special reference to the immunological effects of these agents.

1.8.1 Objectives set for the thesis were to:

1. Study the role of pro-inflammatory cytokines in the pathogenesis of murine cerebral malaria
2. Describe the immune cell kinetics during the early events in the spleen of mice infected with *Plasmodium chabaudi* AS.
3. Investigate the effect of artesunate on experimental malaria (PbA model) and to clarify its effect (if any) on cytokine production.
4. Investigate the *in vivo* antiplasmodial activity (using PbA model) of a selection of plants traditionally used to treat malaria in South Africa and to describe their effects on the immune response.

CHAPTER 2

Materials and Methods

2.1 Experimental model for cerebral malaria

2.1.1 Mice

Mice were bred and housed under specific pathogen free conditions in the animal house of the University of Cape Town. Genetically modified mice ($TNFR^{-/-}$ and IFN-$\gamma^{-/-}$) were obtained from Prof B Ryffel and F Brombacher. Genotypes of the knockout mice were confirmed by PCR analysis of the tail biopsies. A group of 5 mice were used for each experiment.

2.1.2 Infection and examination

Plasmodium berghei ANKA (PbA, Swiss Tropical Institute, Basle) is a highly chloroquine sensitive strain. The parasites kept in liquid nitrogen were thawed and passaged into normal mice as a parasite donor as described before (Rudin W *et al.*, 1997). Parasite infected erythrocytes were collected in heparinized tubes by retro-orbital bleeding and parasitaemia was estimated by counting Giemsa stained thin blood smears. Parasitized erythrocytes (1×10^6 in 0.2 ml of saline) were injected intra-peritoneally. These mice were carefully observed daily and thin blood smears prepared from a drop of tail blood were examined by Giemsa staining.

Neurological examination was performed by three independent observers using three parameters as described (Rudin W *et al.*, 1997): Postural responses (0 to 2), reflex responses (0 to 2), and grip strength (0 to 1) were tested on each mouse. Complete disability in the three tests gives the maximal score of 5. The mean scores are presented here. The body weight of the mice was also measured, as weight loss is an indicator of disease.

2.1.3 Haematology

Blood was collected from the tail vein for the determination of parasiteamia and retro-orbital plexus for the determination of haematological parameters. Parasiteamia was

analysed on a smear preparation stained with Giemsa. The percentage of parasitized erythrocytes was determined from 200-300 erythrocytes. Standard hemograms (red cell count; white cell count; hematocrit; and the whole differential count) were performed from fresh heparinized blood using the Technikon H1 haematology analyser.

2.2 Experimental model for mechanisms of resistance to malaria

2.2.1 Mice

Mice used in this study were female, 8-10 weeks old C57BL/6 mice supplied by the Biomedical Services Unit, University of Oxford. The animals were kept in the animal facility at the University of Oxford and supplied with food and water ad libitum. A group of 5 mice were used for each experiment.

2.2.2 Parasites and infection

Plasmodium chabaudi chabaudi AS was kindly donated by Prof David Walliker (University of Edinburgh, Edinburgh, UK). Blood stage infections with *Plasmodium chabaudi chabaudi* AS was maintained by weekly passage in naïve mice. Experimental blood stage infections were initiated by intraperitoneal inoculation of $1x10^5$ infected red blood cells. Parasitaemia was monitored by daily Diff-Quick (Triangle Biomedical Sciences, UK) stained blood smears made from tail snips. % Parasiteamia was estimated by counting approximately 1000 red blood cells.

2.2.3 Antibodies

The antibodies used for the immunohistochemical analysis were as follows: monoclonal rat anti-mouse CD 4 (553043; Pharmingen), monoclonal rat anti-mouse Mϕ (F4/80) [MCAP497; Serotec], monoclonal rat anti-mouse CD45/B220 (557390; Pharmingen), monoclonal hamster (arm) anti-mouse CD11c (553799; Pharmingen), polyclonal mouse anti-hamster (arm) IgG-HRP (SC-2789; Santa Cruz), polyclonal mouse ant-rat IgG-HRP (553876; Pharmingen). The antibodies used as isotype control were purified Rat IgG2a

(553927; Pharmingen), purified Rat IgG2b (553986; Pharmingen) and purified Hamster IgG1 (553969; Pharmingen).

2.2.4 Processing of spleens from infected and control mice

Control and infected mice were sacrificed at day 0, 1, 3, 5 and 7 post-infection. Spleens were rapidly harvested, snap frozen using dry ice and stored at -70^{0}C for later immunohistochemical analysis. Spleens were fixed in neutral buffered formalin (10% formalin in PBS, pH 7.0). All paraffin-wax sections were prepared and processed by standard histological techniques.

2.2.5 Immunohistochemical analysis

Frozen tissues were embedded on the OCT medium (Cell Path, order no. 005627) and 8 μm thick sections were cut and mounted on multispot microscope slide, PTFE and specialised coatings (C. A. Hendley Essex (LTD)]. The sections were air dried overnight at room temperature, fixed in acetone for 10 minutes and then air dried at room temperature for 2 hours. The sections were then rehydrated with a 5-minute wash of 1x Tris Buffered Saline (TBS) and blocked in endogenous peroxidase block reagent (DAKO, code no. S2001) for 30 minutes at room temperature. The tissues were incubated with the primary antibody diluted in 1x TBS for 45 minutes at room temperature in a humidity chamber. Isotype control and diluent used for each antibody were prepared as negative control. After 5 minutes wash in TBS, the secondary antibody diluted in TBS, was incubated on the sections for 45 minutes at room temperature in a humidity chamber. Slides were washed in TBS for 5 minutes and incubated with DAB substrate (Sigma, D-4293) for approximately10 minutes. After the final wash of 5 minutes in TBS, the tissue sections were counter stained in haematoxylin (Sigma Diagnostics, GHS-3-32). The tissues were mounted in mounting media (BDH, UK, OC 112866). These were viewed under light microscope and captured using progress grabber (Kontron/Prog/Res/3012).

2.3 Oral artesunate prevents *Plasmodium berghei Anka* infections in mice and the effect of artesunate and other artemisinin derivatives on immune response

2.3.1 Drugs

Artesunate powder (Mepha AG, Aesch Switzerland) was dissolved in 5% sodium bicarbonate ($NaHCO_3$) in sterile distilled water. Chloroquine diphosphate salt (Sigma-Aldrich) was diluted in sterile distilled water. The concentrations of these drugs were adjusted so that the final dose in mg/kg body weight was given in 0.2 ml. The animals were treated with both drugs through oral gavage once daily for 7 or 14 days. The drugs were administered as from day 1 post-infection.

2.3.2 Mice

C57BL/6 mice, 7-10 weeks of age (Animal Unit, University of Cape Town) were caged in groups of five animals. The animals were housed under standard conditions and had free access to a standard diet and tap water. A group of 5 mice were used for each experiment.

2.3.3 Infection and examination

Plasmodium berghei ANKA (PbA, Swiss Tropical Institute, Basle) is a highly chloroquine sensitive strain. The parasites kept in liquid nitrogen were thawed and passaged into normal mice as a parasite donor as described before (Rudin W *et al.*, 1997). Parasite infected erythrocytes were collected in heparinized tubes by retro-orbital bleeding and parasitaemia was estimated by counting Giemsa stained thin blood smears. Parasitized erythrocytes (1×10^6 in 0.2 ml of saline) were injected intra-peritoneally. These mice were carefully observed daily and thin blood smears prepared from a drop of tail blood were examined by Giemsa staining. Neurological examination was performed by three independent observers using three parameters as described (Rudin W *et al.*, 1997): Postural responses (0 to 2), reflex responses (0 to 2), and grip strength (0 to 1) were tested on each mouse. Complete disability in the three tests gives the maximal score of 5. The mean scores are presented here.

2.3.4 Clinical sign, parasite clearance and curative efficacy

The "7-day suppressive test" and a "14-day suppressive test" were adopted for the determination of the parasite clearance and curative efficacy respectively. The doses for artesunate given were 1, 10 and 100-mg/kg body weight and 10 mg/kg for chloroquine. Groups of 5 mice each were administered vehicle only, or 1, 10 or 100-mg/kg body weight of artesunate or 10-mg/kg chloroquine by oral gavage route.

The parameters evaluated were: Clinical signs of cerebral malaria (Rudin W *et al.*, 1997), development of parasitaemia, survival and body weight. Neurological signs, and body weight were monitored daily and blood smears were made every 3-4 days to monitor the development of parasitaemia. The effect of the drug was considered *ineffective* if: before and after treatment no significant difference in degrees of parasitaemia or a continuous increase of parasitaemia is noted; *Slight suppression*: parasitaemia was suppressed temporarily and increases thereafter; *Marked suppression*: parasitaemia became negative for more than two days but within 30 days recrudescence occurred; *Complete suppression*: recrudescence occurred between 30-60 days after first negative parasitaemia. Lastly, the mice were considered *Cured*, if no parasite were detected 60 days post-infection.

2.3.5 Histopathology

Cerebrum, cerebellum, brain stem, lungs, livers kidneys and spleen of mice were prepared by fixing in 4% buffered formaldehyde (BDH) before paraffin embedding. Sections were stained with haematoxylin and eosin and were evaluated for degenerative and inflammatory lesions by light microscopy.

2.3.6 Isolation of splenocytes

Mice (C57BL/6, uninfected) were euthanased by intra-peritoneal injection with a lethal dose of anaesthetic. The spleens were aseptically removed and placed in iced 1 ml RPMI 1640 medium (Sigma, Cat. No. R-0883). Spleen cells were isolated by perfusing each spleen with 2-3 ml of RPMI 1640 supplemented with Gentamicin (25 µg/ml) (Sigma, UK), 2 mM L-glutamine (Gibco) and 10% heat-inactivated Fetal Calf Serum (FCS).

Aggregates were left to sediment for a few seconds and the cell suspension transferred into a fresh tube. The cell suspensions were centrifuged for 10 minutes at 1500 rpm at 4^0C and resuspended in 5 ml of lysis buffer and kept on ice for 60 seconds. The cell suspension was centrifuged for 10 minutes at 1500 rpm at 4^0C and resuspended into 20 ml culture medium. The cells were counted and plated at 0.5 x 10^6 cells/ well in 96-well plates (Falcon, Becton Dickinson), [5 x 10^6 cells/ml] at 100 µl/well. Viability was determined by exclusion of trypan blue dye. Splenocytes were pre-treated with chloroquine (Sigma-Aldrich) and artesunate (Mepha AG, Aesch Switzerland) in the following concentrations: 3, 10, 30 and 90 µM for 3 hours at 37^0C and 5% CO_2. Thereafter, the splenocytes were stimulated with 10 mg/ml Concanavalin A (Con A) (Sigma) or lipopolysaccharide (LPS) diluted in RPMI for 24 or 48 hours at 37^0C and CO_2 in the presence or absence of different concentrations of chloroquine and artesunate. Splenocytes culture supernatants were tested for TNF-α (after 24h stimulation), IFN-γ (after 48h stimulation), IL-10 (after 24h stimulation) and IL-12p40 (after 48h stimulation) using ELISA.

2.3.7 Enzyme Linked Immunosorbent Assay.

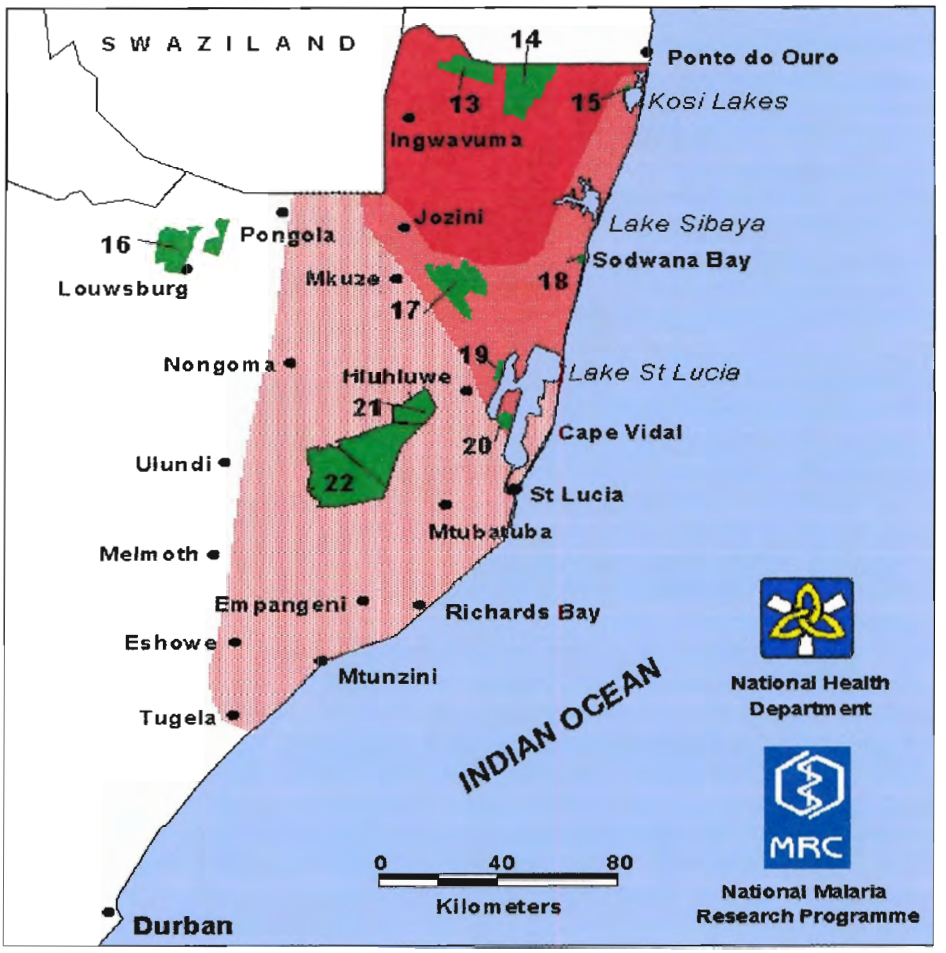
96-well Maxisorb microtiter plates (Nalge Nunc International, Naperville, IL, USA) were incubated overnight with *capturing antibody* (10 µg/ml) at 4^0C. Plates were washed 4x with Washing Buffer and incubated overnight with 200 ml Blocking Buffer at 4^0C. The Blocking Buffer was removed and recombinant mouse cytokine standards were added in three fold dilutions at concentrations ranging from 100 µg/ml – 0.6 pg/ml. Samples were added undiluted and the plates incubated overnight at 4^0C. Plates were washed 4x and incubated overnight with a biotinylated-*detecting antibody* (2 µg/ml) at 4^0C. Plates were washed 4x and incubated with streptavidin-alkaline phosphatase (BD Pharmingen) (1: 1000 dilution) for 2 hours at room temperature. The plates were subsequently washed, incubated with p-nitrophenyl phosphate (1 mg/ml) (Boehringer Mannheim, Germany) and the enzymatic reaction read at 405nm using a microplate spectrophotometer (Molecular Devices, spectra MAXGemini).

2.4 In vivo antiplasmodial activity of plants that are traditionally used to treat malaria in South Africa and their effect on the immune response.

2.4.1 Ethnobotanical Survey

In this thesis, we present ethnobotanical data on medicinal plants that are used to treat malaria and other fevers, based on first-hand information collected by personal contact with traditional healers, ordinary villagers (old people), as well as through personal observations of applications of herbal remedies administered by traditional healers.

The South African Traditional healers, also called (sangoma's) in Zulu, were the main focus and the guides for the study. The other group known as izinyanga, which uses witchcraft, and their treatments, are based mainly on magical rites, sometimes combined with plant therapy; only a few of those were consulted during the study. Old people, who had knowledge of the curative property of plants, were the second most important group of people with whom the research team has worked. It is also very much important to emphasize the importance of the 'mother of family' (mother, grand mother) who is capable of treating the everyday (less significant) ailments of her family using the medicinal plants grown in her own backyard. Our field team, which consisted mainly of research students, worked with traditional healers from Northern Province (now Limpopo), Mpumalanga, Gauteng and KwaZulu Natal. The criterion used to choose these provinces was because of being high endemic areas for malaria in South Africa. The information was collected through informal interviews, which were recorded on tape and filmed during the fieldwork with traditional healers. The gathering and recording of information were repeated with different healers, in different parts of each province. Each individual was asked about the plants used for medicinal purposes in their area, the disease malaria (if they had any knowledge of it), and the symptoms they use to diagnose a malaria patient. For each plant the following descriptors were recorded: vernacular name, part of plant used, the mode of preparation and most importantly, the form of administration of the remedies. Figure 2.1 and 2.2, respectively, show the distribution of malaria in these provinces.



Take preventative measures against mosquito bites throughout the year in ALL RISK areas.

HIGH RISK

Prophylactic medicines are recommended from October to May.

INTERMEDIATE RISK

Prophylactic medicines are recommended for high risk individuals from October to May.

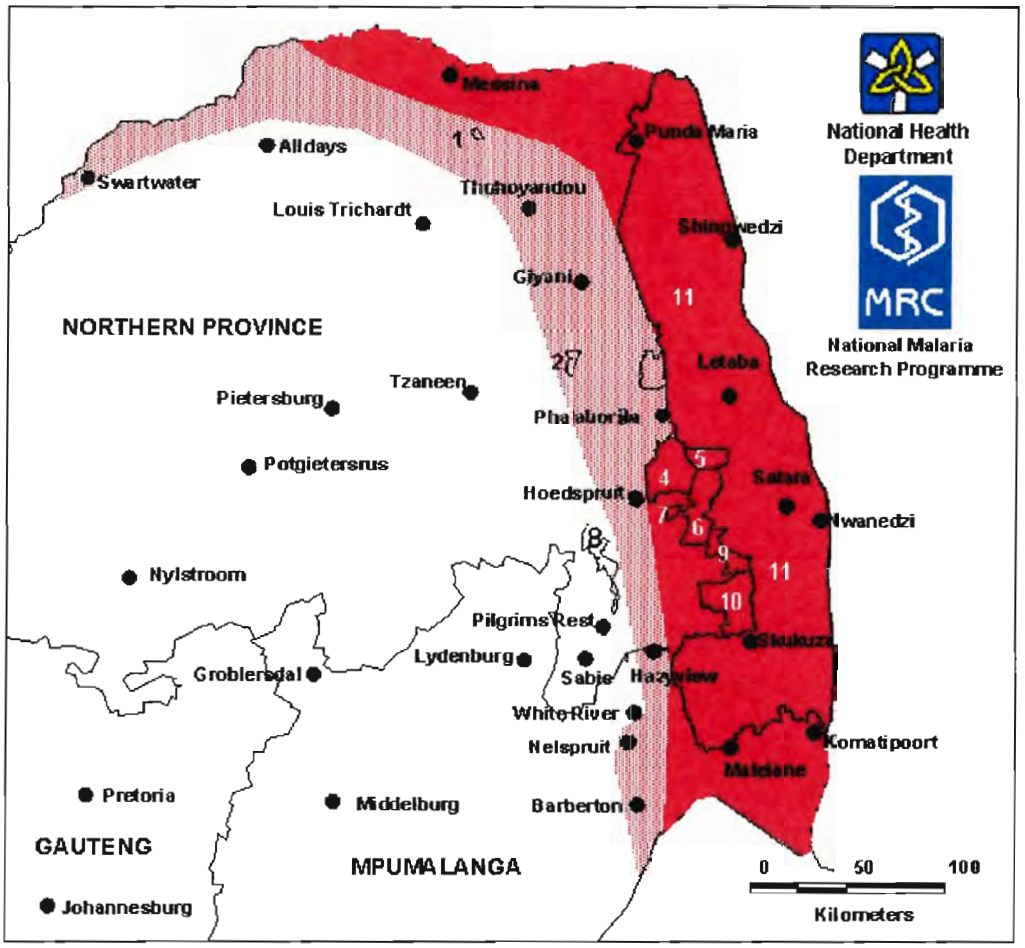
LOW RISK

No prophylactic medicines are recommended.

GAME RESERVES

13. Ndumu
14. Tembe
15. Kosi Bay
16. Itala
17. Mkuze
18. Sodwana
19. False Bay
20. Fanies Island
21. Hluhluwe
22. Umfolozi

Figure 2.1 The distribution of malaria in KwaZulu-Natal Province.
(http://www.malaria.org.za/malaria-Risk/cases/.html); last accessed May 2003



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GAME RESERVES

1. Tshipese
2. Hans Merensky (Eiland)
3. Groot-Letaba
4. Klaserie
5. Sharalumi
6. Timbavati
7. Thornybush
8. Blyderivierpoort
9. Manyeleli
10. Sabie-Sand
11. Kruger National Park
12. Pilanesburg

Figure 2.2 Distribution of malaria in Limpopo (Northern Province) and Mpumalanga. *(http://www.malaria.org.za/malaria-Risk/cases/.html); last accessed May 2003*

2.4.2 Collection of plants

Plant species were selected based on the information gathered from healers around the country. Some of the plants were collected under the guidance of healers. For most of the times, the team obtained the permission from the healers prior to the collection of the desired plants. Voucher specimen of these medicinal plants, with specimen data including vernacular name, voucher number, collecting locality and the date of collection were done together with Department of Botany, University Of Cape Town.
The table below shows some names and origins of the plants that were evaluated.

Table 2.1 Names and origins of plants evaluated.

Vernacular and botanical name	Part evaluated	Collection location in SA
1. Isibaha (Warburgia Salutaris)	cortex or bark	Durban (KZN)*
2. Ikhathazo (Alepidea Amatymbica)	cortex or bark	Durban (KZN)
3 Umlulama (*Nuxia Floribunda*)	bark and leaves	Durban (KZN)
4. Ingwavuma (Cassine transvaalensis)	roots	Nongoma (KZN)
5. Devil's claw (*H. procumbens)*	roots	Grassroots (WP)^
6. Qinghaosu *(Artemisia annua)*	leaves	Grassroots (WP)
7. Umgwenya *(Harpephyllum caffrum)*	bark	Durban (KZN)
8. Inyathelo *(Vernonia adoensis)*	bark and leaves	Durban (KZN)

(KZN)* - denotes KwaZulu Natal; (WP)^ - denotes Western Cape.

2.4.3 Chemical Extraction of Plant Material.

Each plant was extracted using cold water and sequentially with dichloromethane and methanol. Cold or hot extraction were also performed, but this was affected by the method by which the healers use traditionally i.e. either prepared in boiling water or cold water. For the plant prepared in boiling water, we performed the hot extraction in the

plant. The plant material were dried and then chopped into small pieces and extracted with water, dichloromethane and methanol. For water extraction, 500g of material was put into a flat-bottomed flask and extracted by vigorous shaking with 500ml of solvent for 24 hours. The extracts were filtered and the remaining plant material was placed into a soxhlet (for hot extraction procedure) overnight. The filtrates for both hot and cold-water extractions were concentrated by freeze-drying. The hot and cold dichloromethane and methanol extracts were evaporated under vacuum using rotary evaporator. The concentrated extracts were then transferred to pre-weighed vials and stored at 4^0C.

2.4.4 Determination of the *in vivo* anti-malarial activity of plant extracts

For the *in vivo* tests, the 'Four Day Suppressive Test' (4-DST) and the 'Seven Day Suppressive Test' (7-DST) was used. C57BL/6 mice, 7-10 weeks of age (Animal Unit, University of Cape Town) were caged in groups of five animals. The animals were housed under standard conditions and had free access to a standard diet and tap water. *Plasmodium berghei Anka* (PbA, Swiss Tropical Institute, Basle) is a highly chloroquine sensitive strain. The parasites kept in liquid nitrogen were thawed and passaged into normal mice as a parasite donor. Parasite infected erythrocytes were collected in heparinized tubes by retro-orbital bleeding and parasitaemia was estimated by counting Giemsa stained thin blood smears. Parasitized erythrocytes (1×10^6 in 0.2 ml of saline) were injected intra-peritoneally. These mice were carefully observed daily and thin blood smears prepared from a drop of tail blood were examined by Giemsa staining. Chloroquine diphosphate salt (Sigma-Aldrich) was diluted in sterile distilled water. The concentrations of these extracts/drug were adjusted so that the final dose in mg/kg body weight was given in 0.2 ml. The animals were treated with plant extracts and chloroquine through oral gavage once daily for 4 or 7 days. The drugs were administered as from day 1 post-infection.

The effect of the extracts/drug was considered *ineffective* if: before and after treatment no significant difference in degrees of parasitaemia or a continuous increase of parasitaemia is noted; *Slight suppression*: parasitaemia was suppressed temporarily and increases thereafter; *Marked suppression*: parasitaemia became negative for more than two days

but within 30 days recrudescence occurred; *Complete suppression*: recrudescence occurred between 30-60 days after first negative parasitaemia. Lastly, the mice were considered cured, if no parasite were detected 60 days post-infection.

2.4.5 Isolation of splenocytes

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three fold dilutions at concentrations ranging from 100 μg/ml – 0.6 pg/ml. Samples were added undiluted and the plates incubated overnight at 4^0C. Plates were washed 4x and incubated overnight with a biotinylated-*detecting antibody* (2 μg/ml) at 4^0C. Plates were washed 4x and incubated with streptavidin-alkaline phosphatase (BD Pharmingen) (1: 1000 dilution) for 2 hours at room temperature. The plates were subsequently washed, incubated with p-nitrophenyl phosphate (1 mg/ml) (Boehringer Mannheim, Germany) and the enzymatic reaction read at 405nm using a microplate spectrophotometer (Molecular Devices, spectra MAXGemini).

2.4.7 Statistical Analysis

The data is expressed as the mean ± SD. Statistical analysis was performed using the Student's t test. For all tests, a p value of <0.05 was considered significant.

2.5 Reagents:

Red blood cell lysis buffer

8.29 g NH_4Cl (0.15 M)

1 g $KHCO_3$ (1 mM)

37.2 mg Na_2EDTA (0.1 M)

Dissolve the above reagents in 800 ml distilled water and adjust the pH to 7.2-7.4 with 1 M HCL. Make up to 1 litre. Filter sterilize through 0.45 μm filter and store at room temperature.

Phosphate Buffered Saline

10 tablets per 1000 ml. Adjust volume to 1000ml and sterilize at 121^0C for 30 minutes. Store at room temperature.

LPS

Dissolve 1 mg of LPS from *Esterichia Coli* 0127:B8 (Sigma Chemical Co, St Louis, USA) in 2 ml of PBS. Sterilise through a 0.45 μm filter (Millipore Corporation, Bedford, USA) and store at 4^0C.

Lethal Anaesthetic

Add 0.5 ml Rompum (Bayer Pty Ltd, Germany) and 2 ml Anaket-V (Centaur Labs, Premier Pharmaceutical Company, Bryanston, South Africa) to 9 ml PBS. Sterilise through 0.45 μm filter (Millipore Corporation, Bedford, USA) and store at 4^0C.

Buffered Formalin

Add 100ml 40% formaldehyde to 900ml PBS. Store in the dark at room temperature.

ELISA reagents

Table 2.2 Antibodies used in ELISA for cytokine detection

Cytokine	Capturing antibody	Recombinant standard	Detecting antibody	Source
TNF-α	20031D	19321T ***Lot No. M049411***	18122D	Pharmingen
IFN-γ	18181D	554587 ***Lot No. M056503***	18112D	Pharmingen
IL-12p40	18491D	554592 ***Lot No. M063820***	18482D	Pharmingen
IL-10	18141D	19281V ***Lot No. M016672***	18152D	Pharmingen

Coating Buffer

Dissolve 0.2g NaN_3 in 800ml PBS (pH 7.2). Adjust the volume to 1000ml. Sterilize by passing the solution through a 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4°C.

Dilution Buffer

Dissolve 10g Bovine Serum Albumin (Boehringher Mannheim, Germany) and 0.2g NaN_3 in 800ml PBS (pH 7.4). Adjust volume to 1000ml. Filter through a 0.45μm filter (Millipore Corporation, Bedford, USA) and store at 4^0C.

Blocking Buffer

Dissolve 40g Bovine Serum Albumin (Boehringher Mannheim, Germany) and 0.2g NaN_3 in 800ml PBS (pH 7.4) and store at 4^0C.

20X Washing Buffer

Dissolve 20g KCL, 20g KH_2PO_4, 144g NaCl in 4.5 litres distilled H_2O. Add 50ml Tween 20 and 100 ml 10% NaN_3 solution. Adjust volume to 5 litres. Sterilize through a 0.45 μm filter (Millipore Corporation, Bedford, USA) and store at 4^0C.

Substrate Buffer

Dissolve 0.2g NaN_3, and 0.8g $MgCl_2$ in 700ml-distilled water. Add 97 ml liquefied diethanolamine. Adjust to pH 9.8 and then adjust volume to 1000ml-distilled H_2O. Sterilize through a 0.45 mm filter (Millipore Corporation, Bedford, USA) and store at 4^0C.

Immunohistochemistry reagents

Meyers Haematoxylin

Dissolve 1g haematoxylin in 800ml distilled H_2O. Add 50g aluminium ammonium sulphate and dissolve. Add 0.2g sodium iodate, 1g citric acid and 50g chloral hydrate ensuring to dissolve each compound before the addition of the other. Make up to 1000ml, filter through Whatmann filter paper no.1 and store in the dark at room temperature.

Eosin

Add 150ml 1% Eosin solution to 75ml 1% Phloxine solution. Adjust volume to 450 with distilled H_2O. Filter through Whatmann paper no.1 and store at room temperature.

Paraffin wax embedding

All tissue sections were immersed in formalin at least 10x the volume of the tissue. For paraffin wax embedding tissues were dehydrated in an automated tissue processor (Shandon Elliot) according to the following program:

70% alcohol	30 minutes
96% alcohol (2x)	45 minutes
100% alcohol (4x)	45 minutes
Xylol (2x)	60 minutes
Wax [55^0C- 60^0C] (2x)	45 minutes with vacuum

Tissues were sectioned at 2 mm using a microtome (Leica, model RM-2125) and fixed onto a glass slide by overnight incubation at 37^0C. Prior to staining tissue sections were incubated at 60^0C for 2 hours-18 hours to remove wax.

Haematoxylin and Eosin staining

Tissues were rehydrated according to the following procedure:

Xylol	3 minutes
Xylol	1 minute (2x)
Absolute Alcohol	1 minute (2x)
96% Alcohol	1 minute (2x)
70% Alcohol	1 minute
Water	1 minute

Sections were stained with Haematoxylin for 8 minutes and rinsed in water. Sections were immersed in 1% acid alcohol for 10 seconds in running water for 30 minutes. Sections were counterstained with 1% eosin for 2 minutes and rinsed in water. Sections were dehydrated by immersion for 10 seconds in 70% alcohol, 96% alcohol and xylol. Sections were mounted using Canada Balsam.

CHAPTER 3

Experimental murine models

3.1 Role of pro-inflammatory cytokines in the pathogenesis of murine cerebral malaria

Introduction

Cerebral malaria is defined as unrousable coma not attributable to other causes in patients with *P. falciparum* malaria. In African children, coma is very often accompanied by fever, convulsions, seizures, metabolic acidosis and hypoglycaemia (Marsh K *et al.*, 1996). The availability of different mouse strains, including various gene knockout mice, has given insight into molecular mechanisms involved in cerebral pathology. Experimental infection with *Plasmodium berghei* ANKA (PbA) causes fatal cerebral malaria in mice, which at least in part mirrors clinical cerebral malaria (Taylor-Robinson *et al.*, 1995), is associated with elevated blood TNF levels (Grau G *et al.*, 1987). The administration of neutralizing antibodies or inhibitors of TNF synthesis prevents cerebral malaria in mice (Clark I *et al.*, 1992; Garcia *et al.*, 1995). In patients, anti-TNF antibodies reduced the fever of clinical cerebral malaria (Kwiatkowski D *et al.*, 1993). Both human and mouse cerebral malaria are characterized by the plugging of small vessels with erythrocytes and, in the case of PbA infection, with monocytes (Clark I *et al.*, 1994; Turner G *et al.*, 1994). Sequestration of circulating blood cells including thrombocytes is probably due to TNF-induced up-regulation of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on the endothelium and leukocyte functional antigen 1 (LFA-1) on circulating cells. While TNF of monocyte and macrophage origin appears to play a central role in the pathogenesis of cerebral malaria, T-cells and T-cell-derived cytokines, e.g. IL-3, granulocyte/macrophage colony stimulating factor (GM-CSF) and IFN-γ are also required for development cerebral malaria (Grau G *et al.*, 1987; Grau G *et al.*, 1994).

While a pathogenic role for TNF and IFN-γ has been well established, in the present investigation we investigated using the gene knockout mice to confirm the role of pro-inflammatory cytokines in the pathogenesis of murine cerebral malaria. Our results confirm that TNF-α receptor deficient mice and IFN-γ receptor deficient mice are

protected from developing cerebral malaria. Protected mice die at a later time of severe anaemia, leukocytosis and overwhelming parasitaemia.

Results

3.1.1 The incidence of Cerebral Malaria and Survival in wild type, TNF-α/β -/- IFN-γ -/- mice

We tested whether wild type mice (B6X129$^{+/+}$ and WT 129$^{+/+}$) were sensitive to *Plasmodium berghei Anka* (PbA)-induced cerebral malaria. Regular neurological examination was performed, by assessing postural responses, reflex responses and grip strength. We observed some neurological alterations, which included abnormal postural responses and reduced reflexes and grip strength. Mice died quite suddenly from these complications and it was concluded that they died of cerebral malaria. Clinical disease commenced around 5-7 days post- inoculation (Figure 3.1).

There was no significant clinical disease in mutant mice. Although some of mice died as the disease progresses, no significant clinical signs were observed. In particular, there was no evidence of neurological involvement. The significant sign that was observed in these mice was that they started losing the body weight and they succumbed to infection without developing specific clinical symptoms, (Figure 3.2). The mice died mainly between days 10-16 for TNF-α$^{-/-}$ and on days 10-18 for IFN-γ$^{-/-}$ post infection. By days 18-21 post-infection, all the mutant mice that were PbA infected succumbed to infection (Figure 3.3).

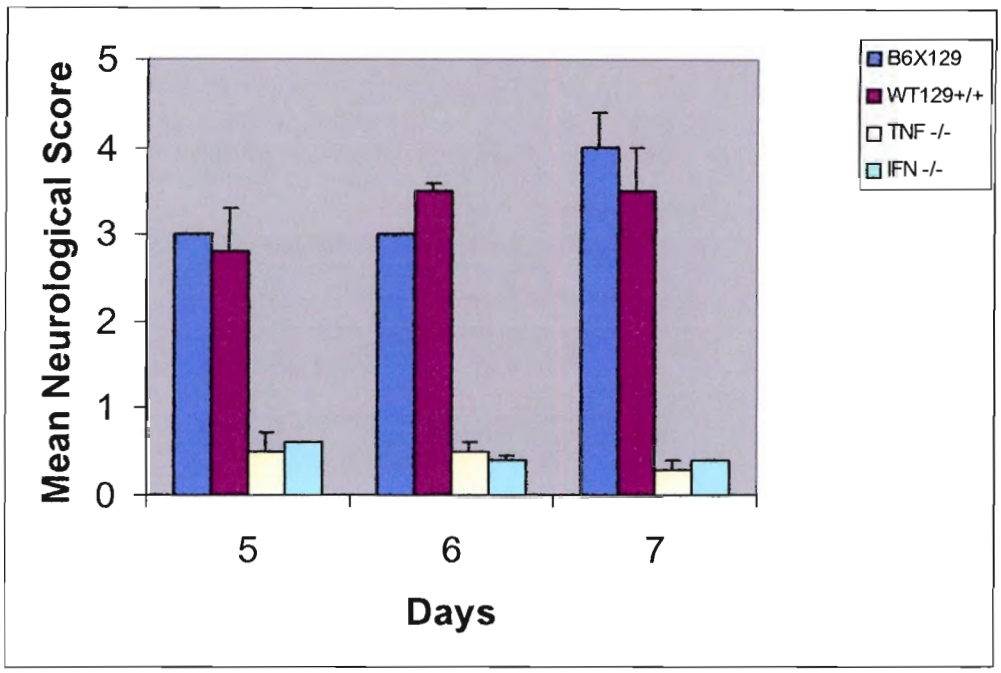

Figure 3.1 CM in PbA-infected wild type and gene knockout mice. Semi-quantitative assessment of neurological symptoms of CM. PbA-infected mice were assessed by a neurological score as described. The results are from a single experiment representative of three independent experiments.

3.1.2 Parasitaemia and Haematological Parameters

In the initial phase of the infection, most of the mice that developed clinical signs had a parasitaemia of about 18-20%. As the diseases progressed, in those mice that survived much longer, a substantial increase in parasitaemia of 30-35% by day 9 was noted. In both wild type and mutant mice, the level of parasitaemia was similar indicating that cytokines have no effect on the parasitization of erythrocytes. The parasitaemia in TNF-$\alpha^{-/-}$ and IFN-$\gamma^{-/-}$ mice increased to a maximum level of 68-70% by days 18 and 20 (Figure 3.4).

As the parasitemia due to PbA infection increases, haematological complications emerged. Severe anaemia is a common complication of malaria infection. However, there is often no close correlation between parasitaemia, anaemia and cerebral malaria

(Rudin W *et al.,* 1997). The main haematological findings observed in our study are anaemia and leukocytosis (Figures 3.5 and 3.6). Anaemia was already quite distinct by day 7 when wild type mice started to die with signs of cerebral malaria. Anaemia in these mice was not significantly different from that in mutant mice. In wild type mice, the erythrocyte count on day 7 was on average 5.36 X 10^{12}/l, haemoglobin 8.0 g/dl and hematocrit 21,3% as compared with 6.04 X 10^{12}/l, haemoglobin 8.1 g/dl and hematocrit of 22% for $TNF^{-/-}$. Anaemia increased sharply at day 14 in $TFN^{-/-}$, (erythrocytes, 3.12 X10^{12}/l; haemoglobin 4.5 g/dl; and hematocrit 15.3%), (Figure 3.5).

Anaemia in $IFN^{-/-}$ mice was comparable to that observed in wild type mice. The erythrocyte count on day 7 was on average 4.24 and 4.16 X 10^{12}/l; haemoglobin content 7.4 and 7.0 g/dl and hematocrit 24% and 21%, respectively. Anaemia increased sharply by day 9 when most of the wild type mice died with erythrocyte count of 2.08 X 10^{12}/l on $IFN^{-/-}$ to 3.02 X 10^{12}/l on wild type mice. Anaemia increased further on $IFN\gamma^{-/-}$ mice by day 14, (erythrocytes at 1.8 X 10^{12}/l; haemoglobin 3.3 g/dl; and hematocrit 11%), (Figure 3.5).

Another manifestation of severe malaria infection is leukocytosis. We have shown a distinct increase in white cell count on days 7 and 14-post infection in $TNF^{-/-}$ and wild type mice which reaches values of 8,20 X10^9/l and 9.41 X 10^9/l respectively and 15,74 X10^9/l by day 14 in mutant mice (Figure 3.6). In $IFN^{-/-}$ mice, the white cell count on day 7-post infection reached 7.8 X10^9/l and 8.6 X10^9/l respectively. By day 9, by which time all wild type mice had died, there was still a distinct increase in white cell counts and by day 14, the white cell count reached 20.23 X 10^9/l in $IFN^{-/-}$ mice (Figure 3.6). In wild type mice, the thrombocyte count was significantly decreased by day 3 and 7 with values ranging from 263.6 X10^9/l to 249,3 X10^9/l. By day 9-post-infection, we observed a significant reduction in thrombocytes ranging at 139 X10^9/l and mainly in mice with neurological signs indicative of cerebral malaria. No similar results were obtained for mutant mice (data not shown).

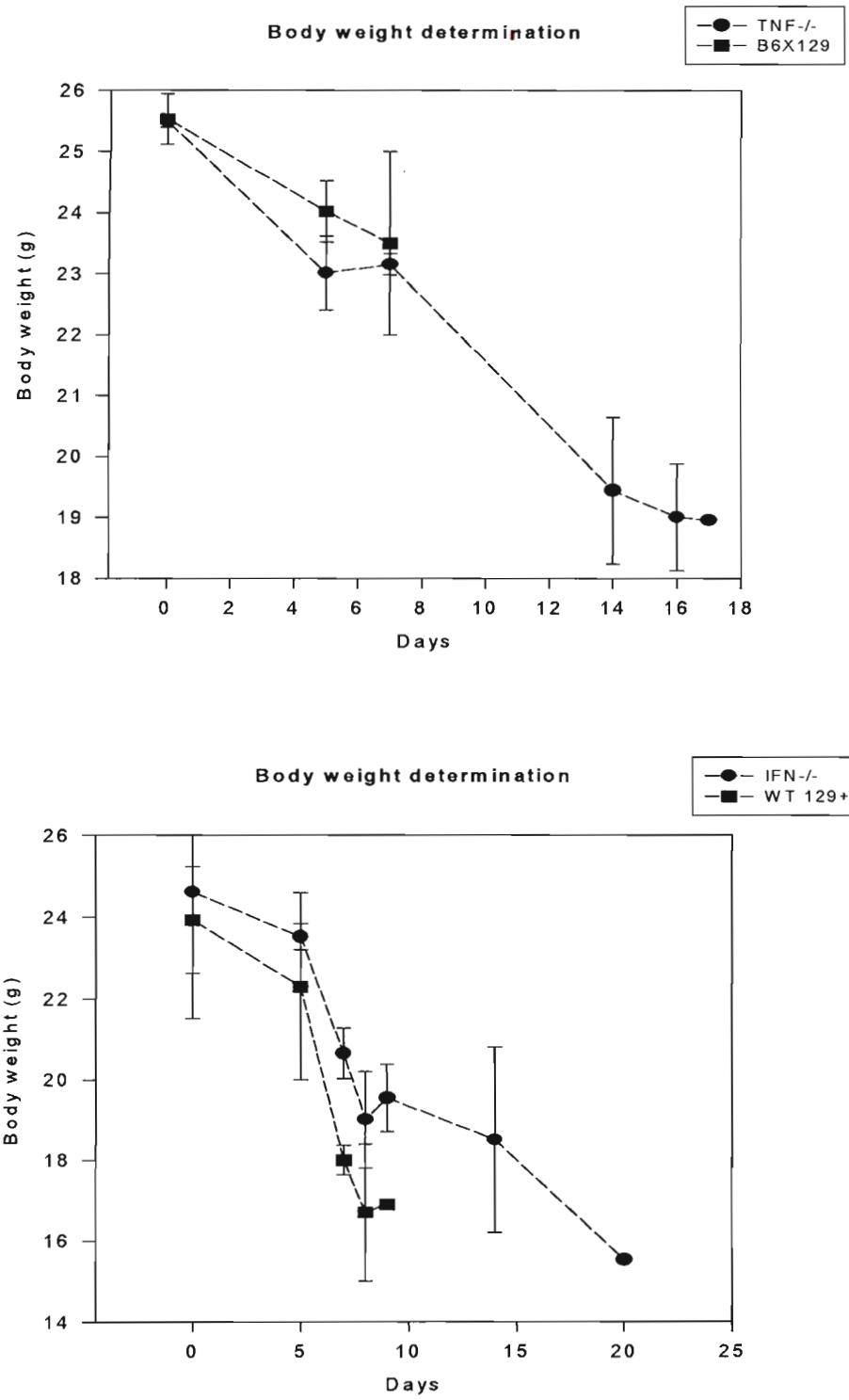

Figure 3.2 Body weight determination of wild type and KO mice after PbA infection. The results are from single experiment (n=5) representative of three independent experiments.

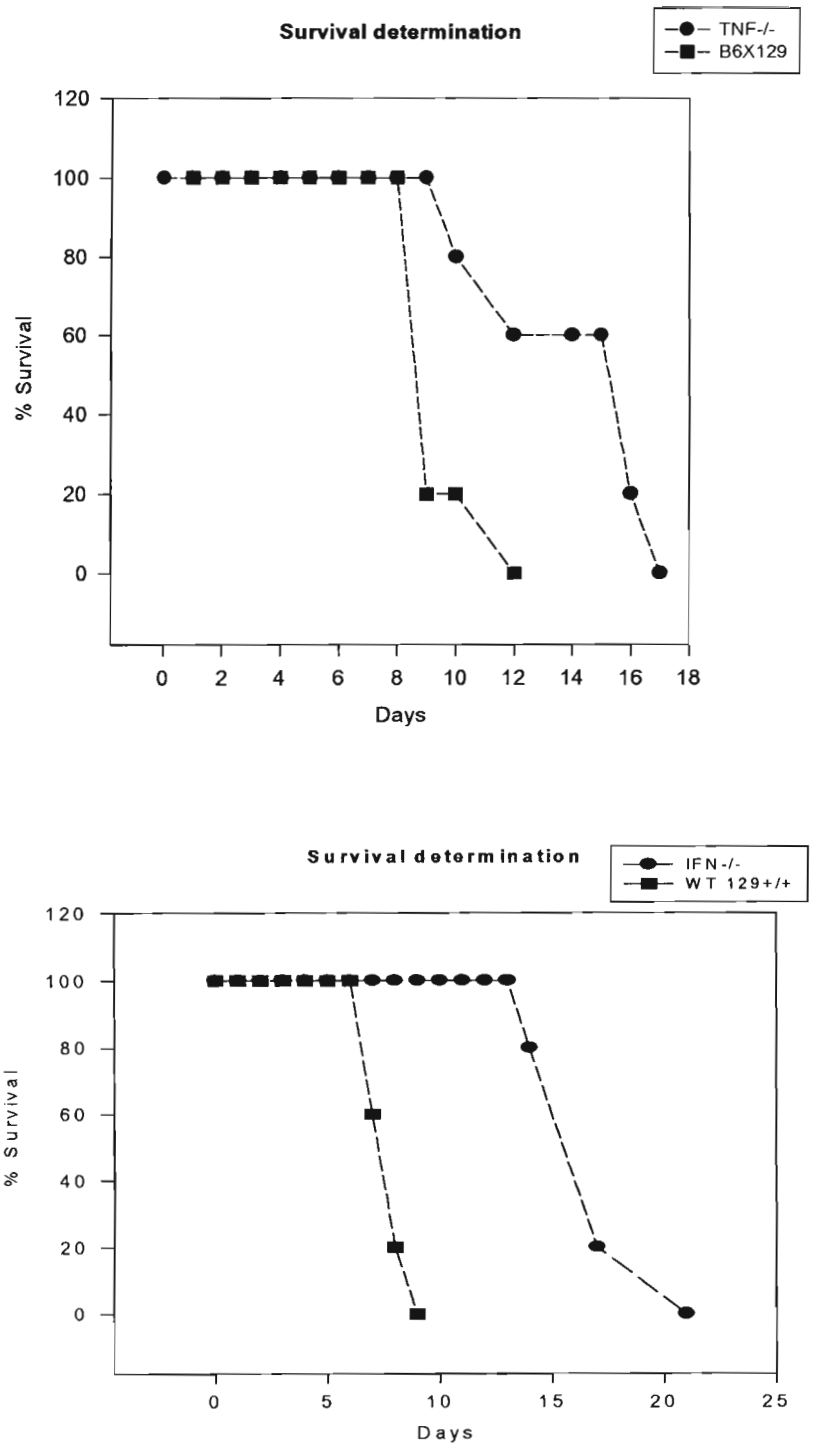

Figure 3.3 Survival of wild type and KO mice upon PbA infection. The results are from a single experiments (n=5) representative of three independent experiments.

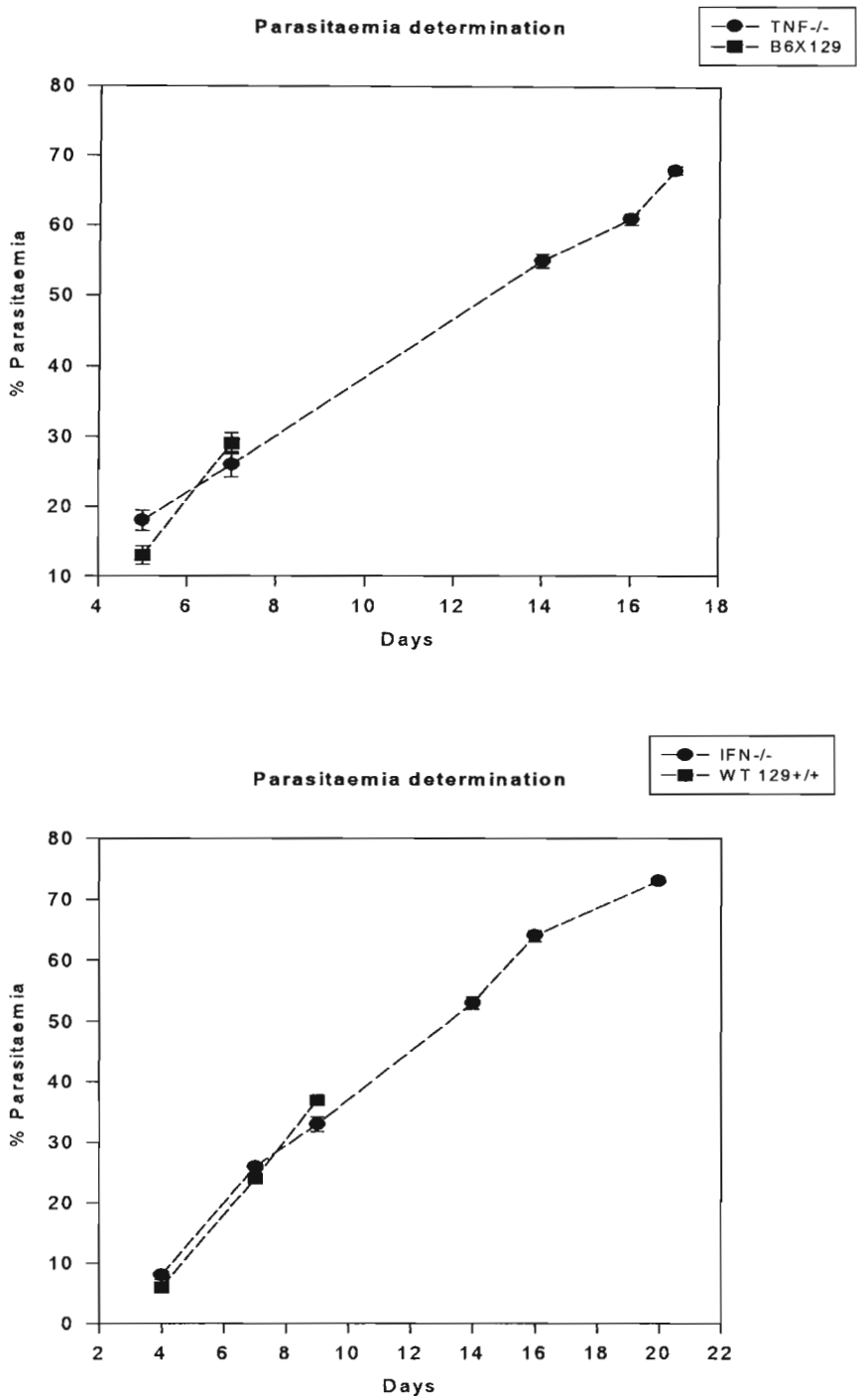

Figure 3.4 Parasitaemia in PbA-infected wild type and KO mice. The percentage of parasitized erythrocytes was determined from 200 erythrocytes; mean values ± SD (n=5) are shown.

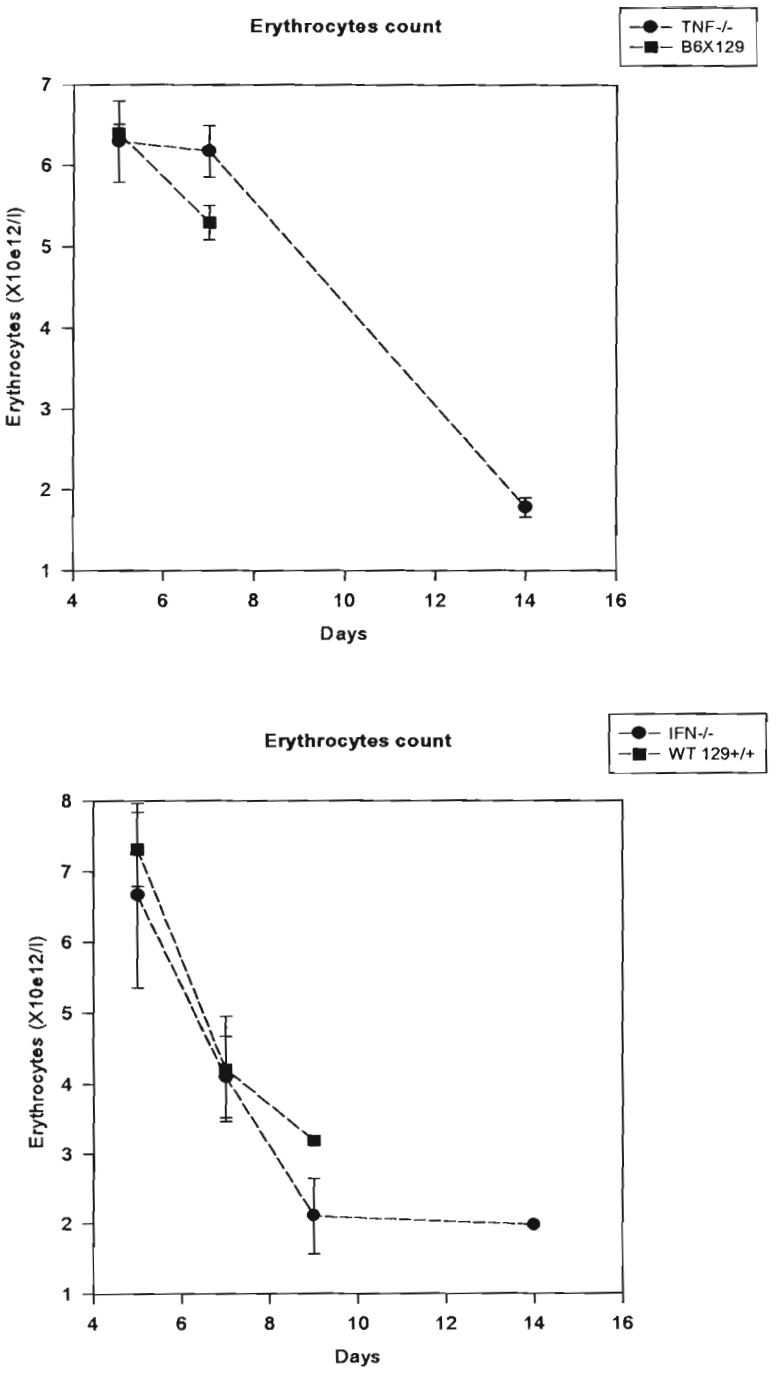

Figure 3.5 Haematological alterations in wild type and KO mice. Erythrocytes counts; mean counts ± SD (n=5) are given. The presented results are from a single experiment representative of three independent experiments.

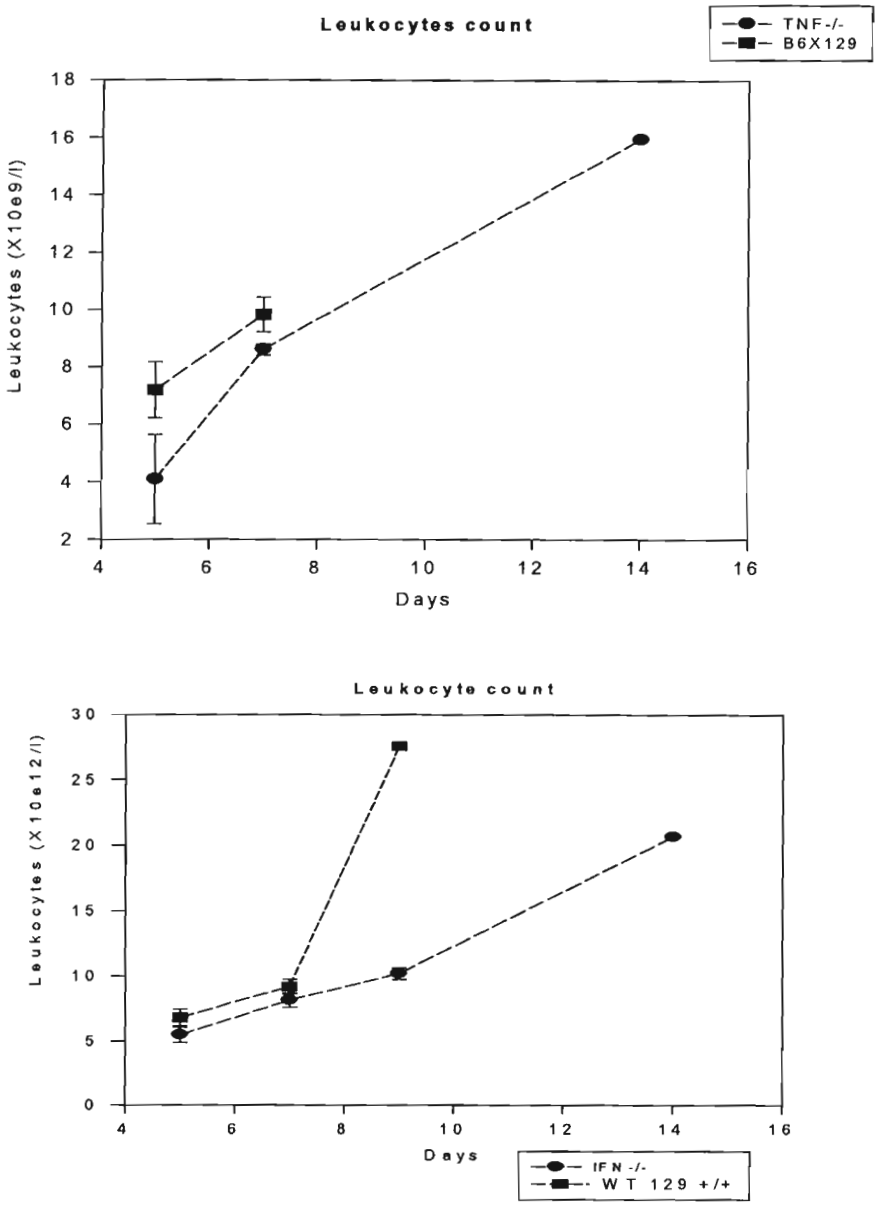

Figure 3.6 Haematological alterations in wild type and KO mice. Leukocyte counts; mean counts ± SD (n=5) are given. The presented results are from a single experiment representative of three independent experiments.

3.1.3 Discussion

Cerebral malaria is a lethal complication resulting from the immune response triggered by PbA infection in susceptible strains of mice. Cerebral malaria does not appear in mutant (TFN and IFN-receptor deficient) mice and only observed in wild type mice (Figure 3.1). We have examined whether endogenous TNF and IFN-γ have any influence on parasitaemia and on haematological alterations in PbA infected mice. PbA-induced TNF synthesis and release might increase the clearance of malaria parasites by enhancing the host defence system, through a more efficient macrophage killing. However, our results show that both wild type and mutant mice develop a similar degree of parasitaemia in the initial phase of the infection (Figure 3.4). Neither TNF nor IFN-γ appears to have an effect on the parasitization of erythrocytes. Mutant mice survived much longer, with parasitaemia reaching approximately 70% at 17-20 days post-infection. The consequent haematological complications are anaemia, leukocytosis and thrombocytopenia. Anaemia and leukocytosis in wild type mice do not differ from mutant mice. Thus both anaemia and leukocytosis are independent of TFN and IFN-γ.

These results are consistent with the hypothesis that there is local production of TNF and IFN-γ during murine PbA infection that plays a crucial role in the pathogenesis of cerebral malaria. Wild type mice are susceptible to neurological complication attributing to cerebral malaria and the mutant mice are resistant. Anaemia and leukocytosis occur in the absence of both TNF and IFN-γ, but thrombocytopenia is influenced by these cytokines. A pathogenic role of both TNF and IFN-γ on the development of cerebral malaria was not unexpected. There is pre-existing experimental evidence supporting the role of TNF in the pathogenesis of cerebral malaria. Medana *et al.*, 2001 showed TNF-α production by central nervous system-resident microglia and astrocytes as well as peripheral monocytes adherent to the meningeal vessels and cerebrovascular endothelial cells. This occurs on or before the onset of cerebral symptoms, suggesting a role in the immunopathology of fatal murine cerebral malaria for TNF-α produced within the brain. Matsuda *et al.*, 1993 showed that injection of TNF-α directly into the brains of mice caused mononuclear cell infiltration around blood vessels, edema, and haemorrhage,

features found in murine cerebral malaria. Mice injected with saline alone exhibited no inflammatory responses. Probert *et al.,* 1995 demonstrated abnormalities in nervous system functions and structure as a result of tissue-specific over-expression of TNF-α in the central nervous system. The condition is manifested by ataxia, seizures and paresis and leads to premature death. Histopathological analysis revealed widespread reactive astrocytosis and microgliosis, and focal demyelination. All these features have been demonstrated in murine (Medana *et al.,* 1996, Medana *et al.,* 1997) and human (Janota *et al.,* 1979) cerebral malaria. In contrast, (Clark *et al.,* 1990) showed that systemic administration of TFN-α to mice induced hypoglycemia, midzonal liver necrosis, and neutrophil adhesion in pulmonary vessels. This pathology is typical of terminal *P. vinckei* infection, in which cerebral malaria does not occur. Thus the difference between non-cerebral malaria and fatal murine cerebral malaria models lie in the location of cytokine regulatory loops during the progression of the infection. During the non-cerebral malaria, the immune response is restricted to the periphery. However, during fatal cerebral malaria, there may be a unique system interposed between the central nervous system and the peripheral immune systems with cytokines, in particular TNF-α, as central effector molecules.

The expression of adhesion molecules is upregulated by cytokines produced during the immune response, such as TNF (Grau *et al.,* 1987). In agreement with this concept, Favre *et al.,* 1999 showed that ICAM-1 was strongly up-regulated in the cortical venules of the C57BL/6 mice. They also observed a stronger expression of VCAM-1 in wild type mice than in ICAM-$1^{-/-}$, probably because of the lower TNF levels in the serum of these mice. TNF, by its effects on endothelial cell adhesion molecules, can increase the sequestration of various cell types, namely macrophages, neutrophils and platelets (Imhof B *et al.,* 1995). The downstream effects of TNF production include upregulation of ICAM-1/CD54, ELAM-1/CD62E (Hviid L *et al.,* 1993), VCAM-1/CD106 (Jackobsen P. *et al.,* 1994), CD36, thrombospondin (Newbold C *et al.,* 1997), PCAM-1/CD31 (Treutiger C *et al.,* 1997), chondroitin-4-sulphate (Pouvelle B *et al.,* 1997) and P-selectin/CD62P (Udomsangpetch R *et al.,* 1997), all of which have been implicated in the adherence of Plasmodium falciparum schizont-infected erythrocytes to endothelial cells.

In *P. falciparum, P.fragile* and *P. coatneyi* adhesion to CD36, thrombospondin and ICAM-1 (CD54) on the vascular endothelium is mediated by knobs on the surface of the mature stages of parasitized erythrocytes (Berendt A *et al.,* 1994). In experimental murine cerebral malaria, adherence of leucocytes occurs via the CD11a alpha chain of LFA-1 binding to CD54 (Mannel D *et al.,* 1997). Endothelial activation also causes platelet-endothelial adhesion via CD11a/CD54 followed by fusion of the leukocyte cell membrane to the endothelium with consequent endothelial damage (Lou J *et al.,* 1997). Our findings of thrompocytopenia in wild type mice suggest that thrombocytes are indeed sequestered in the peripheral vascular beds, and this sequestration is dependent on TNF and IFN-γ. In keeping with human cerebral malaria, ICAM-1 upregulation in murine cerebral malaria is associated with monocytes and parasites sequestration in the brain (Hearn J *et al.,* 2000). Neither monocytes nor parasitized red blood cells sequester in ICAM-1 (CD54) knockout mice (Favre N *et al.,* 1999).

T cells play a central role in the pathophysiology of cerebral malaria. In the absence of T cells, such as in nude mice or by eliminating CD4 T cells by antibody administration, PbA does not cause cerebral malaria (Grau G *et al.,* 1986, Finley *et al.,* 1982). There are several lines of evidence suggesting that sensitivity to develop cerebral malaria in response to PbA is mediated by a cellular or Th1 immune response, e.g., mice susceptible to PbA-induced cerebral malaria have heightened TNF-α and IFN-γ transcripts and reduced IL-4 in the brain, whereas in resistant mice, these gene transcripts are in the normal range (de Kossodo S *et al.,* 1993). Accordingly, neutralizing IFN-γ antibody prevented PbA-induced cerebral malaria (de Kossodo S *et al.,* 1993 and Grau G *et al.,* 1989). Administration of neutralizing TNF antibodies or soluble TNF receptors reduced or largely prevented fever of cerebral malaria in patients (Kwiatkowski D *et al.,* 1993). The administration of neutralizing antibodies and of inhibitors of TNF synthesis prevented cerebral malaria in mice (Clark I *et al.,* 1992). In contrast to these findings in PbA infection, resistance to *P. chabaudi* infection in C57BL/6 mice is associated with sequential activation of Th1 $CD4^+$ T cells followed by activation of the Th2 subset, which results in healing. The susceptible A/J mice, which succumb to a lethal infection with *P. chabaudi* with fulminant parasitaemia, develop immediately a strong Th2

response (Stevenson M *et al.*, 1989). Therefore, an initial Th1 response appears to be necessary to resist *P. chabaudi* infection. This is also demonstrated by the administration of IL-12, which has a protective effect in *P. chabaudi* infection with distinct reduction of parasitaemia (Stevenson M *et al.*, 1995). Our results show that mutant mice are protected against PbA-induced cerebral malaria, suggesting that the immune response may be shifted from a Th1 to a Th2-type immune response. We have demonstrated that resistance to cerebral malaria in mutant mice is associated with reduced Th1 response. We conclude that TNF-α and IFN-γ are absolutely required for the development of murine cerebral malaria. We have shown that $TNF^{-/-}$ and IFN-$\gamma^{-/-}$ mice are resistant to PbA-induced cerebral malaria, and our findings confirm the role that the Th1 cytokines play in the pathogenesis of the disease and in particular its cerebral complications.

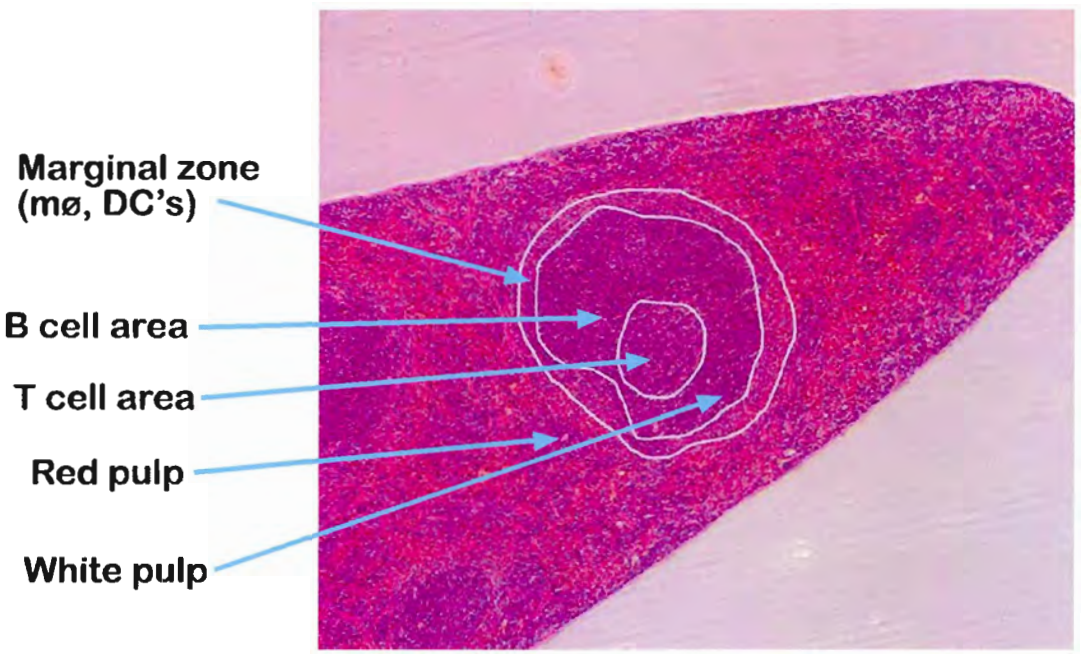

Figure 3.9 Haematoxylin and Eosin stained section from a normal (C57BL/6) spleen where the white and the red pulp are clearly separated. Note the marked B and T cell areas in the white pulp and surrounded by the well distinct marginal zone. (Original magnification: 100x.)

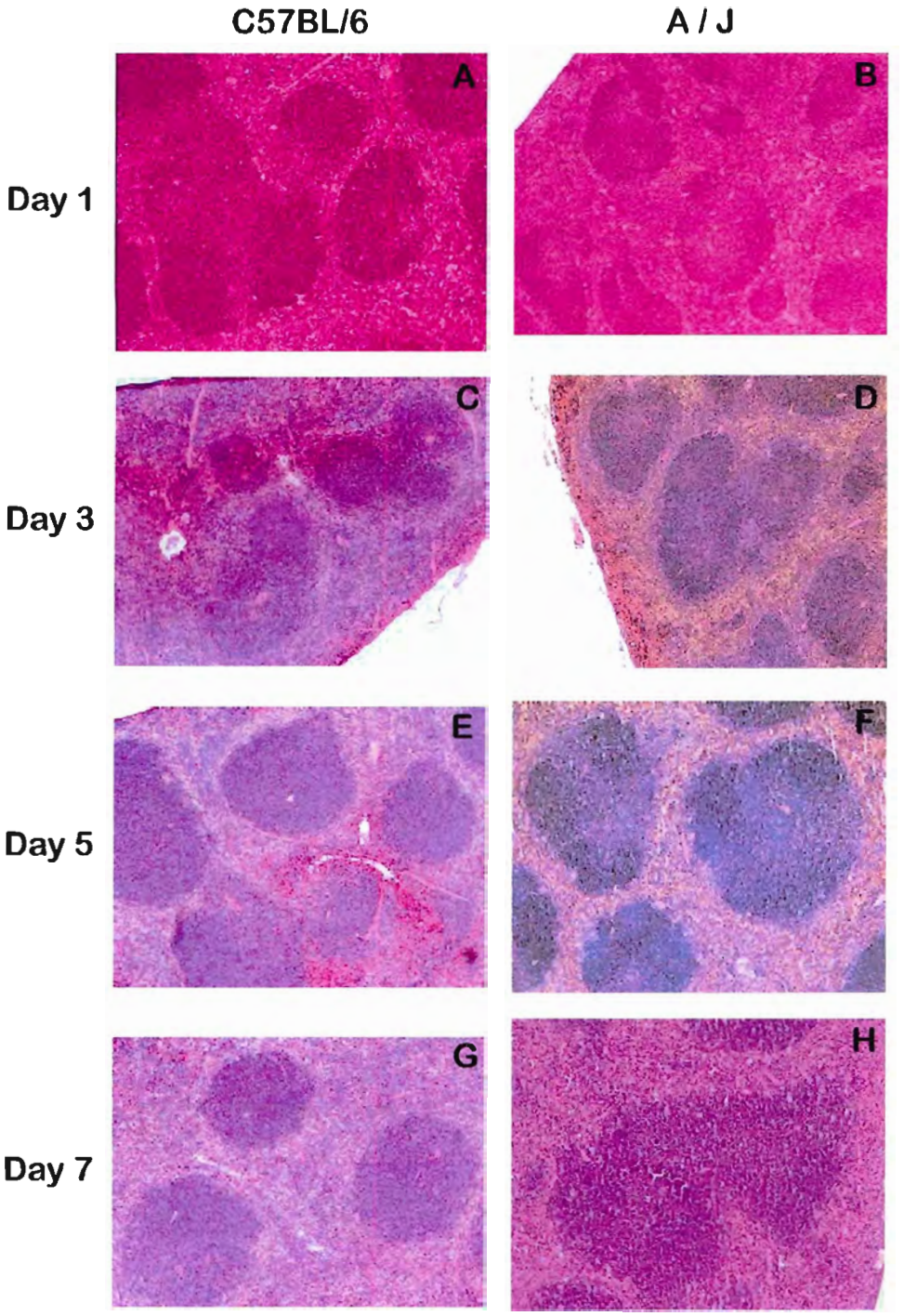

Figure 3.10. Haematoxylin and Eosin stained sections from the wild type C57BL/6 and A/J mouse strains infected with P. chabaudi AS infection and sacrificed at days 1, 3, 5 and 7 post-infection. No organizational changes on both white pulp and the red pulp during the first 4 days of infection. The marginal zone of the white pulp as well as the follicles showed no major difference in both strains. By day 5, we observed white hyperplasia and the marginal zone becomes less distinct and the red pulp becomes less nucleated. This becomes more evident by day 7. (Original magnification: 100x.)

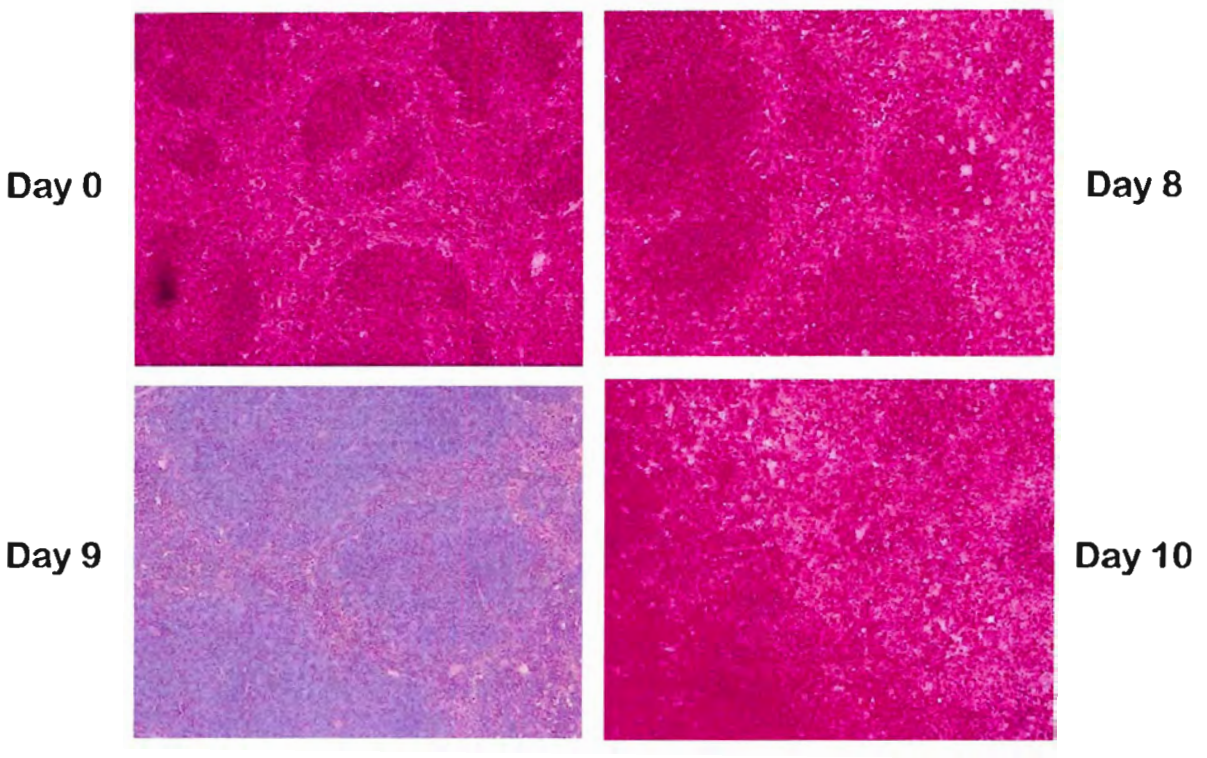

Figure 3.11. H&E stained sections from C57BL/6 mice at day 0, 8, 9 and 10. The white pulp and the red pulp are clearly separated (A), during crisis at day 8-10 post-infection (B-D), splenomegaly at this time is even more apparent with the spleen having increased 5 fold. Note that the white pulp is not longer distinguished as cells from the white pulp appear to have invaded most of the organ and there are only few red blood cells. The spleen becomes more of a homogenous mixture with some obvious dense nucleated cells and the marginal zone is not visible. (Original magnification: 100x).

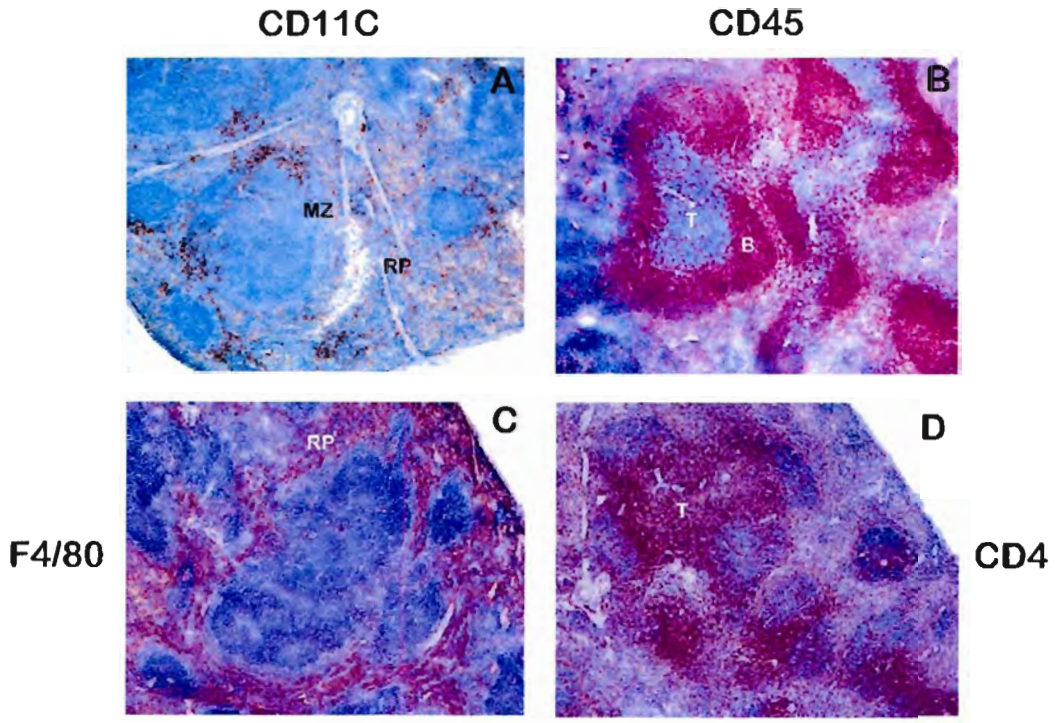

Figure 3.12 Spleen sections from normal C57BL/6 stained with antibodies specific for CD11c (dendritic-cell marker), CD45 (B-cell marker), F4/80 (macrophage marker) and CD4 (T-cell marker). Note the distribution of CD11c marker in the red pulp (RP) and marginal zone (MZ) [A], CD45 in the B-cell area (B) of the white pulp [B], F4/80 solely in the red pulp [C] and the CD4 chiefly into the T cell area (T) of the white pulp with few stained sections of the red pulp [D]. (Original magnification: 100x).

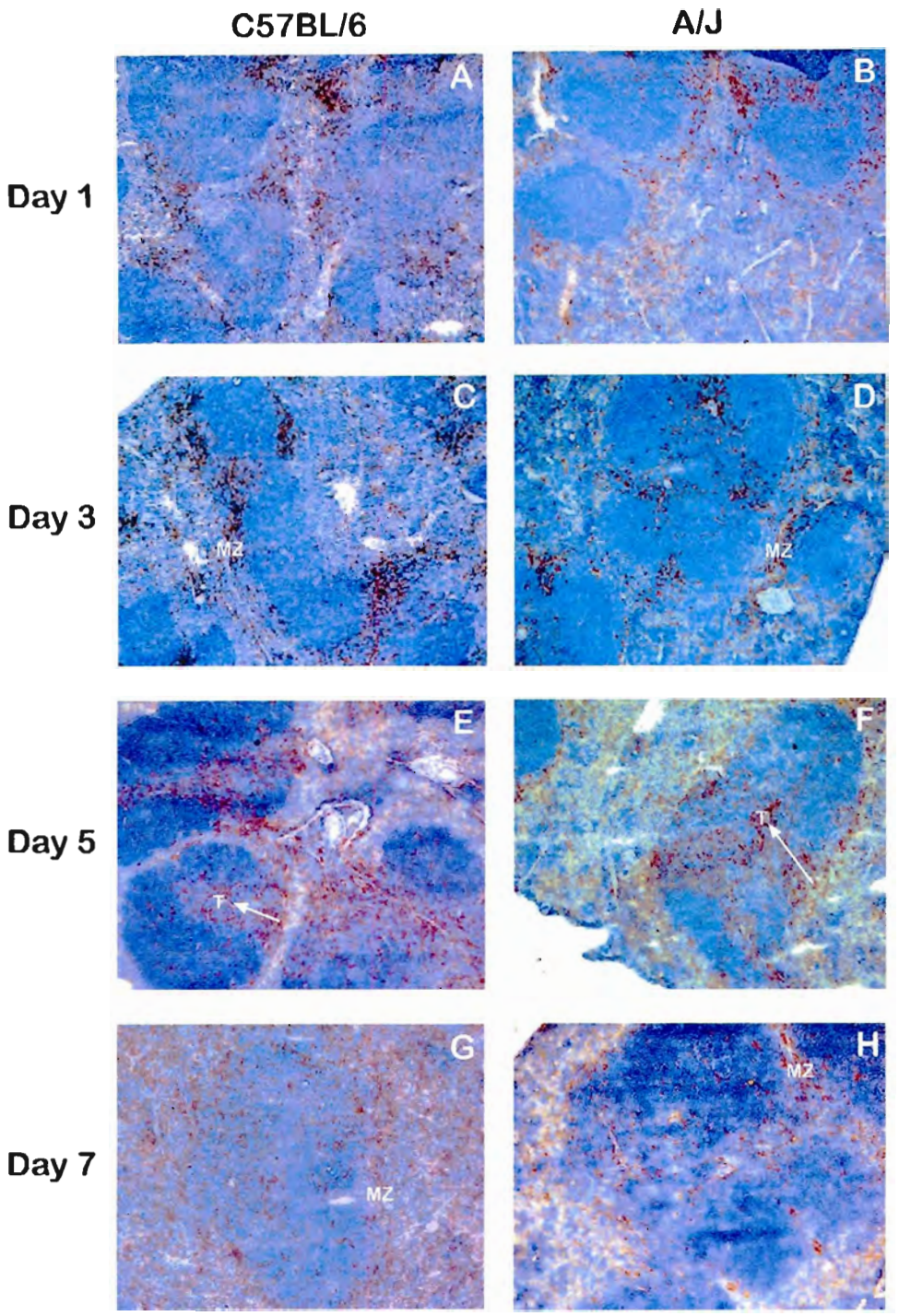

Figure 3.13. Spleen sections from the wild type C57BL/6 and A/J mouse strains infected with P. chabaudi AS infection and sacrificed at days 1, 3, 5 and 7 post-infection. Frozen sections were stained with antibody specific for CD11c (dendritic-cell marker) and note the distribution of brown stain around the marginal zone and red pulp (days 1 and 3). By day 5, note the redistribution of the CD11c positive cells from the red pulp and marginal zone to the T cell area of the white pulp. By day 7, the CD11c positive cells are distributed all over the section with very strong marginal zone staining. (Original magnification: 100x).

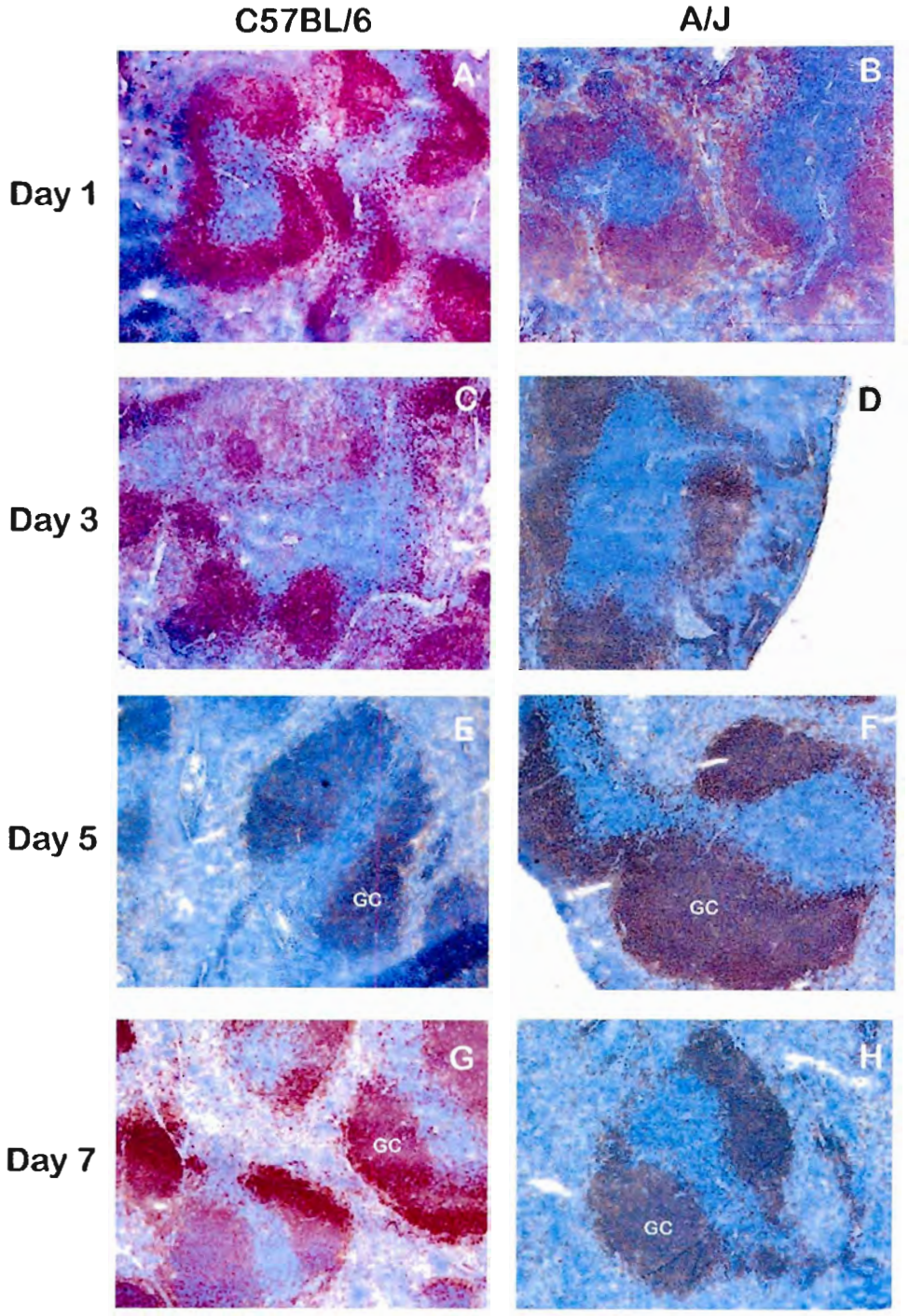

Figure 3.14. Spleen sections from the wild type C57BL/6 and A/J mouse strains infected with P. chabaudi AS infection and sacrificed at days 1, 3, 5 and 7 post-infection. Frozen sections were stained with antibody specific for CD45 (B-cell marker). Note that CD45 positive cells maintained their organization in the B-cell follicles of the white pulp throughout the infection. (Origional magnification: 100x) *GC - Germinal Centre*

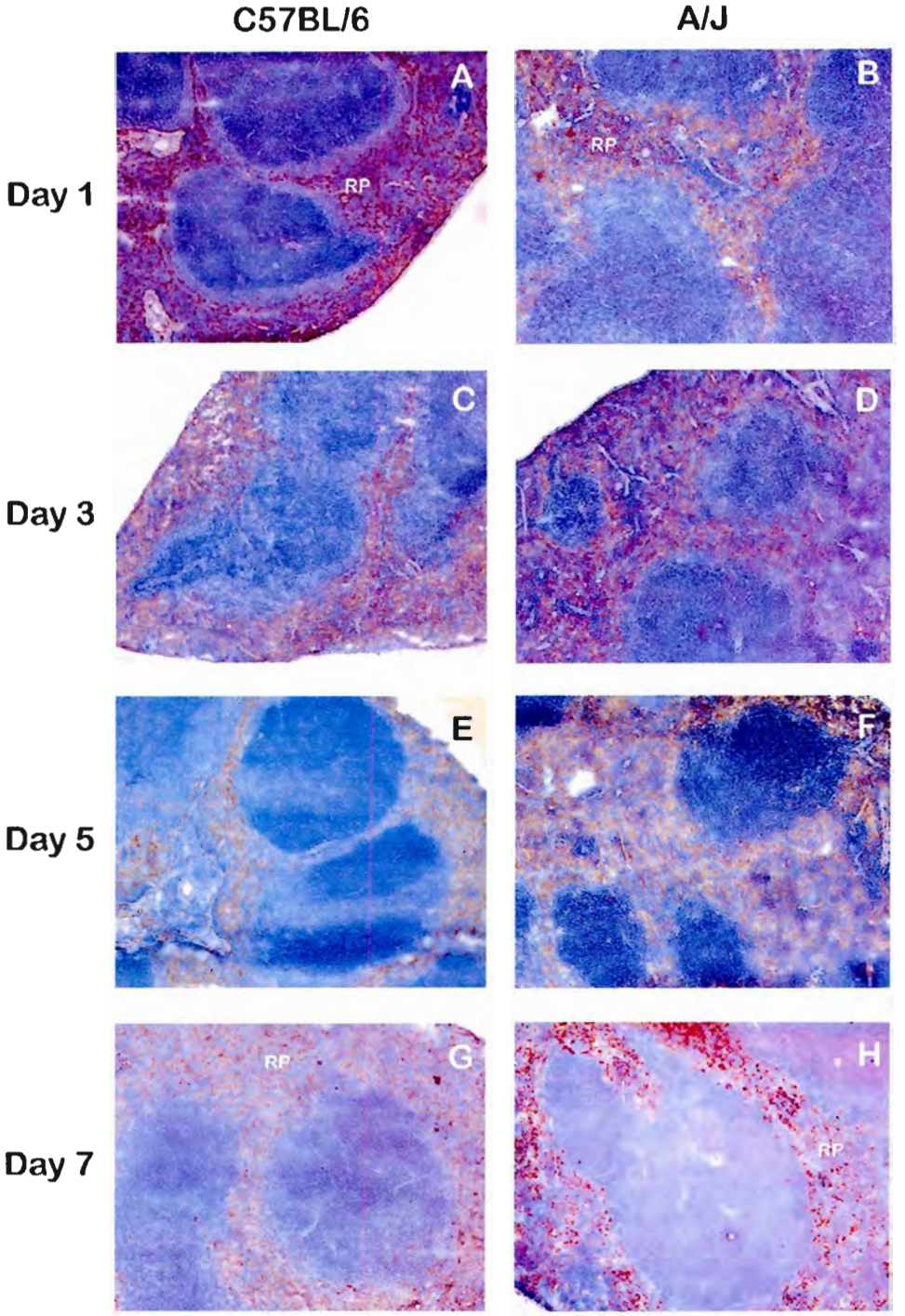

Figure 3.15. Spleen sections from the wild type C57BL/6 and A/J mouse strains infected with P. chabaudi AS infection and sacrificed at days 1, 3, 5 and 7 post-infection. Frozen sections were stained with antibody specific for F4/80 (macrophage marker). Note that F4/80 positive cells maintained their organization in the red pulp throughout the infection. (Origional magnification: 100x)

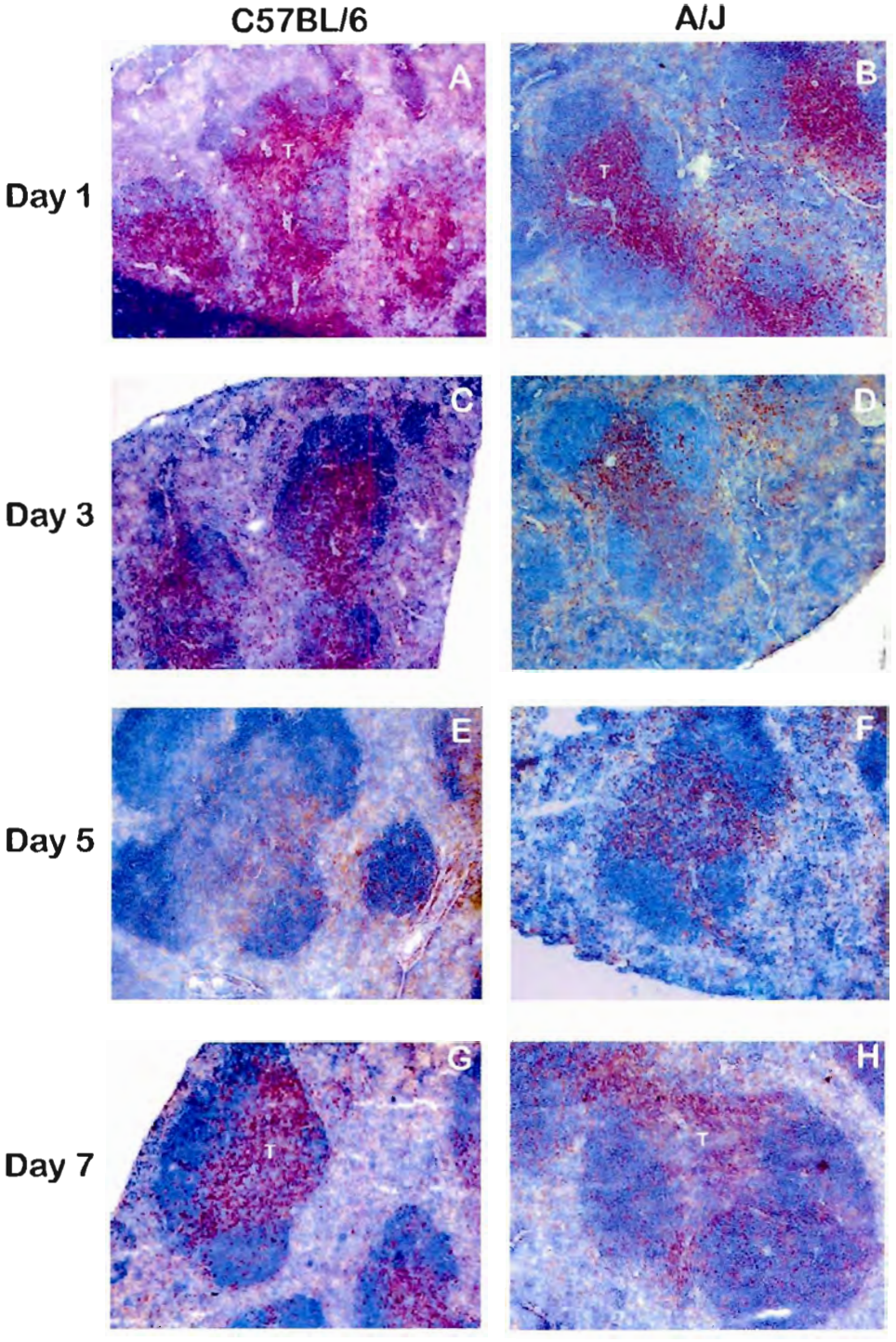

Figure 3.16. Spleen sections from the wild type C57BL/6 and A/J mouse strains infected with P. chabaudi AS infection and sacrificed at days 1, 3, 5 and 7 post-infection. Frozen sections were stained with antibody specific for CD4 (T-cell marker). Note that CD4 positive cells maintained their organization in the T-cell area (T) of the white pulp throughout the infection. (Origional magnification: 100x)

macrophages at this site in the spleen. There was an increase in this cell population in the red pulp. One striking feature was confinement of the distribution of the macrophages to the red pulp. No macrophages were detected in the white pulp including in the T-cell area of the white pulp.

3.2.5 Discussion

Several lines of evidence suggest that the spleen is an organ of major importance during malaria infection in terms of both its haematopoietic and immunological functions (Alves H *et al.*, 1986). Besides undergoing changes in size and function, the spleen is also closely linked with the initial development and maintenance of a protective immune response. Using a spleen transplant technique in a malaria rat model, it has been shown that, after primary infection, the spleen develops all the necessary elements to protect itself against reinfection (Favila-Castillo L *et al.*, 1996). Stevenson M *et al.*, 1996 demonstrated the genetic control of resistance to infection with murine malaria species, *Plasmodium chabaudi* AS. C57BL/6 mice were found to be resistant and 100% survived the malaria infection. A/J mice strains were typed as susceptible and the mean survival time was less than 10 days with death occurring as early as 8 days post-infection. The ability of the C57BL/6 to control the level of parasitaemia within the first 8-10 days of infection appears to be related to the development of splenomegaly, and hence able to eliminate the parasites and survive the malaria infection. In contrast, susceptible A/J mice, which develop only minimal splenomegaly, appear to be unable to control parasite multiplication and succumb to infection (Stevenson M *et al.*, 1989).

The complex interactions between the cells and the signals regulating expansion and changes in the composition of various cell types during this period are poorly understood. The aim of this investigation was to elucidate splenic microanatomy by histology and immunohistochemistry in non-lethal and lethal mouse model of malaria, respectively. We first noted detectable parasiteamia at around day 4 post-infection. The numbers sharply increased (ascending parasiteamia) and reached a maximum at day 8 and then rapidly declined becoming undetectable by light microscopy. When

parasites first became detectable by light microscope (approximately day 4), there were no noticeable changes in spleen histology, size differences, as indicated by cell numbers. White pulp hyperplasia was first noted on day 5 post-inoculation; this correlated with the spleen size and became more evident by day 7. Splenomegaly by day 7 became even more apparent, the spleen having increased 2-3 fold for A/J mice and 5 fold in C57BL/6 mice. This correlated with red and white pulp hyperplasia and disappearance of the marginal zone. Despite observing an apparent rapid disintegration by H&E staining, immunohistochemistry showed a surprising retention of architectural organization of the immune cells making both the red and white pulp of the spleen. What had been thought to be domination of erythropoiesis in the spleen, immunohistochemistry revealed that the spleen became more populated with lymphocytes (B and T), CD11c positive cells (dendritic cells) and macrophages. As the infection progressed, all cells labelled with CD4 (T-cells marker) and CD45 (B-cell marker) followed the same pattern of organization throughout the infection. F4/80 positive cells (macrophages) showed uniform arrangement in both A/J and C57BL/6 mice strains, distributed only in the red pulp throughout the course of infection. Only CD11c positive cells showed movement from the marginal zone of the white pulp and the red pulp into the T-cell area of the white pulp.

The different immune mechanisms through which the spleen exerts its antiparasitic functions remains ill defined. The immune response is likely to be related to the complex structure of the spleen and the peculiarity of its vascular beds and blood flow. Two possible effector sites are the marginal zone and the red pulp, where macrophages and T-cells can act as antiparasitic immune effectors (Yadava A *et al.*, 1996). The position of the T-cell area in and around the follicles of the white pulp could also be the site of immune complexes. In this study, the changing characteristics and localization of immune cells in the hyperplasic white pulp and the red pulp have been traced. This is where immune complexes would take place. Importantly, it was found that CD11c (dendritic cells) moved from the marginal zone to the T-cell area of the white pulp. This juxtaposition of CD11c (dendritic cells) positive cells and the apparent T cells, and to some extent the B cells, in the white pulp might be associated with immune complexes developing in the spleen during malaria infection. Activation of T-cells by antigen-presenting cells is required to initiate specific immune responses. Dendritic cells are a unique type of antigen presenting cells because of their ability to induce primary immune response by

efficient activation of T-cells (Banchereau J *et al.*, 1998). It is possible that in our experimental model system, following activation of the dendritic cells and antigen capture in the marginal zone, they migrate to the lymphoid organs where they prime antigen-specific $CD4^+$ T cells.

The second significant observation in our histological study was the distribution of macrophages in the spleen during the course of the infection. No macrophages were observed migrating into the T-cell area of the white pulp.

Following systemic *Plasmodium chabaudi chabaudi* AS infection in mice, there are a number of striking changes that occur in the nature and architecture of the splenic cellular immune responses that ultimately lead to control of this infection. We were not able to analyse the production of cytokines in our model. The initial host response to a non-lethal blood stage infection with malaria parasites in mice is characterized by the production of interferon-gamma (IFN-γ) and tumour necrosis factor (TNF-α). These cytokines are detectable in infected animals within the first week and after infection with P.c.c (Langhorne *et al.*, 1990; Jacobs P *et al.*, 1996) or *Plasmodium yoelii* blood stage parasites (de Sousa B *et al.*, 1997) and are thought to be important for the development of an effective immune response to blood stage parasites. They may also play a role in severe malaria in humans and in the cerebral and other pathology associated with infections in mice (Kwiatkowski D *et al.*, 1990). Blood stage parasites activate dendritic cells (DC) to produce IL-12, a cytokine known to play an important and crucial role in the initiation and regulation of IFN-γ production by NK cells and $CD4^+$ T cells (Macatonia S *et al.*, 1995; Heufler C *et al.*, 1996). In experimental malaria infections, serum IL-12 levels are already elevated within 2 days after infection in resistant strains of mice (Mohan K *et al.*, 1998). Administration of rIL-12 can protect susceptible A/J mice against *P.c.chabaudi* infections and this protection operates via mechanisms dependent on IFN-γ and TNF-α and nitric oxide (Stevenson M *et al.*, 1995).

In the present study, the T cell area of the white pulp was initially found to be devoid of dendritic cells. It would appear that the dendritic cells play a major role in the antigen presentation to naïve T-cells of the white pulp. This is in agreement with the *in vitro* findings of von Stebut *et al.*, 1998 who showed that DC's rather than

macrophages, initiate the protective T cell response to other protozoan infection, *Leishmania major* in this regard. Indeed, a large body of evidence now indicates that antigen presentation by DC's, which are present in splenic periarteriolar lymphoid sheath (PALS) and marginal zone, is responsible for the initiation of a primary T cell response (Banchereau J *et al.*, 1998). Once activated, the T-cells are driven towards protective Th1 phenotype by the local production of IL-12, initially within the marginal zone and later diffusely throughout the spleen. We have shown that over time, the T cells and dendritic cells are distributed throughout the white pulp. This co-migration of T-cells and antigen presenting cells maybe one way that effector T-cells are able to overcome the infection in the mouse model system. Although we were not able to perform double staining as well as triple staining experiments for co-localization of T cells, dendritic cells and IL-12, we have reason to believe that dendritic cells were the chief IL-12 producing cells (Leisewitz *et al.*, unpublished data). Further work is necessary to elucidate the role of cytokines signals involved in these changes in cellular architecture in our mouse model systems.

CHAPTER 4

4.1 Oral artesunate prevents *Plasmodium berghei* ANKA infections in mice

Introduction

Artemisinin (Qinghaosu) is an anti-malarial drug isolated from the Chinese herb *Artemisia Annua* (Qinghao). Because of the favourable properties, its chemically modified derivatives, such as dihydroartemisinin and sodium artesunate, are extensively investigated for their activity (Dhingra V *et al.*, 2000). The compound has low toxicity and has been shown to be highly potent and rapidly metabolised (Wang T *et al.*, 1985). However, the recrudescence rate with this treatment is high. Different approaches have been used to alter the structure of artemisinin in order to increase its efficacy. For example, the drug has been modified to make it more lipid soluble, leading to the compound artemether, and more water soluble, resulting in the compounds sodium artesunate and sodium artelinate (Klayman D *et al.*, 1991).
The various pharmaceutical forms of artemisinin and its derivatives that have been investigated include tablets, sustained-release tablets, oil and water suspensions, aqueous intravenous preparations, and suppositories (Klayman D *et al.*, 1991). They are rapidly effective and produce faster resolution of parasitaemia and fever than all other compounds (Xuan W *et al.*, 1988). Although knowledge of their pharmacokinetic properties is incomplete, it is apparent that artemisinin and its derivatives (artesunate, artemether) are all metabolised in humans to a common biologically active metabolite (dihydroartemisinin), which is cleared rapidly from the circulation (Newton P *et al.*, 2000). Thus the duration of anti-malarial activity is short. When these drugs are used alone particularly for short courses of 3-5 days, recrudescence rates are high. Despite their excellent intrinsic anti-malarial activity, drug exposure of the infecting parasite population for only two or three life cycles is insufficient to eradicate all the malaria parasites in the body.

In the present study, artesunate, a derivative of a natural occurring anti-malarial artemisinin was compared with chloroquine in murine *Plasmodium berghei* Anka (PbA) infection. A 7-day administration of artesunate prevented parasitaemia at >= 10 mg/kg. However, recrudescence of parasitaemia and cerebral malaria occurred upon

cessation of treatment followed by death within 28 days. A 14-day course of artesunate treatment prevented completely the development of parasitaemia and cerebral malaria with a survival of more than 60-days at 100-mg/kg artesunate or 10-mg/kg chloroquine. This data demonstrate that oral artesunate inhibits PbA and prevents cerebral malaria, but needs to be administered for prolonged time and at higher doses than chloroquine to eradicate PbA infection.

Results

4.1.1 Short-term artesunate retards the development of parasitaemia

Control mice receiving only the vehicle or 1 mg/kg artesunate died within 10 days of acute cerebral malaria (Fig. 4.1.1). Mice given artesunate at 10 and 100 mg/kg had a prolonged survival over control mice ($P<0.05$). Parasitaemia in the 10 and 100 mg/kg artesunate treated mice was completely suppressed from day 1 through 9, and recrudesced in all of them by day 10. A steady increase in parasitaemia (Fig. 4.1.2) occurred by day 14 and the mice were all dead by day 28. By contrast, mice treated with 10-mg/kg chloroquine were cured and no toxicity was detected in these mice. Importantly, no recrudescence was observed after the first negative parasitaemia in the chloroquine group. This was also evident by the body weight gain in mice treated with chloroquine ($P<0.05$) as compared to animals treated with artesunate, which succumbed to infection (Fig. 4.1.3).

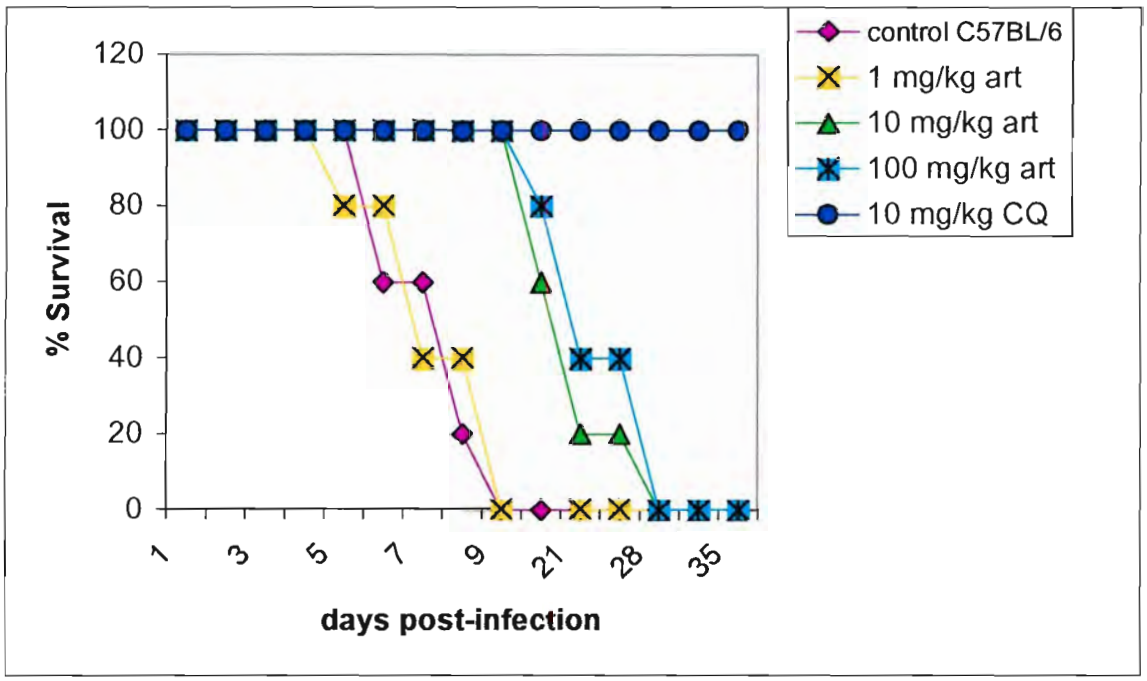

Figure 4.1.1 Survival of artesunate and chloroquine treated mice (evaluated using the 7-day suppressive test) upon PbA infection. The results are from a single experiments (n=5) representative of three independent experiments

4.1.2 Prolonged artesunate administration prevents cerebral malaria and eradicates the parasite

Artesunate at 1mg/kg, even when administered for 14 consecutive days, was ineffective and the mice died as control mice of cerebral malaria (Fig. 4.1.4) with a rapid increase parasitaemia between day 5 and 7 (Figure 4.1.5) and development of cerebral malaria. By contrast, the higher doses of 10 and 100-mg/kg artesunate respectively given for 14 days were effective in preventing parasitaemia. The 10-mg/kg dose of artesunate, however, did not eradicate the parasites completely, and the mice exhibited a recrudescence 30 days post-infection and succumbed after day 42 with a high parasitaemia as high as 63%, but no signs of cerebral malaria (Fig. 4.1.5). By contrast, the 100-mg/kg-artesunate dose killed the parasites and was as effective as chloroquine (10 mg/kg). No relapse of PbA infection occurred in those mice by day 85. Furthermore, the prolonged drug administration at the high dose did not cause any signs of toxicity. In support of the excellent tolerability of artesunate, was absence of body weight loss and of any drug related morphological changes during the post-mortem examination of the major organs (data not shown).

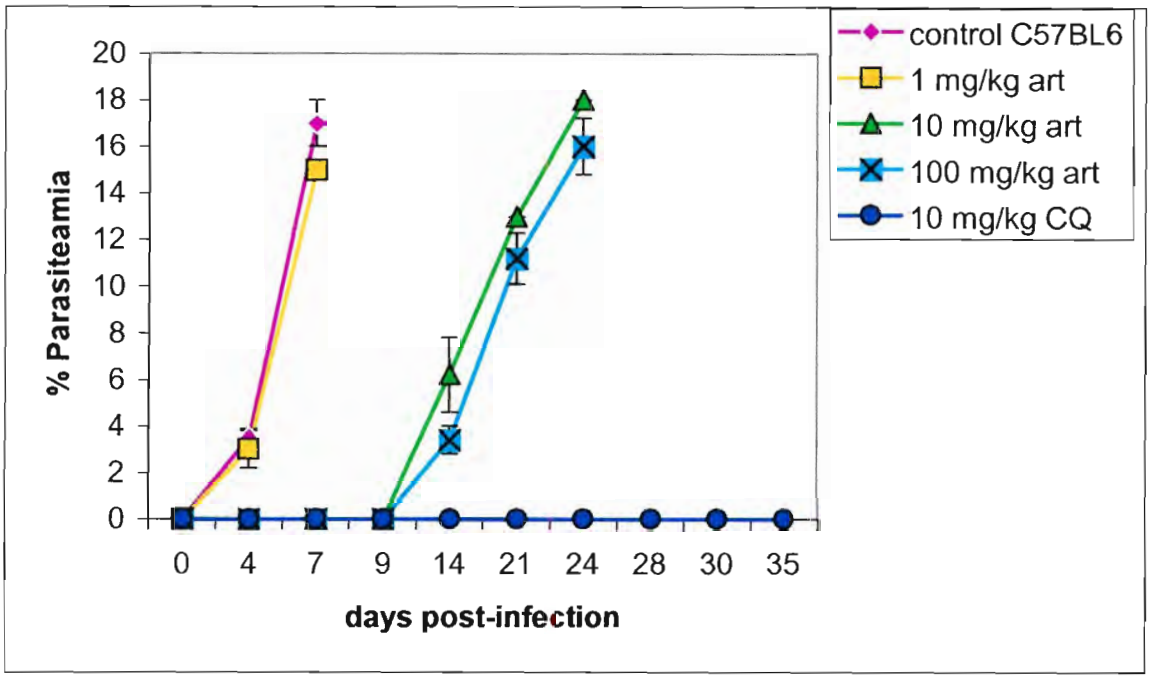

Figure 4.1.2 Parasitaemia in artesunate and chloroquine treated mice (evaluated by a 7-day suppressive test). The percentage of parasitized erythrocytes was determined from 200 erythrocytes; mean values ± SD (n=5) are shown, from a single experiment, representative of three independent experiments.

4.1.3 Artesunate and chloroquine prevent the development cerebral malaria

We further examined whether drug administration influenced PbA-induced cerebral malaria in mice. Untreated mice developed the neurological symptoms of cerebral malaria within 5 to 7 days after infection. The neurological alterations reached a mean score of 2.5 at day 6 and included abnormal postural responses and reduced reflexes as well as grip strength. The control mice died quite suddenly with signs of cerebral malaria between 6 to 8 days. Fig. 4.1.6 demonstrates the clinical severity of cerebral malaria in untreated control in mice and mice treated with the non-protective dose of 1 mg/kg artesunate. Cerebral malaria commenced around day 5 after injection of PbA-infected erythrocytes and was not affected by the 1-mg/kg dose of artesunate. By contrast, 10 and 100 mg/kg artesunate prevented the development of cerebral malaria, as did the administration of chloroquine $P<0.05$). Interestingly, the mice succumbing following a 14 days administration of 10-mg/kg artesunate died with parasitaemia of about 60% (Figure 4.15) without signs of cerebral malaria (Fig. 4.1.6).

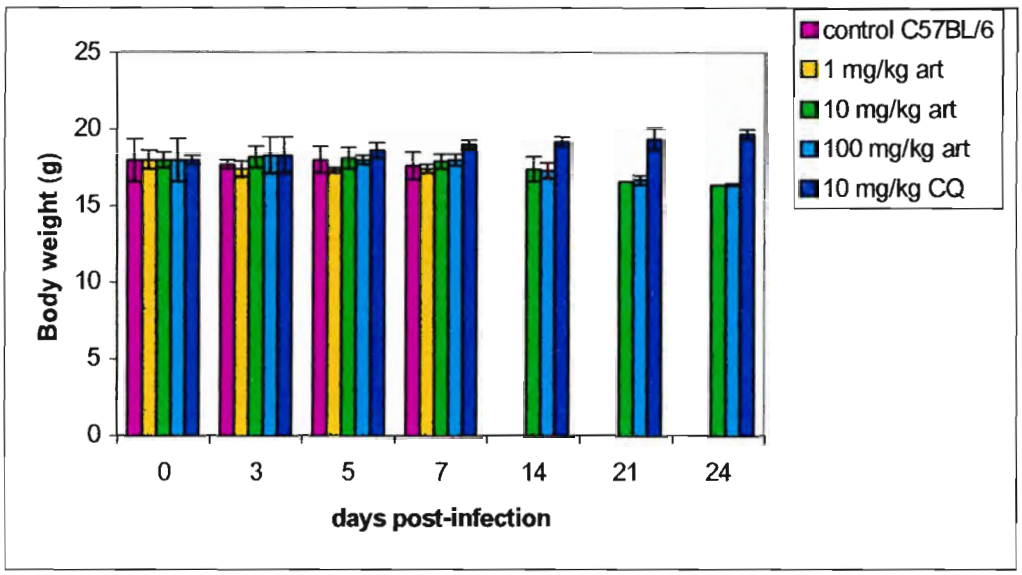

Figure 4.1.3 Body weight determination of artesunate and chloroquine treated mice evaluated using a 7-day suppressive test, upon PbA infection. The results are from single experiment (n=5) representative of three independent experiments.

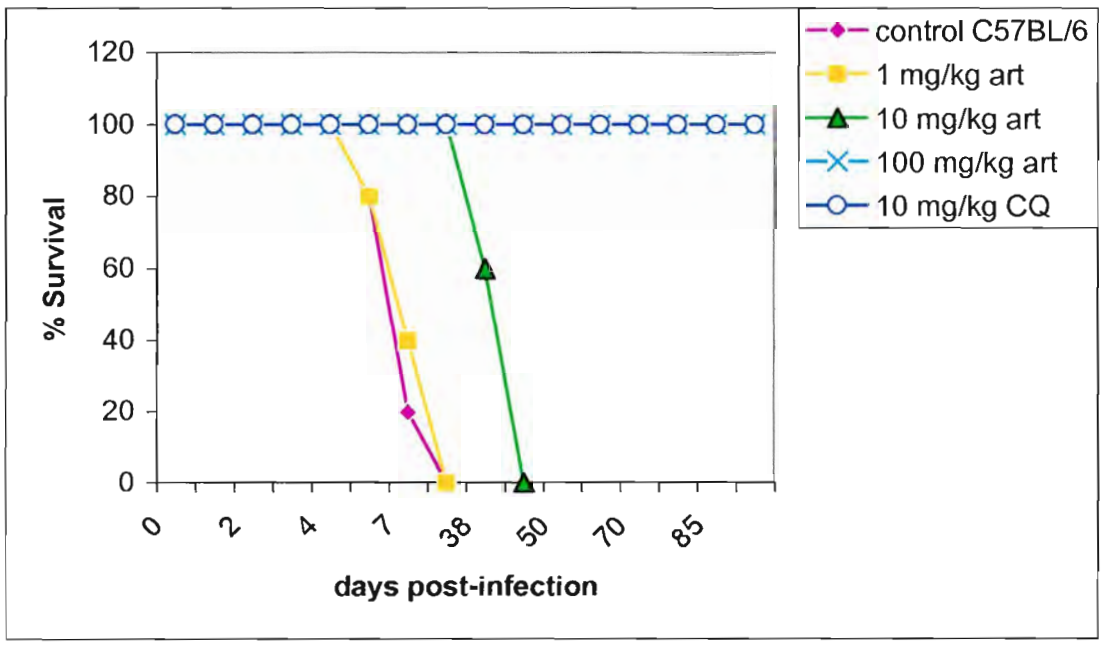

Figure 4.1.4 Survival of artesunate and chloroquine treated mice upon PbA infection evaluated using a 14-day suppressive test. The results are from a single experiments (n=5) representative of three independent experiments

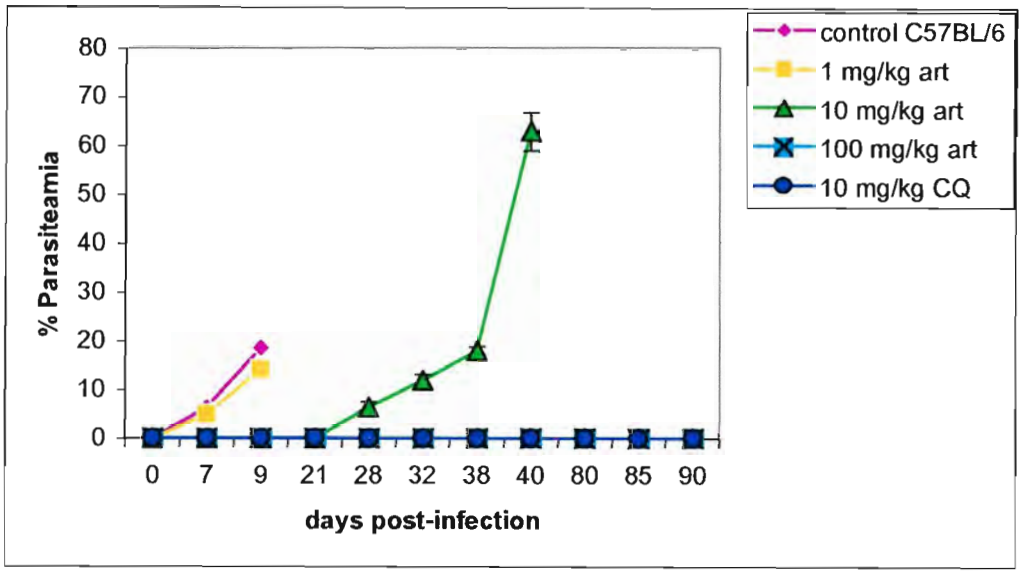

Figure 4.1.5 Parasitaemia in artesunate and chloroquine treated mice evaluated by a 14-day suppressive test. The percentage of parasitized erythrocytes was determined from 200 erythrocytes; mean values ± SD (n=5) are shown, from a single experiment, representative of three independent experiments.

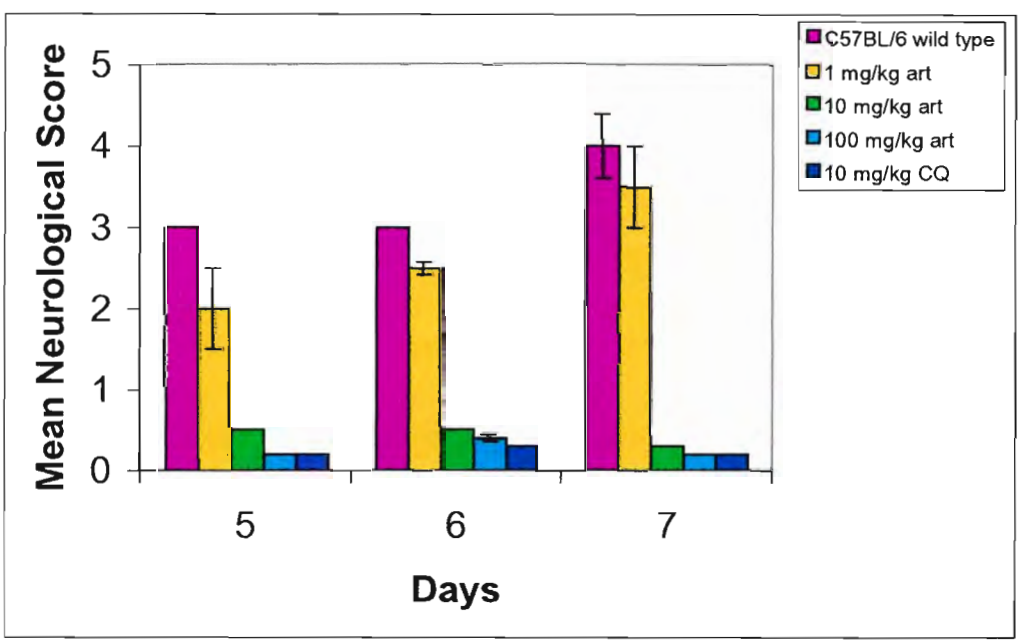

Figure 4.1.6 CM in PbA-infected wild type and 1 mg/kg artesunate treated mice. Semi-quantitative assessment of neurological symptoms of CM. PbA-infected mice were assessed by a neurological score as described. The results are from a single experiment representative of three independent experiments.

4.1.4 Discussion

In the present study, we compared the efficacy of oral artesunate with that of chloroquine in *Plasmodium berghei Anka* infected mice. We confirm that artesunate has potent anti-malarial activity in this murine infection by oral administration, which is comparable to that of chloroquine. Artesunate given for 14 days at 10 and 100 mg/kg was effective in preventing parasites from developing. Mice treated given 10-mg/kg artesunate, however developed recrudescence of parasitaemia after 30 days and most of them succumbed to the infection by about 40 days with a high parasitaemia as high as 60% (as shown in Figure 4.1.5). Both, oral artesunate (100 mg/kg) and chloroquine (10 mg/kg) were able to eradicate PbA infection. Recrudescence did not occur in treated mice at those doses.

In clinical studies, artesunate has been associated with high recrudescence rates (Karbwang J *et al.,* 1994). The maximum dose of oral artesunate used in those studies was 600 mg given over 5 days, which was followed by a recrudescence rate of 10-28% with uncomplicated *P. falciparum* malaria infection (Karbwang *et al.,* 1994).

In the present experimental study, no toxicity was observed at a total dose of artesunate of 1400 mg/kg. In dogs, neurotoxicity was observed after a high dose (400-800 mg/kg) of artemisinin, but the effect was transient and disappeared within 48 hours (Trigg P *et al.,* 1984). Microscopic investigations of the brains of mice receiving the high artesunate dose however did not reveal any morphological alterations. Artesunate is readily hydrolysed to dihydroartemisinin (DHA), probably by blood esterases and the hepatic cytochrome P450 $3A_4$, as is the case with closely related compounds artelinic acid and arteether (Grace J *et al.,* 1999). Artemether is also probably biotransformed by intestinal cytochrome P450 $3A_4$. If artesunate is similarly metabolised, interindividual variability in intestinal P450 $3A_4$ activity may be important in determining bioavailability. The majority of the antimalarial activity can then be explained by DHA, which is cleared predominantly by hepatic biotransformation either to biologically inert glucoronides (such as DHA-glucoronide) or to metabolites which lack the peroxidase bridge necessary for antimalarial activity (Meshnick S *et al.,* 1996). Thus the duration for antimalarial activity is short. Artesunate and artemether and their active metabolite DHA are extremely potent antimalarials with an in vitro range of 50% effective drug concentrations (IC50) of

between 0.1 and 20 nmol/L (mean 2.7) for artemether and 0.1 and 0.15 nmol/L for DHA. Artemether, artesunate and DHA have a broader spectrum of antimalarial activity than any of the other antimalarial drugs (Shmularski M *et al.,* 1993, Basco L *et al.,* 1993). They kill both schizonts and gametocytes. In experimental malaria and clinical infection, cerebral malaria is associated with elevated blood tumour necrosis factor (TNF) levels (Grace G *et al.,* 1987, Rudin W *et al.,* 1997). The administration of neutralising antibodies and of inhibitors of TNF synthesis prevents cerebral malaria in mice (Clark I *et al.,* 1992, Garcia *et al.,* 1995). In patients, TNF antibodies reduce fever of clinical cerebral malaria (Kwiatkowski *et al.,* 1993). Chloroquine and artesunate treated mice were protected against PbA-induced cerebral malaria. It is likely that the effect of these drugs, especially artesunate might be associated with their affect on cytokine synthesis such as TNF associated with cerebral malaria.

In conclusion, short course artesunate is not efficacious. However, a 14 days oral administration of artesunate eradicates PbA infection in mice and prevents the development of cerebral malaria, which is equivalent to the chloroquine effect. Therefore, prolonged administration of oral artesunate is an effective alternative treatment of chloroquine resistant malaria.

Effect of artesunate and chloroquine on cytokine production

4.2 Effect of chloroquine and artesunate on the production of cytokines

Introduction

The efficacy of both chloroquine and artesunate in malaria has been demonstrated in clinical practice. Clinicians working in areas of chloroquine resistance have observed that the clinical response after treatment with sulphadoxine-pyrimethamine may show complete anti-parasite efficacy but is less efficient in abrogating the clinical symptoms as compared with chloroquine (Bojang K *et al.,* 1998). This suggests that the beneficial effects of chloroquine may include pharmacological activity other than anti-parasitic.

Cytokines interact via a network and play a vital role in various types of intercellular communication. The excessive production of distinct pro-inflammatory cytokines is important in the pathogenesis of malaria (Jacobs P *et al.,* 1996; Waki S *et al.,* 1992). In particular, high levels of tumour necrosis factor (TNF)-α correlate with severity of human *Plasmodium falciparum* malaria, for example, in severe anaemia (Clark I *et al.,* 1988; Taverne J *et al.,* 1994) and cerebral malaria (Grau G *et al.,* 1987; Grau G *et al.,* 1989; Kwiatkowski D *et al.,* 1990). A means to elucidate the mechanism of a particular drug is to study its effect on cytokine production. In the literature it is suggested that chloroquine exert different effects on cytokine production. Several *in vitro* studies have shown that chloroquine inhibits the production of pro-inflammatory cytokines such as TNF-α, interferon-gamma (IFN-γ) and interleukin 6 (IL-6) (Picot S *et al.,* 1993; van der Borne B *et al.,* 1997; Landewe R *et al.,* 1992; Karres I *et al.,* 1998; Jeong J *et al.,* 1997). Chloroquine also reduces the proliferative responses to antigen and mitogen, possibly explained by impaired antigen processing and IL-2 production [Landewe *et al.,* 1995]. Artesunate is one of the derivatives of artemisinin isolated from the Chinese herb *Artemisia annua*. It has been found to be a much safer and more potent than chloroquine, and is also effective on chloroquine-resistant malaria (Klayman D *et al.,* 1985). In addition to their antimalarial effect, these compounds were recently reported to modulate immune responses. Experiments *in vivo* and *ex vivo* have shown that sheep red blood cells (SRBC)-driven antibody-forming cell formation, lymphocyte proliferation, and delayed-type hypersensitivity (DTH) were significantly inhibited by artemisinin (in a range from 100 to 600-mg/kg,

ip in mice) and dihydroartemisinin (100-mg/kg, ip in mice) (Lin X *et al.,* 1984, Sun X *et al.,* 1991, Tawfik A *et al.,* 1990). Sodium artesunate, a water-soluble derivative of artemisinin, markedly enhanced anti-SRBC antibody formation when given to mice intraperitoneally at dose of 50-mg/kg daily for 5 days (Chen M *et al.,* 1988). On the other hand, Tawfik *et al.,* 1990, showed that artemisinin and its two derivatives, dihydroartemisinin and arteether, exhibited some suppression of humoral response at the doses ranging from 400 to 600-mg/kg. With these contradictory reports, we hypothesise that these compounds may stimulate the immune response at lower doses, but suppress immune response at higher doses. It is well established that the balance between functionally distinct cytokine-producing T cells of the CD4 and CD8 phenotypes, determines whether an individual will succumb to the disease or not [Abbas A *et al.,* 1996]. These cell types are distinguished by their capacity to regulate pro-inflammatory and anti-inflammatory responses. In pro-inflammatory responses, IFN-g production plays an important role by attracting and stimulating cells of the immune system to kill the parasites [Stevenson M *et al.,* 1995]. On the other hand, IL-10 is a critical component in the down-regulation of the pro-inflammatory immune reactions and the upregulation of the anti-inflammatory responses [Song S *et al.,* 1997; Ding L *et al.,* 1993; Mosmann T *et al.,* 1991].

The purpose of this study was to compare the effects of chloroquine and artesunate in identical *in vitro* conditions with respect to the production of TNF-α, IFN-γ, IL-10 and IL-12. These cytokines are thought to play an important role in the pathogenesis of severe malaria. We compared the effects of artesunate and chloroquine on stimulated splenocytes with respect to cytokine production. We tested for interleukin 12 (IL-12p40), interleukin 10 (IL-10), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) by specific ELISA, after stimulation with lipopolysacharide (LPS) and Concanavalin A (ConA) in the presence or absence of different concentrations of artesunate and chloroquine. We observed a concentration dependent inhibition of LPS induced TNF-α and IL-12p40, and ConA induced IFN-γ by chloroquine. Chloroquine increased the production of ConA induced IL-10 at 10 μM concentration. In contrast, artesunate lacked an inhibitory effect of LPS induced TNF-α and IL-12p40 and ConA induced IFN-γ at conventional doses. We observed a significant increase of IL-10 production upon treatment with artesunate at 10-μM concentrations. Our findings indicate the anti-inflammatory effect of chloroquine by

inhibiting the production of Th1 cytokines and inducing the production of IL-10. Artesunate lacked the inhibitory effect that chloroquine possesses. ConA induced IL-10 was affected by artesunate. These results suggest that chloroquine affects the balance between the pro-and anti-inflammatory cytokines, rather than by general inhibition of cytokine production. These observations might help in understanding the mechanism of action in these drugs in cerebral malaria.

Results

4.2.1 Tumour necrosis factor-alpha (TNF-α) production

From our set of experiments, the maximum production of TNF-α was reached after 24 hours of stimulation with lipopolysacharide (LPS) at 10 μg/ml. No production of this cytokine could be detected in cultures without addition of LPS. Addition of different concentrations of artesunate and chloroquine to the cultured splenocytes stimulated by LPS showed concentration dependent inhibition of TNF-α production (Figure 4.2.1). The most significant inhibition of TNF-α production in chloroquine treated splenocytes was reached over a concentration of 10 μM ($p=0.0304$). At the highest concentration of 90 μM ($p=0.0080$), chloroquine almost completely abrogated this response, reducing TNF-α in response to LPS to negligible levels. No comparable effect on TNF-a production by splenocytes was observed with artesunate, even at higher doses (Figure 4.2.1). To determine whether or not the inhibition of TNF-α production after stimulation with LPS was caused by toxicity of the drugs to the cytokine producing cells, trypan blue staining was done. This showed no increase in cell death on culturing in the presence of either chloroquine or artesunate. The observed inhibition of TNF-α production cannot be explained by cell toxicity.

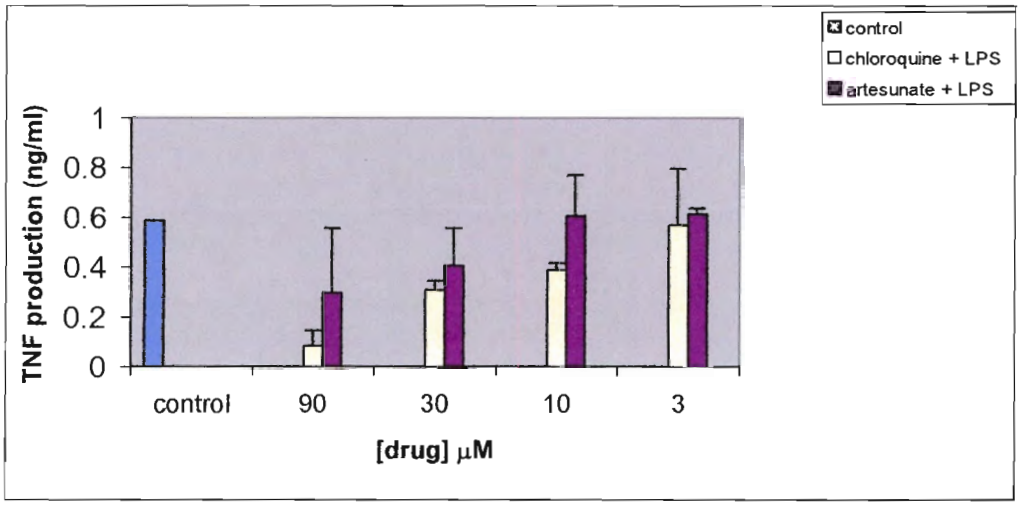

Figure 4.2.1 The effects of a concentration range of chloroquine and artesunate on LPS induced TNF-α production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

4.2.2 Interferon-gamma IFN-γ production

Maximum production of IFN-γ by splenocytes stimulated with Concanavalin A (ConA) was reached after 48 hours. There was no production of this cytokine in our culture without addition of ConA. Addition of different concentrations of artesunate and chloroquine to the cultured splenocytes stimulated with ConA showed concentration dependent inhibition of IFN-γ production (Figure 4.2.2). The most significant inhibition of IFN-γ production in chloroquine treated splenocytes was reached over a concentration range of 3-90 μM ($p<0.05$). At the highest concentration, chloroquine almost completely abrogated this response, reducing IFN-γ in response to ConA to almost zero levels. In contrast to the inhibiting effects on ConA stimulated IFN-γ production by chloroquine, artesunate lacked the inhibitory effect of IFN-γ at concentration range of 3 and 30 μM. There was a slight insignificant inhibitory effect of artesunate on IFN-γ production that was observed at 30 μM ($p=0.342$) concentration. In artesunate treated splenocytes, the most significant inhibition was observed at 90 μM ($p=0.0138$) concentration range (Figure 4.2.2). To determine whether the inhibition of IFN-γ production after stimulation

with ConA was caused by toxicity of the drugs to the cytokine producing cells, trypan blue staining was done. This showed no increase in cell death upon culturing in the presence of either chloroquine or artesunate. This indicates that the observed inhibition of IFN-γ production cannot be explained by cell toxicity.

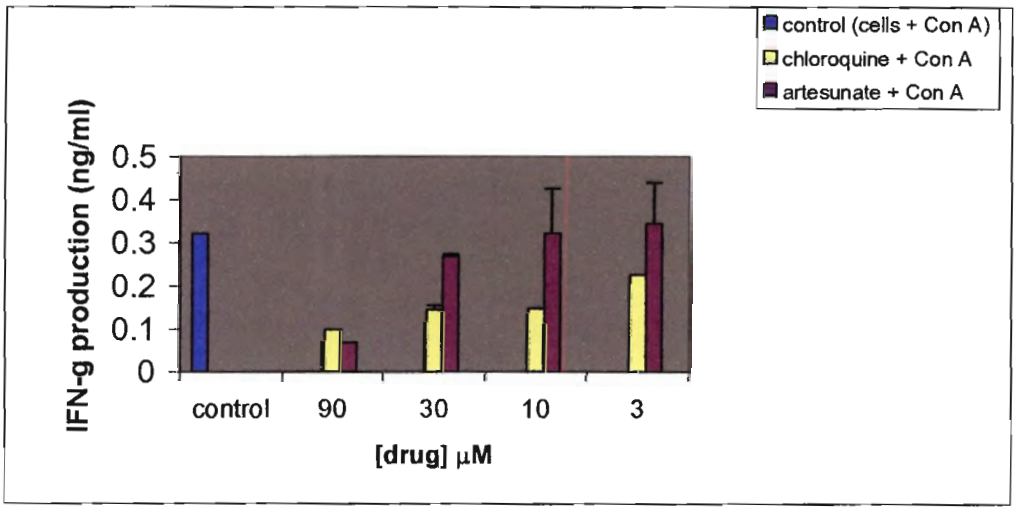

Figure 4.2.2 The effects of a concentration range of chloroquine and artesunate on Con A induced IFN-γ production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

4.2.3 IL-12p40 production

Maximum production of IL-12p40 by splenocytes stimulated with lipopolysacharide (LPS) was reached after 48 hours. There was no production of this cytokine without addition of LPS. Addition of different concentrations of artesunate and chloroquine to the cultured splenocytes stimulated with LPS showed concentration dependent inhibition of IL-12p40 production (Figure 4.2.3). The most significant inhibition of IL-12p40 production in chloroquine treated splenocytes was reached over a concentration range of 10 μM (p=0.0295) and 30 μM (p=0.0244). At the highest concentration (90 μM), chloroquine almost completely abrogated this response, reducing IL-12p40 in response to LPS to almost half that of the control levels. In

contrast to the inhibiting effects on LPS stimulated IL-12p40 production caused by chloroquine, artesunate lacked any inhibitory effect of over the concentration range of 10-30 μM. In contrast, the most significant proliferative effect of artesunate on IL-12p40 production was observed at 3 μM ($p=0.0046$) concentration (Figure 4.2.3). By trypan blue staining it was shown that there was no increase in cell death upon culturing in the presence of either chloroquine or artesunate. The observed inhibition of IL-12p40 production cannot be explained by direct cell toxicity.

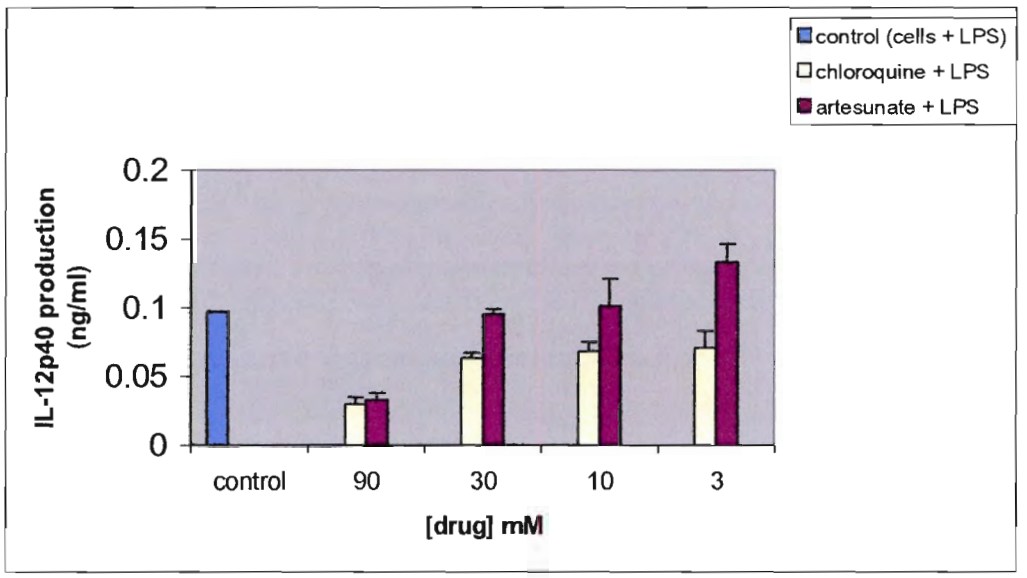

Figure 4.2.3 The effects of a concentration range of chloroquine and artesunate on LPS induced IL-12p40 production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

4.2.4 IL-10 production

Maximum production of IL-10 by splenocytes stimulated with Concanavalin A (ConA) was reached after 24 hours. There was no production of this cytokine without addition of a mitogen. In the presence of chloroquine, an enhanced IL-10 production was found, additive to the mitogen stimulation (Figure 4.2.4). There was no significant effect of chloroquine at 3 μM on IL-10 production. The enhanced additive

effect was observed at 10 μM (p=0.05), whereas at the highest concentration, chloroquine almost completely abrogated this response, reducing IL-10 release in response to ConA to almost half the control levels. Artesunate also showed no effect on IL-10 production at low concentration (3 μM) as compared to a significant enhanced effect, additively to the mitogen stimulation at 10 μM (p=0.0042) (Figure 4.2.4). At the highest concentration, artesunate almost completely abrogated this response, reducing IL-10 release in response to ConA to almost zero (Figure 4). By trypan blue staining, it was shown that the observed inhibition of IL-10 production cannot be explained by cell toxicity.

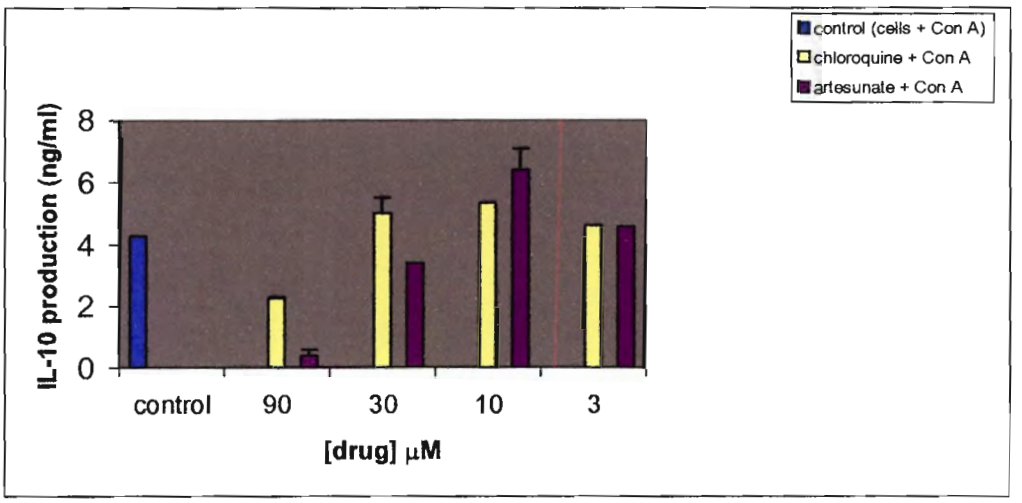

Figure 4.2.4 The effects of a concentration range of chloroquine and artesunate on Con A induced IL-10 production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

4.2.5 Discussion

Today it is well established that the balance between functionally distinct cytokine-producing T cells of the CD4 or CD8 phenotype, determines whether an individual will succumb to the disease or not [Abbas A *et al.*, 1996]. These cell types are distinguished by their capacity to regulate pro-inflammatory and anti-inflammatory responses. In the present study, we compared the effects of both chloroquine and

artesunate in identical in vitro conditions with respect to the production of TNF-α, IFN-γ and IL-12 (pro-inflammatory cytokines) and IL-10 (anti-inflammatory cytokine). These are cytokines that are thought to play an important role in the pathogenesis of severe malaria. Our results show that artesunate has no anti-inflammatory activity, as it shows no inhibition of pro-inflammatory cytokines. We observed that at 10 μM concentrations, artesunate enhances IL-10 production. In contrast, chloroquine showed a significant concentration dependent inhibition of TNF-α, IFN-γ and IL-12p40. At 10 μM concentrations, chloroquine enhanced IL-10 production. This data therefore suggest that chloroquine, at concentrations corresponding to levels reached during standard treatment for malaria patients (Hellgren U *et al.,* 1989), interferes with the balance of Th1 and Th2 cells by affecting the state of the cytokines produced by these cells. Artesunate on the other hand, does not show any anti-inflammatory activity but does enhance IL-10 production.

These findings therefore might suggest that artesunate can exert its anti-inflammatory activity by enhancing IL-10 production. IL-10 is a critical component in the down-regulation of the pro-inflammatory immune reactions and the upregulation of the anti-inflammatory responses. Thus, artesunate probably influences the direction of an emerging immune response by indirectly affecting anti-inflammatory cytokine production. Artemisinin and its derivatives have recently been reported to modulate the immune responses. Artemisinin at doses ranging from 50-100-mg/kg, ip were found to increase the phagocytosis capacity of peritoneal macrophages and interferon production in serum, and to enhance the delayed type hypersensitivity response and acid phosphatase activity of macrophages (Qian R *et al.,* 1987, Ye X *et al.,* 1982, Qian R *et al.,* 1981). This is however in contrast to our findings, in that we couldn't observe any increase interferon production from ConA and LPS stimulated splenocytes treated with artesunate. Sodium artesunate, a water-soluble derivative of artemisinin (and the same drug used in our study), markedly enhanced anti-SRBC antibody formation when given to mice intraperitoneally at dose of 50-mg/kg daily for 5 days (Chen *et al.,* 1988). Artemisinin and its two derivatives, dihydroartemisinin and arteether, exhibited some suppression of humoral response at the doses ranging from 400 to 600-mg/kg (Tawfik A *et al.,* 1990). Our results are partially in agreement with what has been observed on the effect of artemisinin and its derivatives on dose

related response. We observed in our study that at higher doses, artesunate exhibited a significant suppression of cytokines tested. This could not be attributed to toxicity of the drug as viability tests showed no toxicity at the doses used. This then confirms one of our hypotheses that this drug may stimulate the immune response at lower doses (enhanced effect of IL-10 by 10 μM concentration in our study), but suppresses immune response at higher doses (inhibitory effect of all cytokines tested in our study at 90 μM concentration). Different effects of chloroquine on Th1 and Th2 cytokines have been reported. In agreement with observed effects of chloroquine on IFN-γ production in study, a dose dependent inhibition of IFN-γ production was reported in isolated clonal T cells (Landewe *et al.,* 1992). The concentration dependent inhibitory effect of chloroquine on LPS induced TFN-α production was first reported by Picot *et al.,* 1991. The same group showed that chloroquine induced inhibition of TNF-α production was not mediated through lysosomotropic mechanism; rather TNF secretion was probably inhibited by the disruption of iron homeostasis.

Our results are in agreement with the observed effect of chloroquine on TNF production. We observed a concentration dependent inhibitory effect of chloroquine on LPS induced TNF-α production in splenocytes. Kwiatkowski *et al.,* 1995 showed that at conventional doses, the TNF-suppressive properties of chloroquine are greater than those of quinine, and much greater than those of artemether and artesunate. Jeong J *et al.,* 2002 showed that chloroquine down-regulates surface expression of TNF-receptor (TNF-R) in U-937 cells and human monocytes. These results suggest that chloroquine not only suppress the production of TNF but also inhibit TNF signalling by reducing cell surface expression of TNF-R. With respect to IL-12, this cytokine has been proven to be effective in conferring protection against bacterial, viral and intracellular parasitic infections (Locksley R *et al.,* 1993, Trinchieri G *et al.,* 1994). This pleiotropic cytokine not only enhances cell mediated immune responses but also influences humoral immunity by inducing isotype switching through both gamma interferon-γ-dependent and independent mechanism (Metzger D *et al.,* 1997). IL-12 also appears to stimulate enhanced antibody production in switched B cells (Metzger *et al.,* 1997). Mohan *et al.,* 1999, demonstrated that low dose chloroquine plus IL-12 treatment of mice with established blood stage infection of *Plasmodium chabaudi* AS induced a protective Th1 immune response and efficient upregulation of

erythropoiesis during primary infection and a higher antimalarial antibody production following infection. In our present study, we observed a concentration dependent inhibitory effect of this cytokine. High production of IL-12 has been shown to result in pathology in during malaria. The pathogenic effect of IL-12 is likely related to the dose of exogenous IL-12 administered in vivo or the level of endogenous IL-12 produced during infection (Stevenson M *et al.,* 1995).

IL-10 is a critical component in the down-regulation of the pro-inflammatory immune reactions and the upregulation of the anti-inflammatory responses. In the present study, we demonstrate that chloroquine enhances the production of this cytokine at 10-μM concentration ($p=0.049$). This is agreement with the observation made by Hugosson *et al.,* 2002, that chloroquine enhances the number of IL-10 producing cells. This enhancement coincided with an increase in the $CD14^+$ populations and the expression of the costimulatory and adhesion molecules B7-2 and ICAM-1. The findings that chloroquine induces IL-10 production in cultures suggest that the drug can act selectively on cytokine production rather than causing an overall cellular inhibition. Thus, chloroquine probably influences the direction of an emerging immune response by directly or indirectly affecting anti-inflammatory and/or pro-inflammatory cytokine production. In contrast, artesunate probably influences the direction of the immune response by indirectly affecting the anti-inflammatory cytokine production. The question whether the effects of chloroquine and artesunate that have been demonstrated in vitro are relevant for the clinical situation is difficult to answer. The therapeutic serum concentration of chloroquine is reported to be 0.6-0.9 μM in man at a clinically safe dose (Mackenzie A *et al.,* 1983). However, the levels of chloroquine in liver, spleen and leucocytes are much higher and therefore concentrations of chloroquine and artesunate used in this study might therefore resemble intracellular leucocytes concentrations that are potentially achievable under therapeutic conditions. These findings on the effect of chloroquine and artesunate on cytokine production, have implications for the management of cerebral malaria. Chloroquine, which is falling into disuse because of its declining anti-parasitic efficiency, may provide a simple and inexpensive way of suppressing pro-inflammatory cytokines playing a crucial role in the pathogenesis of severe malaria. Extrapolation of *in vitro* results to clinical situation requires some consideration. An

in vivo system, as in a patient, is far more complex than *in vitro* system, which is closely controlled. This implies that evaluation of the effects of chloroquine and artesunate *in vitro* provides an opportunity to help define the *in vivo* modulatory action of the drugs, provided the concentrations used *in vitro* are not toxic for the cells. Excessive production of pro-inflammatory cytokines in severe malaria cases; cerebral malaria and anaemia, is associated with disease severity. The *in vitro* inhibitory effect of chloroquine on pro-inflammatory cytokines production and the enhanced production of anti-inflammatory cytokine by artesunate and chloroquine would predict a beneficial effect on disease activity of severe malaria and could explain the ameliorative action of both drugs on disease activity in severe malaria. Taken together, our data show that artesunate and chloroquine enhances IL-10 production, and in addition chloroquine inhibits pro-inflammatory cytokines and thereby affects the balance between Th1 and Th2 type cytokine response.

CHAPTER 5

5.1 *In vivo* antiplasmodial activity of plants traditionally used to treat malaria in South Africa

Introduction

Malaria is still regarded as one of the deadly parasitic infections in the tropical areas. In the last decades resistance to several anti-malarials became widely disseminated, while the cost of effective treatment is prohibitive for the large majority of the populations in these areas. There is therefore an urgent need for new chemotherapeutic compounds, which are easy to administer and store, and which are of low cost. One possible source for such affordable treatments lies in the use of traditional herbal remedies. In South Africa alone, 80% of the country's population use plants as their primary source of medicinal agents. Not surprisingly as the use of traditional medicine extends over at least three continents, including several countries in Africa, Asia and Americas (WHO, 2000). However, very little data is available to assess the extent to which these plant remedies are used in the health care systems of tropical countries. The recognition and validation of traditional medical practices and the search for plant-derived drugs could lead to new strategies in malaria control.

Since many modern antimalarial drugs such as quinine (*Cinchona*) and artemisinin (*Artemisia annua*) originate from plants, it is essential that other medicinal plants that have traditional folklore reputation for antimalarial properties should be investigated, to establish their safety and efficacy, and to determine their potential as sources of new anti-malarial drugs. To overcome some of the most common problems in this field, such as identification of plant material and variability of chemical content of the locally used plant remedies, we have evaluated some of the plants used traditionally in South Africa to treat malaria and fever. The results are presented in this thesis of an investigation conducted on eight medicinal plants used to treat malaria and fever in South Africa. The aqueous, methanolic, and dichloromethane extracts of eight medicinal plants commonly used to treat malaria in South Africa were evaluated for their antimalarial activity *in vivo*, by a 7-day, suppressive test against *Plasmodium berghei* Anka in mice. No toxic effect or mortality was observed in mice treated orally with any of the extracts daily for 7 days at a dose of 500 mg/kg-body weight. The dichloromethane extract of the bark of *W. salutaris* reduced parasitaemia by >50% at an oral dose of 100 and 500-mg/kg-body weight. The petroleum ether

extract of *H. procumbens* exhibited mild chemosuppression of 35.50% given in a dose of 200-mg/kg-body weight. The aqueous extract of the leaves of *A. annua* reduced parasitaemia by 64% at a dose of 200-mg/kg-body weight. These results appear to justify the use of these medicinal plants as traditional medicines for the treatment of malaria in South Africa.

Results

5.1.1 Testing of the toxic effect of the crude extracts

The toxic effect of the crude extracts used in our study was determined by using two mice in each group. Uninfected mice were given the test extract in a dose of 500 mg/kg, once daily for 7 consecutive days. None of the mice treated with the extracts showed any signs of toxic effect of the test extracts. There was continued gain in body weight at the same rate as untreated controls (Table 5.2a and b). As no external toxic effects or mortality were noted in the 30 days following extract treatment, the median lethal dose is taken to be greater than the highest total dose used in our system.

5.1.2 Antimalarial activity

The *in vivo* antimalarial activities of plants used traditionally to treat malaria in South Africa of the aqueous, methanolic and dichloromethane extracts are shown in Figures 5.1.1, 5.1.2 and 5.1.3 and Table 5.3a and b. The main emphasis was on the aqueous extracts in accordance with the advice of the traditional healers. Control mice receiving only the vehicle or mice treated with *Cassine transvaalensis, Alepidea Amatymbica, Harpephyllum caffrum, Vernonia adoensis* died within 10 days of infection (Table 2a). The chemosuppression observed with each extract on day 7 post-infection was not significant compared with control mice. While the level of parasitaemia in the untreated controls and in the extract treated groups increased throughout the period of observation, parasitaemia in the positive controls given artesunate (100 mg/kg) was completely suppressed to 0% on day 7. Mice given *W. salutaris, A. annua,* and *H. procumbens* had prolonged survival compared with control mice. The dichloromethane extract of *W. salutaris* at doses of 100 and 500 mg/kg respectively showed 53.25% and 58.40% chemosuppression by day 7.

Although there was recrudescence in these mice after the 7DST course, the extract shows some promising activity.

The aqueous extract of *A. annua* at 100 and 200 mg/kg showed 54% and 64% chemosuppression respectively by day 7. This was significantly lower than controls ($p<0.05$) (Figure 5.1.1). Mice treated with *H. procumbens* showed 5% and 36% chemosuppression respectively at 100 and 200 mg/kg of petroleum ether extract. This extract showed mild parasite suppression at 200-mg/kg dose that was considered significant ($p<0.05$). Recrudescence was observed in all treated mice, and all in each treatment group succumbed to the infection by day 14 (Figure 5.1.3). No recrudescence was observed after the first negative parasitaemia in the chloroquine (10 mg/kg) treated group. There was also slight body weight gain in this group (Figure 5.1.2).

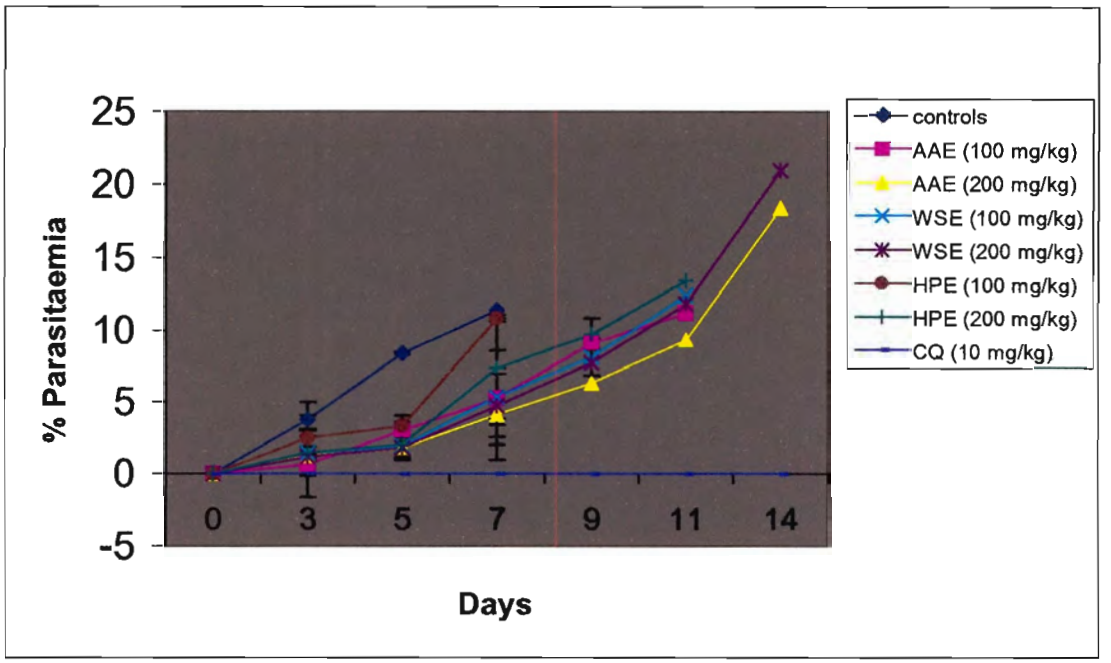

Figure 5.1.1 Time course changes of parasitaemia in the blood stream of mice in response to treatment with hot water extract of *A. annua* (100 and 200 mg/kg b.wt); dichloromethane extract of *W. salutaris* (100 and 200 mg/kg b.wt) and petroleum ether extracts of *H. procumbens* (100 and 200 mg/kg b.wt). *P. berghei Anka* infected mice were orally given extracts from day 1-7 after inoculation intraperitoneally with 1 X 10^6 parasitized erythrocytes. The control group of mice received an equivalent volume of 5% sodium bicarbornate.
AAE- A. annua Extract; WSE- Warburgia Salutaris Extract; HPE- H. procumbens Extracts; Chloroquine

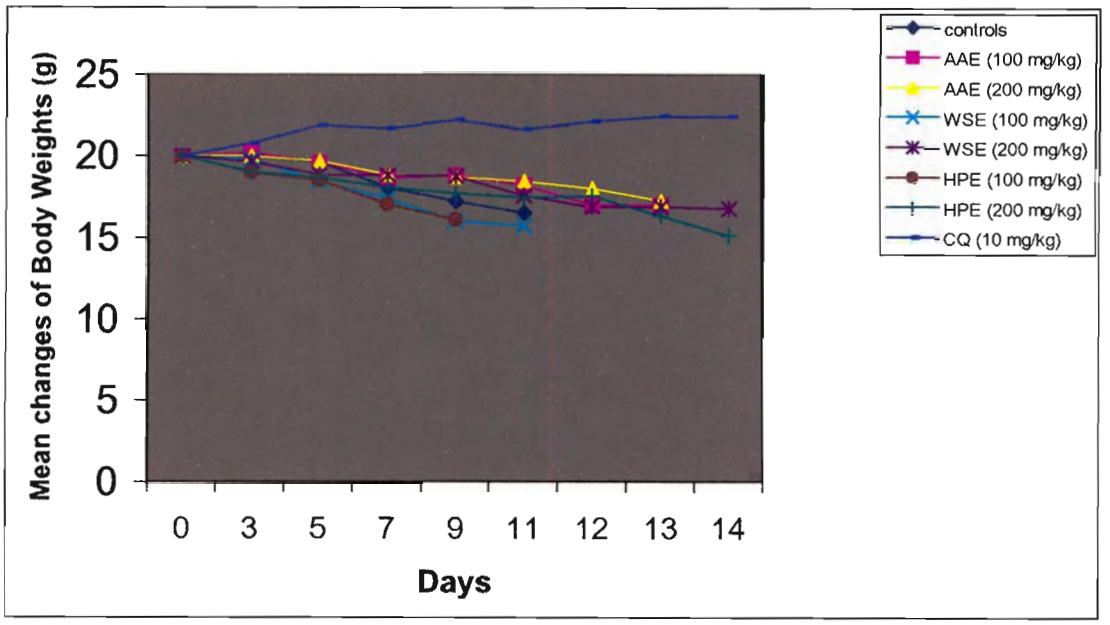

Figure 5.1.2 Time course changes of body weight of mice in response to treatment with hot water extract of *A. annua* (100 and 200 mg/kg b.wt); dichloromethane extract of *W. salutaris* (100 and 200 mg/kg b.wt) and petroleum ether extracts of *H. procumbens* (100 and 200 mg/kg b.wt). *P. berghei Anka* infected mice were orally given extracts from day 1-7 after inoculation intraperitoneally with 1 X 10^6 parasitized erythrocytes. The control group of mice received an equivalent volume of 5% sodium bicarbornate.

AAE- A. annua Extract; WSE- Warburgia Salutaris Extract; HPE- Harpagophytum procumbens Extract; CQ-Chloroquine

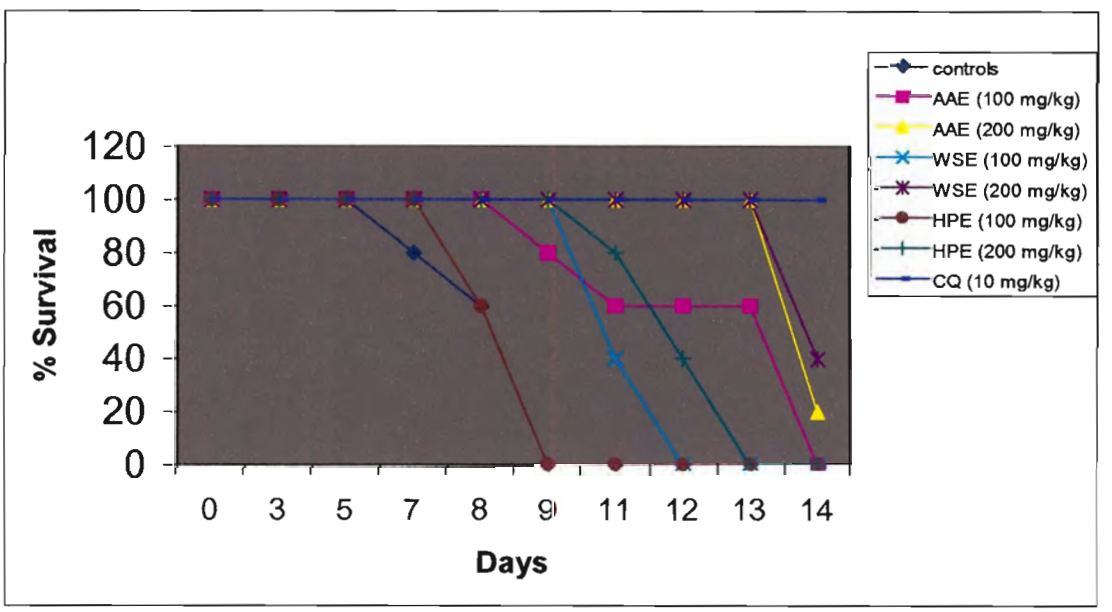

Figure 5.1.3 Time course changes of survival rate of mice in response to treatment with hot water extract of *A. annua* (100 and 200 mg/kg b.wt); dichloromethane extract of *W. salutaris* (100 and 200 mg/kg b.wt) and petroleum ether extracts of *H. procumbens* (100 and 200 mg/kg b.wt). *P. berghei Anka* infected mice were orally given extracts from day 1-7 after inoculation intraperitoneally with 1 X 10^6 parasitized erythrocytes. The control group of mice received an equivalent volume of 5% sodium bicarbornate.

AAE- A. annua Extract; WSE- Warburgia Salutaris Extract; HPE- H. procumbens Extract; CQ-Chloroquine

Table 5.1
Characteristics of the plant extracts investigated

Plant name (Vernacular name)	Part extracted	Plant origin	Dry weight of extracts (% yield)			
			Methanol	Dichloromethane	Aqueous (Hot)	Aqueous (Cold)
Warburgia Salutaris. **(Isibaha)**	Bark	Durban (KZN)	4.62	3.60	3.20	4.00
Alepidea Amatymbica **(Ikhathazo)**	Bark	Durban (KZN)	1.80	1.20	2.94	2.80
Harpephyllum caffrum **(Umgwenya)**	Bark	Nongoma (KZN)	1.70	0.40	1.80	1.37
Vernonia adoensis **(Inyathelo)**	Bark and Leaves	Durban (KZN)	1.50	1.45	5.25	3.56
Cassine transvaalensis **(Ingwavuma)**	Roots	Nongoma (KZN)	1.82	1.25	4.50	3.00
Artemisia annua **(Qinghaosu)**	Leaves	Grassroots (WP)^	Nd*	Nd*	2.50	2.20
Harpagophytum procumbens **(Devil's claw)**	Roots	Grassroots (WP)	Nd*	Kind gift from Cailean Petroleum ether extract: 0.26%	Nd*	Nd*
Nuxia Floribunda **(Umlulama)**	Leaves	Nongoma (KZN)*	Nd*	Nd*	2.60	4.55

Percentage yields of plant extracts. Note the different yields for each extraction method showing the chemical diversity of each plant.
Nd*-denotes extraction not performed/ not determined.
(KZN)* - KwaZulu/Natal Province
(WP)^ - Western Province

Table 5.2a

Determination of oral toxicity of plant extracts

Treatment	Extract	Dose (mg/kg.day)	Body Weight (g) (S.D.) Day 0	Day 7
Cassine transvaalensis	Aqueous	500	19.4 (1.2)	22.0 (1.8)
	Dichloromethane	500	19.4 (1.2)	21.7 (1.1)
Alepidea amatymbica	Aqueous	500	19.46 (1.7)	21.9 (2.3)
	Dichloromethane	500	19.46 (1.7)	21.6 (1.7)
Harpephyllum caffrum	Aqueous	500	20.0 (0.2)	22.0 (2.2)
	Dichloromethane	500	20.0 (0.2)	21.8 (0.21)
Vernonia adoensis	Aqueous	500	19.6 (1.1)	21.6 (0.33)
	Methanol	500	19.6 (1.1)	21.10 (1.4)
Artesunate		100	20.0 (0.1)	22.23

Table 5.2b

Determination of oral toxicity of plant extracts

Treatment	Extract	Dose *mg/kg.day*	Body weight (g) (S.D.)	
			Day 0	Day 7
Nuxia floribunda	Aqueous	500	19.8 (1.3)	20.13 (0.2)
	Dichloromethane	500	19.8 (1.3)	21.07 (0.8)
Warburgia salutaris.	Aqueous	500	20.0 (0.4)	21.9 (0.1)
	Dichloromethane	500	20.0 (0.4)	22.05 (1.3)
Artemisia annua	Aqueous	500	20.0 (0.38)	21.70 (1.1)
	Methanol	500	20.0 (0.38)	21.65 (0.8)
Harpagophytum procumbens	Petroleum Ether	500	20.0 (0.6)	21.7
Chloroquine		10	20.0	22.2

Table 5.3a

In-vivo antimalarial activity of the plant extracts

Treatment	Extract	Dose (mg/kg.day)	Mean (S.D.) value (%)	
			Parasitaemia (%)	Chemosuppression (%)
Cassine transvaalensis	Aqueous	100	16.00 (2.2)	1.80 (1.0)
		200	15.74 (3.4)	3.43 (1.1)
	Methanol	100	Nd*	Nd*
		500	Nd*	Nd*
	Dichloromethane	100	15.21 (4.0)	6.68 (2.2)
		500	15.33 (3.0)	5.95 (1.0)
Alepidea amatymbica	Aqueous	100	14.70 (2.1)	9.80 (1.3)
		200	15.02 (6.2)	7.85 (1.1)
	Methanol	100	Nd*	Nd*
		500	Nd*	Nd*
	Dichloromethane	100	15.93 (5.1)	2.26 (1.8)
		500	15.26 (3.6)	6.38 (1.5)
Harpephyllum caffrum	Aqueous	100	16.40 (1.1)	-0.61 (6.7)
		200	15.55 (0.98)	4.60 (1.3)
	Methanol	100	Nd*	Nd*
		500	Nd*	Nd*
	Dichloromethane	100	14.44 (1.0)	11.41 (2.2)
		500	14.24 (1.2)	12.63 (1.1)
Vernonia adoensis	Aqueous	100	15.30 (3.3)	6.13 (1.7)
		200	15.15 (2.8)	7.05 (1.0)
	Methanol	100	14.89 (2.7)	8.65 (1.4)
		500	15.00 (1.8)	7.97 (1.0)
	Dichloromethane	100	Nd*	Nd*
		500	Nd*	Nd*
None			16.3 (2.7)	
Artesunate		100	0	100 (0.0)

Nd*- denotes not determined/ not performed

Table 5.3b

In-vivo antimalarial activity of the plant extracts

Treatment	Extract	Dose *mg/kg.day*	Mean (S.D.) value (%)	
			Parasitaemia (%)	Chemosuppression (%)
Nuxia floribunda	Aqueous	100	10.75 (2.3)	5.53 (2.4)
		200	10.55 (2.6)	7.29 (3.3)
	Methanol	100	Nd*	Nd*
		500	Nd*	Nd*
	Dichloromethane	100	11.21 (4.0)	1.50 (1.8)
		500	11.30 (4.1)	0.70 (2.6)
Warburgia salutaris.	Aqueous	100	10.95 (2.0)	4.00 (1.7)
		200	10.70 (2.2)	6.00 (2.2)
	Methanol	100	Nd*	Nd*
		500	Nd*	Nd*
	Dichloromethane	100	5.32 (4.7)	53.25 (3.2)
		500	4.73 (3.7)	58.40 (2.5)
Artemisia annua	Aqueous	100	5.26 (3.3)	53.78 (3.1)
		200	4.10 (3.2)	64.00 (3.9)
	Methanol	100	11.15 (2.4)	2.02 (1.3)
		500	10.90 (2.6)	4.20 (2.1)
	Dichloromethane	100	Nd*	Nd*
		500	Nd*	Nd*
Harpagophytum procumbens	Aqueous	100	Nd*	Nd*
		200	Nd*	Nd*
	Methanol	100	Nd*	Nd*
		500	Nd*	Nd*
	Petroleum Ether	100	10.81 (5.2)	5.00 (3.6)
		200	7.33 (3.5)	35.50 (4.3)
None			11.38 (2.1)	
Chloroquine		10	0	100 (0.0)

Nd*- denotes not determined/ not performed

5.1.3 Discussion

Altogether fifteen extracts representing eight plant species were investigated. Table 5.1 shows the botanical names, vernacular names and plant parts tested. The summary of the results of the anti-malarial activity is shown on Table 5.3a and b. None of the extracts in Table 5.3a showed any activity against *Plasmodium berghei* ANKA (PbA) infections in mice. The negative results were surprising as this was a chloroquine sensitive strain. Of the fifteen extracts tested for toxicity in the C57BL/6 mice, none of these extracts showed any toxic effect (Table 5.2a and b). Three plant extracts showed promising anti-malarial activity *in vivo*. The aqueous extract of *A. annua*, dichloromethane extract of *W. salutaris* and the petroleum ether extract of *H. procumbens* showed promising anti-malarial activity. Most of the antiplasmodial activity was present in the organic solvent extracts. Traditionally, plant extracts are prepared with water (for example, infusions, decoctions and poultices), and it is impossible that traditional healers would produce extracts in petroleum ether, dichloromethane or in methanol.

5.1.3.1 W. salutaris

W. salutaris, commonly known as *isibhaha* in isiZulu and pepper-bark tree in English has been used as a medicinal plant for many years. The pinch of a powdered bark is taken in a spoonful of cold water or smoked, mixed with *Cannabis savita* leaves, for treatment of dry cough (Hutchings *et al.*, 1999). The bark of this plant is used in emetics or purgatives for febrile complaints and for rheumatism or for ailments known as *isibhobo* or *amanxeba*, which are traditionally thought to be caused by sorcery. Powdered bark mixed with fat is used as ointment for penial irritation. Lotions, made from pounded leaves with stalks of *Hibiscus surattensis*, are applied to the penis in cases of inflammation of the urethra, sores ad other irritation. Roots and stembark are widely used in Southern Africa as expectorants or smoked for coughs and colds (Watt and Breyer-Brandwiljk *et al.*, 1962). The Vhavenda (in the Limpopo province of South Africa) use the bark for colds, chest complaints, malaria, venereal diseases, purgatives, backache, skin complaints, stomach ulcers and aphrodisiacs. This plant is widely used in other parts of Africa, as the bark and the roots are used in

West Africa for influenza, fevers, pains, stomachache and other gastro-intestinal disorders (Hutchings *et al.*, 1999). Despite its frequent use in Southern Africa as a medicinal plant, preliminary screening of extracts from the inner bark show highly toxic effects in experiments currently being undertaken at the University of Stellenbosch (South Africa). In our study, no toxic effect or mortality was observed in mice treated for 7 consecutive days with 500 mg/kg of aqueous and dichloromethane extracts. As no external toxic effects or mortality were observed within the period of extract administration, the median lethal dose of the extract is presumably higher than the highest total dose tested. In the present study, treatment of PbA-mice infected with dichloromethane extract of the bark, in oral dose of 100 and 500 mg/kg, led to significant suppression in parasitaemia of 53% and 58%, respectively ($p<0.005$), compared with controls, see Table 5.3b and Figure 3. Further studies are required to determine if the antimalarial activity is attributable to the compounds that have been reported to be present in the bark of this plant. Taniguchi and Kubo *et al.,* 1993 reported the bark to contain tannin and about 3% of mannitol. Sesquiterpenoid dialdehydes, also isolated from the bark has been reported. *W. salutaris* has been reported to possess the antibacterial activity. The methanol extract of *W. salutaris* inhibited the growth of gram-positive and of *Escherichia coli* (Rabe *et al.*, 1997). Despite its wide use in traditional medicine, this appears to be the first study of its antimalarial activity conducted on this plant.

5.1.3.2 H. procumbens

H. procumbens (Devil's claw) is found only in Southern Africa. The natural habitats are Kalahari savannas and dessert areas in Namibia and parts adjacent to Republic of South Africa, Botswana, Angola, and Zimbabwe. The plant belongs to the botanical family *Pedaliaceae*. The medicinal material consists of the cut and dried secondary root tubers of the plant. The primary vertical root contains the same constituents but at lower levels than the secondary roots. Medicinal preparations of the secondary root tubers have been used in traditional medicine in Southern Africa for centuries. The plant is traditionally used for the relief of fevers and other blood diseases and to treat digestive disorders, arthritic and rheumatic conditions (Watt and Breyer-Brandwijk *et al.*, 1962 and van Wyk *et al.*, 1997). The roots are also used as a general analgesic and are applied in an ointment for to sores, ulcers and boils. Dried roots are

administered during pregnancy and continued after labour for pain relief (Watt and Breyer-Brandwijk *et al.*, 1962). *H. procumbens* was introduced into Europe as a herbal tea in the 20th century. This occurred via a German soldier who studied the traditional system of the Namibian people (Wegener *et al.*, 2000). Western use of this plant is generally in line with the traditional application, although attention has been focused on its anti-inflammatory and anti-rheumatic use. Decoctions, tinctures and tablet forms of the dried roots are readily available in pharmacies and health food shops and are used to stimulate appetite, relieve indigestion and to treat a range of joint and muscular problems. This would include rheumatic and arthritic conditions, back pain, gout and fibrositis (Chevallier, 1996). Clarkson *et al.*, PhD thesis 2002 demonstrated the *in vitro* antiplasmodial activity of *H. procumbens*. The crude petroleum ether extract showed *in vitro* antiplasmodial activity against a chloroquine sensitive strain of *P. falciparum*. An active principle was isolated and its chemical formula determined to be $C_{20}H_{28}O_3$. The active component showed significant *in vitro* antiplasmodial activity against both chloroquine (CQ)-sensitive and CQ-resistant strains of *P. falciparum*. The *in vitro* cytotoxicity assays indicated that the compound was not toxic to Rat-1-cells at the concentrations required to kill *P. falciparum* parasite. This led to *in vivo* antiplasmodial screening of this extract in the present study. Mice treated with a daily oral dose of 200-mg/kg-body weight produced a significant (36%) chemosuppression of parasitaemia ($p<0.005$). At a daily oral dose of 500-mg/kg-body weight given to uninfected mice for 7 days, this extract was well tolerated and not toxic. It would have been interesting to evaluate the *in vivo* activity of the aqueous extract of this plant, even though it did not show *in vitro* antiplasmodial activity. But research on many medicinal plants has shown that water extracts used in traditional medicine do not necessarily contain the active principles isolated through *in vitro* bioassay guided fractionation of organic solvents (Phillipson *et al.*, 1993). In some instances, precursors or structural analogues of the active principles are present in the aqueous extract and have to be modified in some way before activity is exhibited. This implies that precursors of the active principles may be present in aqueous extract but have to be converted into an active metabolite or modulate the immune system before the activity is exhibited. The present study demonstrates the antiplasmodial activity of *H. procumbens* in mice even though it was observed in an organic solvent extract. Traditionally, aqueous extracts are used to

treat fevers and related illnesses; these would contain very little if any non-polar compounds extracted by organic solvents.

5.1.3.3 A. annua

In 1970, Professor Wei, of the institutes of traditional Chinese medicines, made an extract of the leaves of the plant *A. annua* (sweet wormwood), a common weed, and observed that this juice acted against the malaria parasite (Jansen *et al*, 2002). *A. annua* had been used for 2000 years in traditional Chinese medicine for the treatment of sores and diseases including fever. Further investigations yielded the isolation of a crystalline product. It was given the name Qinghaosu, which is the Chinese word for Artemisinin. Owing to the wide use of these compounds and their effect as anti-malarial drugs, we took advantage of the indigenous *A. annua* grown at Grassroots in the Western Province of South Africa. We prepared methanol and aqueous extract of the leaves and tested them in our mouse model system for antimalarial activity. The methanol extract did not show any activity in the mice infected with PbA and treated with 100 and 500-mg/kg body weight dose. However, the aqueous extract at a dose of 100 and 200-mg/kg body weight produced significant (>50%) suppression of parasitaemia ($p<0.05$), Table 5.3b and Figure 5.1.3. The compound Artemisinin was isolated from *A. annua* leaves by hexane extraction (Jansen *et al*, 2002). This compound acts as a precursor for its derivatives artesunate and artemether. Artemether is very lipophilic and artesunate itself is not soluble in water. The antimalarial activity displayed of the aqueous extract shows that the active ingredient is unlikely to be artemisinin in this regard. Research on the chemical composition of *A. annua* extracts has resulted in the isolation of a number of compounds, including several flavonoids which are structurally not related to artemisinin, that can enhance the *in vitro* antiplasmodial activity of artemisinin (Elford *et al.*, 1987). Hence, low levels of artemisinin in an aqueous extract could exhibit antimalarial activity if a synergistic relationship exists with other compounds. In addition, other studies on *A. annua* tea, prepared by traditional methods, have shown that the levels of artemisinin are surprisingly higher than would be expected (Mueller *et al.*, 2000). It has since been suggested that other plant constituents might help extract and keep the lipophilic compound in an aqueous solution. These various factors might account for the

antiplasmodial activity of the aqueous extracts used in traditional medicine and, as such, explain the current observation in our study. We suggest then that whatever the effect of the compound in this extract, it might be converted into an active metabolite *in vivo* or modulate the immune system. *A. annua* is a wild growing cosmopolitan species with relatively low artemisinin contents, ranging from less than 0.1 to 1% of the plant dry weight, depending on the geographical origin of the plant (Haynes R *et al.*, 1994; Laughlin C *et al.*, 1994). Montanus L *et al.*, (Honours project 1999 Department of Pharmacology) did not identify the presence of artemisinin, in the same batch of plant material. Further studies have been conducted on this plant extract, to elucidate possible immunomodulatory effect based on cytokine production. Phytochemical investigation will also have to be conducted to determine the compounds that might be responsible for antimalarial activity.

In conclusion, our toxicological experiments indicate that mice tolerated all the extracts tested. This confirms the safety of these medicinal plants as they are widely used in traditional healing settings. The dichloromethane extract of the bark of *W. salutaris* reduced parasitaemia by >50% at an oral dose of 100 and 500-mg/kg-body weight. The petroleum ether extract of *Harpagophytum procumbens* exhibited mild parasite suppression of 36% at a dose of 200-mg/kg-body weight. The aqueous extract of the leaves of *Artemisia annua* reduced parasitaemia by up to 64% at high dose of 200-mg/kg-body weight. These results of the present study appear to justify the use of these medicinal plants as traditional medicines for the treatment of malaria in South Africa.

Effect of plant extracts on cytokine production

5.2 Effects of plant extracts on cytokine production in splenocytes stimulated with different mitogens.

Introduction

Medicinal plant products, commonly known as herbal drugs or traditional medicines, are known to have immunomodulatory properties. These may act either by stimulating both cell mediated and humoral immunity or only cell mediated immune response while suppressing the humoral component of the immune system (Atal *et al.* 1986). Traditional medicines in South Africa are largely based upon herbal or plant preparations and have specific diagnostic and therapeutic principles. Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of traditional healing is sometimes based on related principles. Immunostimulation in a drug-induced immunosuppression model and immunosuppression in an experimental hyper-reactivity model by the same preparation can be said to be true immunomodulation (Patwardhan *et al.* 1990). Apart from being specifically stimulatory or suppressive, certain agents have been shown to possess activity to normalize or modulate pathophysiological processes and are hence called immunomodulatory agents (Wagner *et al.* 1983). In the present study we investigated 3 extracts from 3 different medicinal plants used to treat malaria in South Africa for their effect on the immune response. These extracts had previously been screened for their activity *in vivo* in mouse model system for *Plasmodium berghei* ANKA and have been found to show promising antimalarial activity.

To elucidate the immunobiological effects of these extracts, we examined their effects on TNF-α, IFN-γ, IL-12 and IL-10 production on ConA and LPS induced splenocytes. These cytokines are thought to play a crucial role in the pathogenesis of severe malaria; cerebral malaria and anaemia.

Results

5.2.1 Tumour necrosis factor-alpha (TNF-α) production.

The maximum production of TNF-α was reached after 24 hours of stimulation with lipopolysacharide (LPS) at 10 μg/ml. There was no production of this cytokine without addition of LPS. Addition of different concentrations of plant extracts ranging from 1.56-100 μg/ml to the cultured splenocytes stimulated by LPS showed contrasting inhibition and enhanced additive effect on TNF-α production (Figure 5.2.1). The addition of different concentrations of *H. procumbens* to our test system showed a concentration dependent inhibition of TNF-α production. The most significant inhibition of TNF-α production in *H. procumbens* treated splenocytes was reached over a concentration of 25-100 μg/ml ($p<0.05$). The addition of different concentrations of *A. annua*, showed contrasting effect on TNF-α production. There was no significant effect observed at low concentration of 1.56 μg/ml. Significant inhibition at 6.25 and 25 μg/ml ($p<0.05$) of this extract was noted. At the highest concentration, *A. annua* had no effect on TNF-α production in response to LPS and was comparable to the controls. A similar finding was observed with *W. salutaris* treated splenocytes, a dose dependent inhibition of TNF-α production at 6.25 and 25 μg/ml ($p=0.001$). There was significant additive effect observed at low concentration (1.56 μg/ml). In contrast, no significant effect was observed at high (100 μg/ml) concentration of this extract (Figure 5.2.1). To determine whether the observed inhibition of TNF-α production after stimulation with LPS was caused by toxicity of the extracts to the cytokine producing cells, trypan blue staining was done, which showed no increase in cell death on culturing in the presence of plant extracts. This indicates that the observed inhibition of TNF-α production cannot be explained by toxicity.

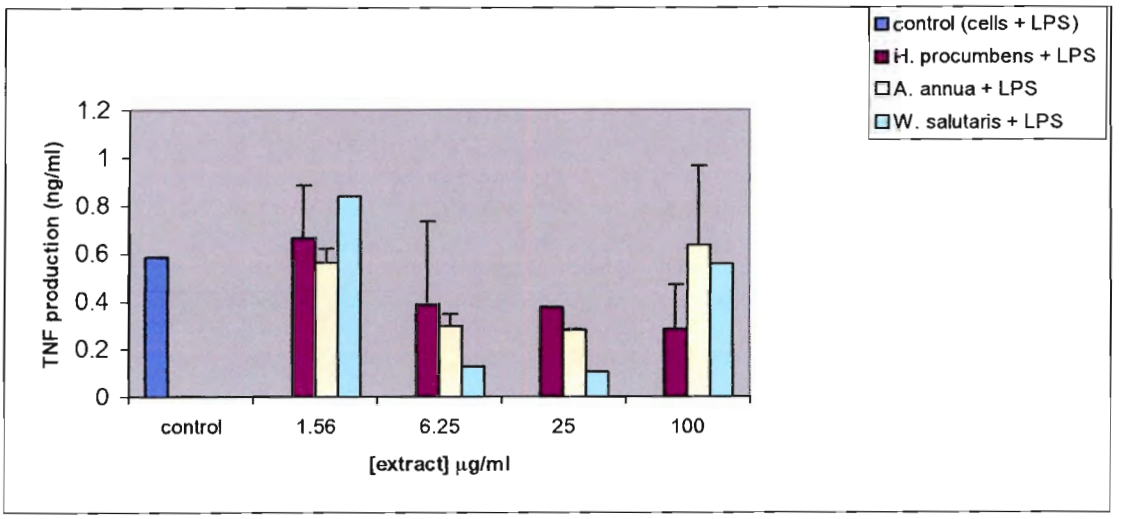

Figure 5.2.1 The effects of a concentration range of extracts on LPS induced TNF-α production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

5.2.2 Interferon gamma IFN-γ production

Maximum production of IFN-γ by splenocytes stimulated with Concanavalin A (ConA) was reached after 48 hours. No production of this cytokine could be detected in our culture system without addition of ConA. The addition of different concentrations of *H. procumbens* to our test system showed a significant inhibition of IFN-γ production. The most significant inhibition of IFN-γ production in *H. procumbens* treated splenocytes was reached over a concentration range of 1.56-25 µg/ml ($p=0.01$) with no effect observed at100 µg/ml. The addition of different concentrations of *A. annua*, showed contrasting effect on IFN-γ production. There was no significant effect observed at low concentration of 1.56-25 µg/ml ($p>0.05$). In contrast, at the highest concentration (100 µg/ml), *A. annua* almost completely abrogated this response, reducing IFN-γ in response to ConA to almost zero. The addition of different concentrations of *W. salutaris* to our test system showed a significant inhibition of IFN-γ production. We observed a concentration dependent inhibition of IFN-γ production in *W. salutaris* treated splenocytes was over a concentration range of 1.56-100 µg/ml ($p<0.05$). To determine

whether the observed inhibition of IFN-γ production after stimulation with ConA was caused by toxicity of the extracts to the cytokine producing cells, trypan blue staining was done. No increase in cell death upon culturing in the presence of plant extracts. This indicates that the observed inhibition of IFN-γ production cannot be explained by toxicity.

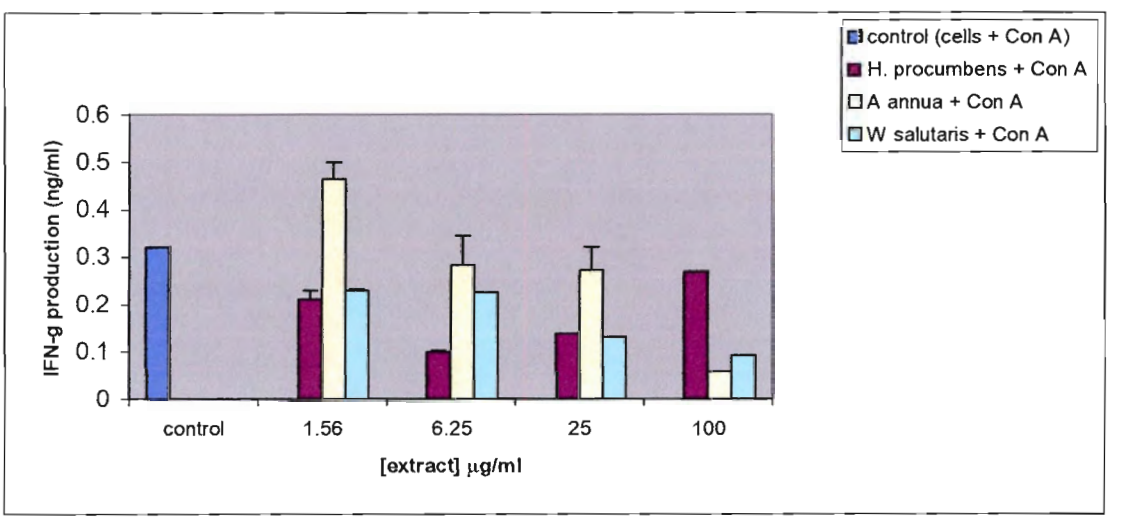

Figure 5.2.2 The effects of a concentration range of extracts on Con A induced IFN-γ production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

5.2.3 IL-12p40 production

Maximum production of IL-12p40 by splenocytes stimulated with lipopolysacharide (LPS) was reached after 48 hours. No production of this cytokine could be detected in our culture system without addition of LPS. Addition of different concentrations of *H. procumbens* to the cultured splenocytes stimulated with LPS showed concentration dependent inhibition of IL-12p40 production (Figure 5.2.3). The most significant inhibition of IL-12p40 production in *H. procumbens* treated splenocytes was reached over a concentration range of 25-100 μg/ml ($p<0.05$). In contrast to the inhibiting effects on LPS stimulated IL-12p40 production by *H. procumbens*, *A. annua* lacked the inhibitory effect of IL-12p40 at concentration range of 1.56-25 μg/ml but instead showed a significant enhanced additive effect on LPS stimulated splenocytes (Figure 5.2.3). At 100 μg/ml ($p=0.01$), there was a significant inhibitory effect of this extract. Almost similar results were observed with *W. salutaris* treated splenocytes. The most significant enhanced additive effect was reached at a concentration range of 1.56-25 μg/ml ($p<0.05$). There was no significant effect observed at high concentration of this extract (Figure 5.2.3). To determine whether the observed inhibition of IL-12p40 production after stimulation with LPS was caused by toxicity of the drugs to the cytokine producing cells, trypan blue staining was done. There was no increase in cell death upon culturing in the presence of plant extracts. This indicates that the observed inhibition of IL-12p40 production cannot be explained by toxicity.

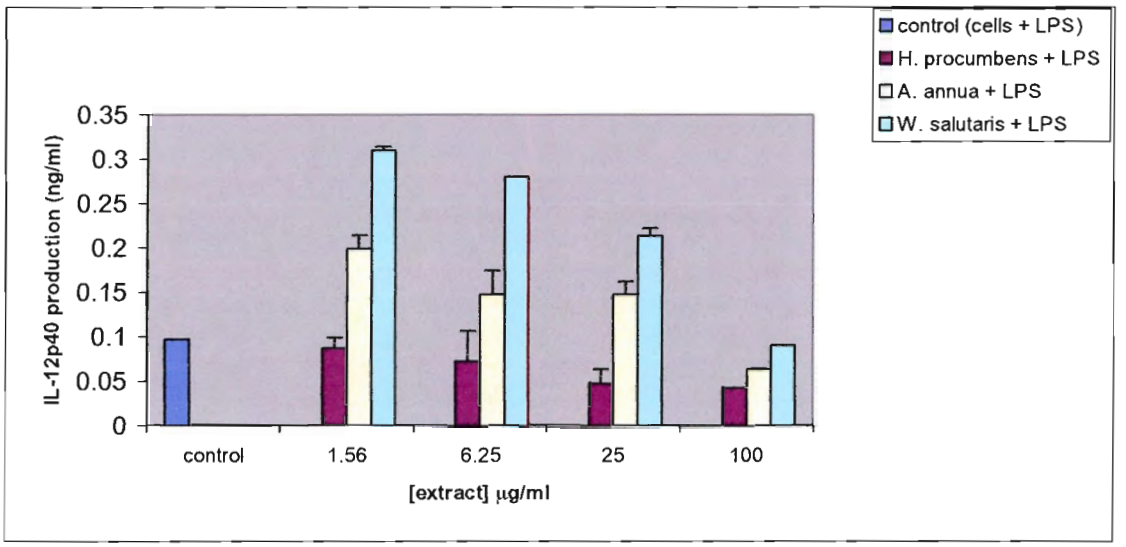

Figure 5.2.3 The effects of a concentration range of extracts on LPS induced IL-12p40 production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

5.2.4 IL-10 production

Maximum production of IL-10 by splenocytes stimulated with Concanavalin A (ConA) was reached after 24 hours. There was no production of this cytokine without addition of a mitogen. In the presence of *H. procumbens*, we observed an enhanced IL-10 production, additively to the mitogen stimulation (Figure 5.2.4). There was a significant enhanced effect of *H. procumbens* at 25 and 100 µg/ml ($p<0.05$) on IL-10 production. In contrast, at the lower concentration, *H. procumbens* showed a significant inhibitory effect of this cytokine, reducing IL-10 release in response to ConA to almost half the control levels (6.25 µg/ml [$p=0.017$]). No effect was observed on *A. annua* treated cultures within a concentration range of 1.56-25 µg/ml and in contrast this extract showed a significant enhanced effect, additively to the mitogen stimulation on IL-10 production at concentration of 100 µg/ml ($p=0.016$) (Figure 5.2.4). In contrast to the enhanced effect of *H. procumbens* and *A. annua, W. salutaris* showed a concentration dependent

inhibition of this cytokine, with the most significant effect observed at a concentration range of 25 and 100 μg/ml ($p<0.05$). To determine whether the observed inhibition of IL-10 production after stimulation with ConA was caused by toxicity of the drugs to the cytokine producing cells, trypan blue staining was done. No increase in cell death upon culturing in the presence of either chloroquine or artesunate. This indicates that the observed inhibition of IL-10 production cannot be explained by toxicity.

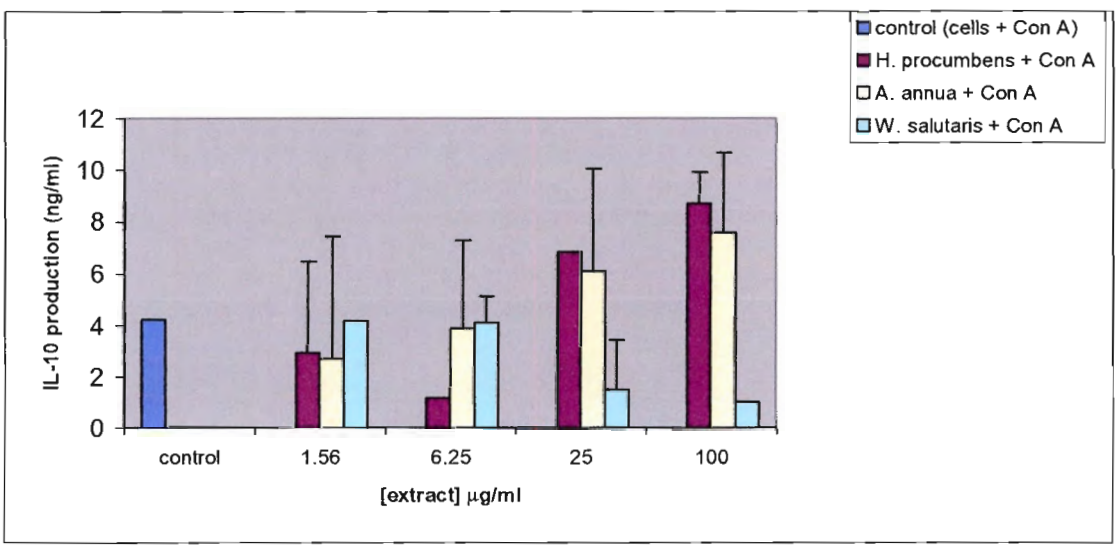

Figure 5.2.4 The effects of a concentration range of extracts on Con A induced IL-10 production for 24 hours. Results are expressed as means ± standard error of the mean (SEM) of duplicates from a single experiment representative of three independent experiments.

5.2.5 Discussion

Three extracts prepared from *H. procumbens, A. annua* and *W. salutaris* were tested for their effect on cytokine production by ELISA. Only three plant extracts showed some promising antimalarial activity *in vivo*. The aqueous extract of *A. annua*, dichloromethane extract of *W. salutaris* as well as the petroleum ether extract of *H. procumbens* showed some promising antimalarial activity in our *in vivo* test system. This then motivated to test one of our hypotheses that these medicinal plants might modulate the immune system. To elucidate the immunobiological effects of these extracts, we examined their effects on TNF-α, IFN-γ, IL-12 and IL-10 production on ConA and LPS induced splenocytes.

5.2.5.1 H. procumbens.

The petroleum ether extract of this plant showed some significant inhibitory effects of TFN-α, IFN-γ and IL-12p40 at doses of 6.25-100 μg/ml. The results of this study demonstrate that *H. procumbens* has immunomodulatory properties and that its action might be mediated by anti-inflammatory activity. This was confirmed by its effect on the enhanced production of IL-10 upon treatment with this extract. At 25-100 μg/ml, we observed a significant enhanced effect of ConA induced IL-10 production. It seems that this extract probably influences the direction of an emerging immune response by directly or indirectly affecting anti-inflammatory and/or pro-inflammatory cytokine production. Many plant extracts used in traditional medicines have been reported to have immunomodulatory properties. While some stimulate both cell mediated and humoral immunity, others activate only cellular immune components without affecting the humoral immune system; others suppress both components of the immune system. These results are in agreement with the anti-inflammatory effect of this plant that has been reported previously. Lanhers M *et al.* 1992, demonstrated in a study where he assessed the anti-inflammatory properties of *H. procumbens* when administered by different routes in the carrageenan-induced rat paw edema test. Aqueous extracts administered intraperitoneally and intraduodenally significantly reduced edema. Extracts of *H.*

procumbens have shown activity in various experimental models (Manez *et al.* 1990). In the croton oil-induced granuloma pouch test and formaldehyde-induced arthritis test in rats, the reduction in inflammation produced by 10 to 12 days of intraperitoneal administration of extract, isolated harpagoside, or its aglycone harpagogenine and by oral administration of the extract was similar to that of the conventional drug phenylbutazone. In a double-blind study, 50 patients with arthrosis received three doses totalling 2,400 mg per day of *H. procumbens* (each dose was two capsules of 400 mg cryoground dried root material, standardized to 1.5% iridoid glycosides) up to three times per week for a three week period (Grahame R *et al.* 1981). Severity of pain was assessed 10 days after treatment completion. Compared with placebo, the extract significantly decreased the severity of the patients' pain. In a double-blind study (Ghisalberti E *et al.* 1998) of 89 patients with rheumatic articulation joint pain, the efficacy and tolerance of a daily dose of 2000 mg of powdered extract (three times daily, 2 capsules, each 335 mg of powdered cryoground material, standardized to 3% of iridoid glycosides) for two months was assessed. The clinical parameters measured on days 0, 30, and 60, severity of Visual Analog Scale (VAS) pain and joint mobility determined by finger-floor distance, revealed a significant drop in the intensity of pain and a significant increase in mobility in the treatment group. Neither side effects nor changes in laboratory parameters were observed during the two-month study. Forty-three patients with osteoarthritis and rheumatoid arthritis were enrolled in an uncontrolled study with a daily dosage of 750 mg powdered secondary tubers of *H. procumbens* for a course of 30 days (Chantre P *et al.* 2000). At the end of treatment, patients reported significant improvement of symptoms, mobility, and morning stiffness. Adverse events were not reported. All these observations on this extract, confirm its anti-inflammatory effect observed in our study. The anti-inflammatory response observed in our study is in agreement with almost similar effect observed in other studies even though different extracts were used. Secondary roots of this medicinal plant are interesting therapeutic remedies for inflammatory conditions.

5.2.5.2 W. salutaris

In the present study, this extract showed a significant inhibition of TNF-α, IL-10 and IFN-γ but in contrast, we observed an enhanced effect of IL-12p40. These conflicting results do not seem to be specific with the kind of effect this extract might possess. Although the immunomodulatory effects of *W. salutaris* have not been reported, the bark of this extract has been used as emetic or purgative for febrile complaints and for rheumatism. This shows the anti-inflammatory effect that the extract might possess. Watt and Breyer-Brandwiljk *et al.* 1962 showed that *W. salutaris* in combination with the stalks of *hibiscus surattensis* are applied to the penis in cases of inflammation of the urethra, which also confirms its anti-inflammatory activity. It has been demonstrated that excessive production of pro-inflammatory cytokines, TNF-α and IFN-γ results to the pathogenesis of severe malaria (Grau *et al.* 1987, Kossodo *et al.* 1988). The extract of *W. salutaris* was able to inhibit the production of TNF and IFN-gamma and might contribute to prevention of disease severity. This then indicates the ability of the extract to possess anti-inflammatory activity. These results add weight to the use of this medicinal plant for the alleviation of ailments like rheumatism and inflammatory conditions. *W. salutaris* is widely used in South Africa for different ailments. These results imply that it can also modulate immune responses and thus improve clinical outcomes of patients with disorders that can benefit from immunological modulation, either stimulation or suppression.

5.2.5.3 A. annua

A. annua has long been used in herbal medicine particularly in ancient China. In 1972, artemisinin also called qinghaosu was isolated as the plant's active principle. Artemisinin is the compound isolated from this plant leaves, using hexane for extraction. We have shown that an aqueous extract produced a significant (>50%) chemosuppression of parasitaemia in mice infected with PbA and this prompted for further investigation of this extract for its effect on immune response. In this study, *A. annua* showed a

significant inhibition of pro-inflammatory cytokines and an enhanced effect of IL-10 at high concentration (100 μg/ml). Thus, *A. annua* probably influences the direction of an emerging immune response by directly or indirectly affecting anti-inflammatory and/or pro-inflammatory cytokine production. Artemisinin is *A. annua's* active principle. Artemisinin and its derivatives have recently been reported to modulate the immune responses. Artemisinin at doses ranging from 50-100-mg/kg, intraperitoneally, were found to increase the phagocytosis capacity of peritoneal macrophages and interferon production in serum, and to enhance the delayed type hypersensitivity response and acid phosphatase activity of macrophages (Qian R *et al.* 1987, Ye X *et al.* 1982, Qian R *et al.* 1981). This is however in contrast to our finding in that we couldn't observe any increase interferon production from ConA stimulated splenocytes treated with *A. annua*. Sodium artesunate, a water-soluble derivative of artemisinin, markedly enhanced anti-SRBC antibody formation when given to mice intraperitoneally at dose of 50-mg/kg daily for 5 days (Chen *et al.* 1988). Tawfik A *et al.* 1990 showed that artemisinin and its two derivatives, dihydroartemisinin and arteether, exhibited some suppression of humoral response at the doses ranging from 400 to 600-mg/kg. Our results are partially in agreement with what has been observed on the effect of artemisinin and its derivatives on dose related response. We observed in our study that at higher doses (100 μg/ml), *A. annua* exhibited a significant suppression of cytokines tested. This could not be attributed to toxicity of the drug as viability tests showed no toxicity at the doses used.

In conclusion, these studies suggest that medicinal plants used to treat malaria and other ailments in South Africa, exhibit some immunological modulation. Extrapolation of *in vitro* results to clinical situation requires some consideration. An *in vivo* system, as in a patient, is far more complex than *in vitro* system, which is closely controlled. This implies that evaluation of the effects of these extracts *in vitro* provides an opportunity to help define the *in vivo* modulatory action of these medicinal plants, provided the concentrations used *in vitro* are not toxic for the cells. The immunomodulatory effects of these extracts in this study, may explain, at least some of the known therapeutic effects of these plant products. *H. procumbens, W. salutaris* and *A. annua*, probably acts by stimulating cell-mediated immune mechanisms. The anti-inflammatory effect exhibited

by *H. procumbens* confirms its wide use for rheumatoid arthritis and related inflammatory disorders. The present study is the first one to show the *in vivo* antimalarial activity of this extract and as such, these results would justify the anti-inflammatory activity obtained as being beneficial in alleviating severe malaria symptoms as pro-inflammatory cytokines play a crucial role in the pathogenesis of disease. *W. salutaris* is widely used in South Africa, but this is the first time this extract have been investigated for its antimalarial activity and its effect on immune response. The present results, showing anti-inflammatory activity, are consistent with the favourable testimonials of patients and traditional healers who have used this plant. The results of this study support to certain degree the traditional medicinal uses of the plants evaluated and reinforce the concept that the ethnobotanical approach to screening plants as potential sources of bioactive substances is successful.

CHAPTER 6

6.1 Conclusions

In acute malaria infection in humans, the sequence of cellular and immunological events and molecular determinants of protection and immunity, as well as pathogenic mechanisms and eventually to death in malaria are still insufficiently understood. A better understanding of these mechanisms will benefit on the development of new drugs and therapies and eventually a vaccine for malaria. The studies performed in this thesis, are focused around understanding the sequence of events leading to cerebral malaria, determinants of immunity, intervention with known antimalarial drugs and a selection of traditional medicines used to treat malaria in South Africa and their effect on immune response.

The role of the pro-inflammatory cytokines in the pathogenesis of murine cerebral malaria has been investigated in this study. Different wild type mice and receptor knockout mice were investigated in the course of infection with *Plasmodium berghei* ANKA, which induces murine cerebral malaria. The early events in the spleen of mice infected experimentally with *Plasmodium chabaudi chabaudi* AS infections, have been studied histopathologically in detail. Immune cell kinetics have been characterised by immunohistochemistry, to evaluate population size, timing and anatomical localization of various immune cells in the spleen of the self-resolving *Plasmodium chabaudi* AS infection in C57BL/6 mice and the fatal infection in A/J mice. The effects of intervention in experimental murine malaria with known antimalarial drugs artesunate and chloroquine are described. Artesunate, a derivative of the natural occurring antimalarial artemisinin, was compared with chloroquine in murine *Plasmodium berghei* ANKA (PbA) infection. The comparative effects of artesunate and chloroquine in identical *in vitro* conditions in their respective influence on production of pro-inflammatory and anti-inflammatory cytokines, thought to play an important role in the pathogenesis of severe malaria, have been studied. Finally, the study investigated the *in vivo* antiplasmodial activity of several plants traditionally used to treat malaria in South Africa. The effects of the three promising extracts (with demonstrated antiplasmodial activity) on the production of pro-and-anti-inflammatory cytokines have been evaluated.

6.1.1 Pro-inflammatory cytokines in the pathogenesis of murine cerebral malaria

Cerebral malaria induced in mice by *Plasmodium berghei* ANKA only partly mirrors the infection (cerebral malaria) in humans. Th1 cytokines, mainly TNF-α and IFN-γ have been shown to play a crucial role in the pathogenesis of cerebral malaria (Grau G et al., 1990). We attempted to verify the involvement of these cytokines by following the course of infection in a PbA mouse model system. We used TNF-double deficient receptor mice (TNF-α/β) as well as mice deficient in IFN-γ receptor. Our results revealed that mutant mice (TNF-$\alpha/\beta^{-/-}$ and IFN-$\gamma^{-/-}$) were resistant to cerebral malaria. No neurological symptoms were displayed by these mice; only by wild type mice. In the present study, we asked whether endogenous TNF and IFN-γ has any influence on parasitaemia and on haematological alterations in PbA infected mice. Both the wild type and the mutant mice developed a similar degree of parasitaemia in the initial phase of infection, showing that both TNF and IFN-γ have no effect on the parasitization of erythrocytes. Mutant mice surviving much longer, reached a parasitaemia of approximately 70% by 17-20 days post-infection. The main haematological complications observed were anaemia, leukocytosis and thrombocytopenia. Anaemia and leukocytosis in wild type mice are not different from that occurring in mutant mice. Both anaemia and mobilization of leukocytes occur in the absence of TNF and IFN-γ. This study therefore is consistent with the hypothesis that there is local production of TNF and IFN-γ during murine PbA infection, playing a crucial role in the pathogenesis of cerebral malaria. Wild type mice are susceptible to neurological complications attributed to cerebral malaria and the mutant mice are resistant. Anaemia and mobilization of leukocytes occur in the absence of both TNF and IFN-γ, but thrombocytopenia is greatly influenced by these cytokines. We conclude that TNF-α and IFN-γ are necessary for the development of murine cerebral malaria. This study has shown that $TNF^{-/-}$ and IFN-$\gamma^{-/-}$ mice are resistant to PbA-induced cerebral malaria, confirming the role of Th1 cytokines in the pathogenesis of cerebral malaria.

6.1.2 Early events in the spleen of mice infected with *Plasmodium chabaudi* AS infection

Experiments using murine malaria models have shown the spleen to be an important organ involved in the control of the blood stage (the disease causing stage) of the disease. The microstructure of the spleen provides an environment, which compartmentalises cells and allows for specific cell-cell interaction over time course of blood borne antigenic exposure. Little is known about the movement or kinetics of spleen immune cells during malaria. Splenomegaly is a hallmark of malaria infection in inbred mouse strains that self resolve infection. In this study, it has been possible to follow the changing characteristics and localization of immune cells in the hyperplasic white pulp and in the red pulp where immune complexes would take place. Despite an apparent rapid deterioration in splenic architecture as observed by H&E staining, IHC confirmed a surprising level structure and organization. Of special interest was the movement of DC's (CD11c positive cells) from the marginal zone of the PALS into the $CD4^+$ T cell area (where their antigen presenting function would be maximal). Because of this juxtaposition of the CD11c (dendritic cells) positive cells and the apparently T cells, could be associated with the immune complexes taking place in the spleen during malaria infection. Activation of T-cells by antigen presenting cells is required to initiate specific immune responses.

Dendritic cells are a unique type of antigen presenting cells because of their ability to induce primary immune response by efficient activation of T-cells. It could be possible that in our model system following activation of the dendritic cells and antigen capture in the marginal zone, they migrate to the lymphoid organs where they prime antigen-specific $CD4^+$ T cells. Other APC's (B cells and macrophages defined as $CD45^+$ and $F4/80^+$ respectively) showed no migration over time. This suggests a critical role of $CD11c^+$ cells in antigen presentation to naïve T cells and a limited role for other APC phenotypes during murine malaria infection. The dynamic CD11c change in location within the spleen is especially interesting in the light of the early (day 3 post-infection) IL-12 production by $CD8\alpha^+$ subset of DC (Leisewitz *et al.*, personal communication). Similar migration and IL-12 production have been described in response to soluble extracts of *Toxoplasma gondii*. $CD8\alpha^+$ DC have been

shown to be important in inducing a shift to a shift a Th1 response in mice. These findings have not been observed in murine malaria infections.

6.1.3 Oral artesunate prevents *Plasmodium berghei* ANKA infections in mice and the effect of artesunate on cytokine production

The efficacy of oral artesunate was compared with that of chloroquine in *Plasmodium berghei* ANKA infected mice. It was confirmed that artesunate has potent anti-malarial activity in this murine infection by oral administration, which is comparable to that of chloroquine. A 7-day administration of artesunate prevented parasitaemia at >= 10 mg/kg. However, recrudescence of parasitaemia and cerebral malaria occurred upon cessation of treatment followed by death within 28 days. A 14-day course of artesunate treatment prevented completely the development of parasitaemia and cerebral malaria with a survival of more than 60-days at 100-mg/kg artesunate or 10-mg/kg chloroquine. This data demonstrated that oral artesunate inhibits PbA and prevents cerebral malaria, but needs to be administered for prolonged time and at higher doses than chloroquine to eradicate PbA infection. We conclude that short course artesunate is not efficacious. However, a 14 days oral administration of artesunate eradicates PbA infection in mice and prevents the development of cerebral malaria, which is equivalent to the chloroquine effect. Therefore, prolonged administration of oral artesunate is an effective alternative treatment of chloroquine resistant malaria.

There have been very few direct comparisons of artesunate and chloroquine in their effect on cytokine production. Cytokines interact via a network and play a vital role in various types of intercellular communication. The excessive production of distinct pro-inflammatory cytokines is important in the pathogenesis of malaria. Today it is well established that the balance between functionally distinct cytokine-producing T cells of the CD4 or CD8 phenotype, determines whether an individual will succumb to the disease or not [Abbas A *et al.*, 1996]. These cell types are distinguished by their capacity to regulate pro-inflammatory and anti-inflammatory responses. In the present study, the effects of both chloroquine and artesunate in identical *in vitro* conditions were compared with respect to the production of TNF-α, IFN-γ and IL-12

(pro-inflammatory cytokines) and IL-10 (anti-inflammatory cytokine), by ELISA. Our results show that artesunate causes no inhibition of pro-inflammatory cytokines. We observed that at 10 μM concentrations, artesunate enhances IL-10 production. In contrast, chloroquine showed a significant concentration dependent inhibition of TNF-α, IFN-γ and IL-12p40. At 10 μM concentrations, chloroquine enhanced IL-10 production. This data therefore suggest that chloroquine, at concentrations corresponding to levels reached during standard treatment for malaria patients (Hellgren U et al. 1989), interferes with the balance of Th1 and Th2 cells by affecting the state of the cytokines produced by these cells. These findings suggest that artesunate exerts anti-inflammatory activity by enhancing IL-10 production, a critical component in the down-regulation of the pro-inflammatory immune reactions and the upregulation of the anti-inflammatory responses. Thus, artesunate probably influences the direction of an emerging immune response by indirectly affecting anti-inflammatory cytokine production.

6.1.4 *In vivo* antiplasmodial activity of plants traditionally used to treat malaria in South Africa and their effect on immune response

Traditional remedies have been the source of important antimalarial drugs (e.g. quinine and artemisinin) and might continue to provide novel and effective treatments, both where pharmaceuticals are not available and also where the disease is highly resistant to commonly prescribed drugs. There is an urgent need to develop new antimalarial drugs since the treatment and the control of the disease relies mostly on chemotherapeutic agents. There are an estimated 200 000 indigenous traditional healers in South Africa, and up to 80% of South Africans consult these healers, usually in addition to using modern biomedical services. In this thesis, ethnobotanical data on medicinal plants that are used to treat malaria and other fevers are presented, based on first-hand information collected from traditional healers, ordinary villagers, as well as through personal observations of applications of herbal remedies administered by traditional healers. Most of the traditional healers interviewed had an understanding of malaria. There were some similarities that we observed amongst the traditional healers as far as the understanding of malaria, throughout the areas visited. A series of plants used by traditional healers were collected and evaluated for their

antimalarial activity and safety. The aqueous, methanolic, and dichloromethane extracts of eight medicinal plants used to treat malaria in South Africa were evaluated for their antimalarial activity *in vivo*, in a 7-day, suppressive test against *Plasmodium berghei* ANKA in mice. No toxic effect or mortality was observed in mice treated orally with any of the extracts daily for 7 days at a dose of 500 mg/kg-body weight. The dichloromethane extract of the bark of *W. salutaris* reduced parasitaemia by >50% at an oral dose of 100 and 500-mg/kg-body weight. The petroleum ether extract of *H. procumbens* exhibited mild chemosuppression of 36% and a dose of 200 mg/kg-body weight. The aqueous extract of the leaves of *A. annua* reduced parasitaemia by up to 64% at high dose of 200 mg/kg-body weight.

These results of the present study appear to justify the use of these medicinal plants as traditional medicines for the treatment of malaria in South Africa. But most of the plants tested did not show any antimalarial activity. Of the fifteen extracts tested for toxicity in the C57BL/6 mice, it was interesting to note that none of these extracts showed any toxic effect in our test system. It was interesting to note that the majority (two of three extracts) of the anti-plasmodial activity was present in organic solvent extracts. Traditionally, plant extracts are prepared with water (e.g. infusions, decoctions and poultices). Bearing in mind that the biological activity in our *in vivo* test system, serves as a guide whether these plant exhibits any activity, a conclusion based on our results that traditional medicines in South Africa have no role in controlling malaria could be wrong. It is therefore necessary for the traditional healers and scientific community to work in collaboration for the eradication of malaria in South Africa. Clarkson *et al.*, 2002 demonstrated the *in vitro* antiplasmodial activity for *H. procumbens* and this motivated for the determination of the *in vivo* antiplasmodial activity. Despite wide range use of *W. salutaris* and *H. procumbens* in South Africa, this is the first study to confirm the *in vivo* antiplasmodial activity of these medicinal plants.

To test whether medicinal plant products, have immunomodulatory properties, we studied the immunobiological effects of three extracts with promising antimalarial activity, by examining their effects on TNF-α, IFN-γ, IL-12 and IL-10 production on ConA and LPS induced splenocytes. These may act either by stimulating both cell mediated and humoral immunity or only cell mediated immune response while

suppressing the humoral component of the immune system. The results demonstrate that *H. procumbens* has immunomodulatory properties and that its action might be mediated by anti-inflammatory activity. *W. salutaris* showed a significant inhibition of TNF-α, IL-10 and IFN-γ but in contrast, we observed an enhanced effect of IL-12p40. These conflicting results do not seem to be specific with the kind of effect this extract might possess. *A. annua* showed a significant inhibition of pro-inflammatory cytokines and an enhanced effect of IL-10 at high concentration (100 μg/ml). In conclusion, these studies suggest that medicinal plants used to treat malaria and other ailments in South Africa, exhibit some immunological modulation, which may contribute to the eradication of symptoms, related to malaria.

Extrapolation of *in vitro* results to the clinical situation is not straightforward. An *in vivo* system, as in a patient, is far more complex than *in vitro* system, which is closely controlled. This implies that evaluation of the effects of these extracts *in vitro* provides an opportunity to help define the *in vivo* modulatory action of these medicinal plants, provided the concentrations used *in vitro* are not toxic for the cells. Immunomodulatory activity of these extracts, may explain, at least some of their known therapeutic effects. *H. procumbens, W. salutaris* and *A. annua*, probably act by stimulating cell-mediated immune mechanisms in addition to direct antimalarial activity.

In conclusion, the results obtained in this thesis indicate exciting new research possibilities for future investigations, of malaria treatment and understanding the pathogenesis of cerebral malaria pathogenesis and molecular determinants of malaria immunity.

CHAPTER 7

7. Future investigations

Animal models have been paramount in our understanding of the pathogenesis of cerebral malaria. They have established that cerebral malaria is caused not just by changes in the brain but rather other factors, in particular high levels of pro-inflammatory cytokines. Cerebral malaria is therefore a complicated neurological syndrome involving interactions between cytokines and cellular environment. High levels of the pro-inflammatory cytokines TNF and gamma interferon up-regulate a number of adhesion molecules, including ICAM-1 (Hviid *et al.*, 1993), CD36 and thrombospondin (Newbold C *et al.*, 1997) on vascular endothelia of the major organs of the body, including the brain. However, the ideal animal model displaying the precise clinical characteristics of human malaria is not yet available. However, Hearn J *et al.*, 2000 have shown that the murine model of PbA in (BALB/c X C57BL/6)F_1 has several features in common with human cerebral malaria and may be useful in future work unravelling the underlying features of the disease. Careful time course studies to correlate the host immune response with micro-environmental changes that occur at the onset of, and/or during the development of the cerebral disease, may reveal processes that are amenable to novel therapeutic strategies.

Some rodent malaria infections are lethal only in certain strains of mice. This allowed us to investigate the mechanisms leading to susceptibility or resistance to infection using the *P. chabaudi* AS. In non-lethal strain C57BL/6 and susceptible strain A/J mice, resistance and susceptibility is associated with responses in the spleen. Development of splenomegaly in C57BL/6 has proven to be a phenotypic marker for resistance in this mouse strain. Susceptible A/J mice only develop minimal splenomegaly and higher parasitaemias than C57BL/6 mice. Our study has shown that the immune cell kinetics in both strains is similar and that resistance may be associated with cytokines produced by both strains. Treatment of susceptible A/J mice with either recombinant TNF or rIL-12 can rescue them from an otherwise lethal infection of *P. chabaudi* AS (Moham K *et al.*, 1999). Further work is necessary to elucidate the role of cytokine signals involved in the changes in cellular architecture in the *P. chabaudi* AS mouse model system. We have shown over time that T-cells and dendritic cells are distributed throughout the white pulp. We believe that dendritic cells may be the principal IL-12 producing cells (Leisewitz *et al.*, personal

communication) as early as day 3 post-infection. Further work with double or triple-staining to identify the co-localization of T-cells, dendritic cells and IL-12 would add to understanding of these events.

Previous studies have shown that chloroquine inhibits the production of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-6 (Picot S *et al.*, 1993, Landewe R *et al.*, 1992, van den Borne B *et al.*, 1997 and Jeong J *et al.*, 1997). Our data confirms this and support the observation that chloroquine inhibits the production of pro-inflammatory cytokines. Hugosson *et al.*, 2002 demonstrated that chloroquine enhances the number of IL-10 producing cells, which also coincided with an increase in the $CD14^+$ populations and the expression of costimulatory molecules and adhesion molecules. In our study both chloroquine and artesunate at 10 μM concentrations, enhanced the production of IL-10. Monocytes, Th2 cells, mast cells and B cells produce IL-10. To demonstrate which cells are induced to produce this cytokine upon chloroquine and artesunate treatment, will be of great interest. Landewe *et al.*, 1995 demonstrated that chloroquine inhibited the production of IL-2, which is important for proliferation of $CD4^+$ T-cells. Different immune cells produce different cytokines. The evaluation of the production of different cytokines by Fluorescent Activated Cell Sorter (FACS) analysis, would demonstrate the type of cells that produce the cytokines. Different spleen cell might be evaluated, including CD3, CD4, CD8, macrophages (F4/80), DC's (CD11c), B-cell (CD45/B220) and natural killer cells (N.K1.1). It would be advisable to use methods other than ELISA to detect cytokine production at different level. ELISA measures protein secretion, it will therefore be useful to use RT-PCR; assessing gene transcription and allows gene expression to be quantified and ELISPOT, which assesses protein secretion and/or membrane bound expression.

This thesis has shown for the first time the promising antimalarial activity of *W. salutaris* and *in vivo* antimalarial activity of *H. procumbens*. *A. annua* has been shown to possess *in vivo* antimalarial activity and this was attributed to the presence of artemisinin. We have shown the antimalarial activity of the South African grown *A. annua* and more interestingly of the aqueous extract. The extract of *H. procumbens* has been shown to possess *in vitro* antimalarial activity and bioassay-

guided fractionation yielded two active diterpenes that were identified as $C_{20}H_{30}O_2$ and $C_{20}H_{30}O$ (Clarkson C *et al.*, 2002). Further work on screening of this compound for its *in vivo* activity using the PbA mouse model system is necessary. Previous screening of the South African medicinal plant *W. salutaris* showed that the plant had promising antibacterial activity (Rabe T *et al.*, 2000). Fractionation of the ethyl acetate extract of the stem bark by chromatographic techniques yielded a sesquiterpenoid, which exhibited antimicrobial activity against gram-positive bacteria. The antimalarial activity observed in our study warrants further studies using bioassay-guided fractionation to identify the active compound. Further work on *A. annua* by bioassay-guided fractionation should be conducted to identify active principles.

Evaluation of these compounds for their effect on immune response could also be studied. It is also possible that medicinal herbal remedies once ingested, are converted into an active metabolite. Metabolism of these compounds might be tested *in vivo* to determine if they are metabolised into an active form.

References

References

Abbas A. K., Murphy K. M., Sher A., Functional diversity of helper T lymphocytes, Nature 387 (1996) 787-793

Aikawa M., Iseki M., Barnwell J., Taylor D., Maung Maung OO and Howard R, The pathology of human malaria, Am. J. Trop. Med. Hyg. 43 (2) (1990) 30-37

Akdis C. A., Blaser K., IL-10-induced anergy in peripheral T-cell and reactivation by microenvironmental cytokines: two key steps in specific immunotherapy. FASEB J. (1999); 19:603-609.

Angus B. J., Chotivanich K., Udomsangpetch R and White N. *In vivo* removal of malaria parasites from red blood cells without their destruction in acute falciparum malaria. Blood (1997); 90: 2037-2040

Atal C., Sharma M., Kaul A., and Khajuria A., Immunomodulating agents of plant origin 1: Preliminary screening. J. Ethnopharmac. (1986); 18: 133-141.

Austyn J. M. Antigen-presenting cells. Am. J. Respir. Crit. Care Med. (2000); Vol 162: S146-S150.

Balint G. A., Artemisinin and its derivatives: An important new class of antimalarial agents. Pharmocology and Therapeutics. (2001); 90: 261-265.

Banchereau J., Briere F., Caux C., Davoust J., Lebecque S., Liu Y., Pulendran B., and Palucka K., Immunobiology of dendritic cells. Annu Rev. Immunol. (2000); 18: 767-811

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

Bell D., Young J. W., Banchereau J. Dendritic cells. Adv. Immunol. 72: 255-324.

Berendt A. R., *et al.*, Molecular mechanisms of sequestration in malaria. Parasitology. (1994); 108: S19-S28.

Bojang K., Palmer A., Boele van Hensbroek M., Banya W and Greenwood B. Management of severe malarial anaemia in Gambian children. Trans R Soc trop Med Hyg. (1997) Sep-Oct; 91 (5): 557-561

Bojang K., Scheider G., Forck S., A trial of Fansidar plus chloroquine or Fansidar alone for the treatment of uncomplicated malaria in Gambian children. Trans. R. Soc. Trop. Med. Hyg. (1998); 92: 73-76.

Bouharoun-Tayoun H., Oeuvray C., Lunel F., Druilhe P., Mechanisms underlying the monocytes-mediated antibody-dependent killing of *P. falciparum* asexual blood stages. J. Exp. Med. (1995); 182: 409-418.

Brewster D. R., Kwiatkowski D., White N. J., Neurological sequelae of cerebral malaria in children. Lancet. (1990); 336: 1039-1043

Bruna-Romero O., Rodriguez A., Dendritic cells can initiate protective immune responses against malaria. Infection and Immunity, Aug. 2001; 5173-5176.

Calvosa V. S., Adagu I. S., and Povoa M. M., *Plasmodium falciparum*: emerging mefloquine resistance *in vitro* in Para State North of Brazil. T. Roy. Soc. Trop. Med. Hyg. (2001); 95: 330-331

Caniato R., and Puricelli L., Natural antimalarial agents (1995-2001). Critical Reviews in Plant Sciences. (2003); 22(1): 79-105.

Carvalho L. H., Sano Gi. G., Hafalla J. C., Morrot A., de Lafaille M. A., Zavala F., IL-4 secreting $CD4^+$ T-cells are crucial to the development of $CD8^+$ T-cell responses against malaria liver stages. Nat. Med. (2002); 8: 166-170.

Chantre P., Cappalaere A., Leblan D., Guedon D., Efficacy and tolerance of *Harpagophytum procumbens* versus diacerhein in treatment of osteoarthritis. Phytomedicine. (2000); 7: 177-183.

Chavallier A., The Encyclopaedia of Medicinal Plants. Dorling Kindersley Limited, London. (1996).

Chen M., Zhu Z., Wang Z., Mo H., Yang Z., and Zhang M., Effect of sodium artesunate on immune function of animals. Acta Guangxi Med. Coll. (1988); 5: 42-44.

Chiwakata C. B. *et al.*, High levels of inducible nitric oxide synthase mRNA are associated with increased monocytes counts in blood and have a beneficial role in *Plasmodium falciparum* malaria. Infect. Immun. (2000); 68: 394-399.

Chotivanich K., Udomsangpetch R., McGready R., Proux S., Newton P., Pukrittayakamee S., Looareesuwan S., and White N. J., Central role of the spleen in malaria parasite clearance. J. Infect. Dis. (2002); 185: 1538-1541.

Clark AI, Cowden WB,. Roles of TNF in malaria and other parasitic infections. Immunol Ser, (1992); 56: 365-407.

Clark I. A. *et al.*, The biological basis of malarial disease. Int. J. Parasitol. (1997); 27: 1237-1249.

Clark I. A., Cowden W. B., Roles of TNF in malaria and other parasitic infections. Immunol Ser. (1992); 56: 365-407.

Clark I. A., Hunt N. H., Butcher G. A., Cowden W. B., Inhibition of murine malaria *Plasmodium chabaudi in vivo* by recombinant interferon-gamma or tumour necrosis factor, and its enhancement by butylated hydroxyanisole. J Immunol. (1987); 139: 3493-3496.

Clark I., Ilschner S., MacMicking J., Cowden W., TNF and Plasmodium berghei ANKA-induced cerebral malaria. Immunol. Lett. (1990); 25: 195-198.

Clark I., Tumour necrosis factor may contribute to the anaemia of malaria by causing dyserythropoiesis and erythrophagocytosis. Br. J. Haematol. (1988); 70: 99-103.

Clark I., and Rockett K., The cytokine theory of human cerebral malaria. Parasitol. Today. (1994); 10: 410-412.

Clarkson C., Isolation and characterisation of two antiplasmodial diterpenes from *Harpagophytum Procumbens* (Devil's Claw) and chemical modification of a related analogue. PhD thesis. (2002); University of Cape Town, South Africa.

Crutcher J. M., Stevenson M. M., Sedegah M., Hoffman S. L., Interleukin-12 and malaria. Res Immunol. (1995); 146: 552-559.

de Kossodo S., Grau G., Role of cytokines molecules in malaria immunopathology. Stem Cells (Dayt) (1993); 11: 41-48.

de Kossodo S., Monso C., Juillard P., Velu T., Goldman M., Grau G. E., Interleukin-10 modulates susceptibility in experimental cerebral malaria. Immunology. (1997); 91: 536-540.

de Souza B., Williamson K., Otani T., and Playfair J., Early gamma interferon responses in lethal and non-lethal murine blood stage malaria. Infect. Immun. (1997); 65: 1593-1598.

Dhingra V., Rao K. W., and Narasu M. L., Current status of artemisinin and its derivatives as antimalarial drugs. Life Sciences. (2000); 66: 279-300.

Ding L., Linsley P., Huang L., Germain R., Shevach E., IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J. Immunol. (1993); 151: 1224-1234.

Dondorp A. M. et al., Nitric oxides in plasma, urine, and cerebrospinal fluid in patients with severe falciparum malaria. Am. J. Trop. Med. Hyg. (1998); 59: 497-502.

Eckwalanga M., Marussig M., Tavares M. D., et al., Murine AIDS protects mice against experimental cerebral malaria: down-regulation by interleukin-10 of a T helper type 1 $CD4^+$ cell-mediated pathology. Proc. Natl. Acad. Sci. USA. (1994); 91: 8097.

Elford B., Roberts M., Phillpson J., and Wilson R., Potentiation of the antimalarial activity of qinghaosu by methoxylated flavones. Trans. Roy. Soc. Trop Med. Hyg. (1987); 81: 434-436.

Eling W. M. C., Sauerwein R. W., Severe cerebral malaria: common or distinct pathophysiology? Rev. Med. Micro. (1995); 6: 17-25

Essner R., Rhoades K., McBride W. H., Morton D. L., Economou J. S., IL-4 down-regulates interleukin-1 and TNF gene expression in human monocytes. J. Immunol. (1989); 142: 3857-3861.

Favila-Castillo L., Monry-Ostria A., Kobayashi E., Hirunpecharat C., Kamada N. Good M. Protection of rats against malaria by a transplanted immune spleen. Parasite Immunol. (1996); 18: 325-331.

Favre N., Da Laperousaz C., Ryffel B., Weiss N., Imhof B., Rudin W., Lucas R., and Piguet P., Role of ICAM-1 (CD54) in the development of murine cerebral malaria. Microbes and Infection. (1999); 1: 961-968.

Finley R., Mackey and Lambert P., Virulent P. berghei malaria: prolonged survival and decreased cerebral pathology in T-cell deficient nude mice. J. Immunol. (1982); 129: 2213-2218.

Fiorentino D. F., Zlotnik A., Mosmann T. R., Howard M., O'Garra A., IL-10 inhibits cytokine production by activated macrophages. J. Immunol. (1991); 147: 3815-3822.

Fitch C. D., Antimalarial schizontocides: ferriprotoporphyrin IX interaction hypothesis. Parasitology Today. (1986); 2: 330-331

Francois G., Passreiter C. M., Woerdenbag H. J., and van Looveren M. Antiplasmodial activities and cytotoxic effects of aqueous extracts and sesquiterpene lactones from *Neurolaena lobata*. Planta Medica. (1996); 62: 126-129.

Francois G., Timperman G., Eling W., Ake Assi L., Holenz J., and Bringmann G., Naphthoisoquinoline alkaloids against malaria: evaluation of the curative potentials of dioncophylline C and dioncophylline A against *Plasmodium berghei in vivo*. Antimicrobial Agents and Chemotherapy. (1997); 41: 2533-2539.

Garcia I, Miyazaki Y, Lesslauer W, Vasalli P,. Transgenic mice expressing high levels of TNF-R1 fusion protein are protected from lethal septic shock and cerebral malaria, and are highly sensitive to *Listeria monocytogenes* and *Leshmania major* infections. Eur J Immunol, (1995); 25: 2401-2407.

Gately M. K., Renzetti L. M., Magram J., Stern A. S., Adorini L., Gubler U., Presky D. H., The interleukin-12/interleukin-12 receptor system: role in normal and pathogenic immune responses, Annu. Rev. Immunol. (1998); 16: 495-521.

Geary T. G., Divo A. D., Jensen J. B., Zangwill M., and Ginsburg H., Kinetic modelling of the response of *Plasmodium falciparum* to chloroquine and its experimental testing *in vitro*. Implications of mechanism of action and resistance to the drug. Biochem. Pharmacol. (1990); 40: 685-691.

Ghisalberti E., Biological and pharmacological activity of naturally occurring iridoids and secoiridoids. Phytomedicine. (1998); 5: 147-163.

Good M. F., Kaslow D. C., Miller L. H., Pathways and strategies for developing a malaria blood-stage vaccine, Annu. Rev. Immunol. 16 (1998) 57-87.

Grace J. M., D. J. Skanchy, and A. J. Aguilar,. Metabolism of artenilic acid to dihydroartemisinin by human liver cytochrome P450 3A. Xenobiotica. (1999); 29:703-717.

Grahame R., Robinson B., Devil's Claw (*Harpagophytum procumbens*): pharmacological and clinical studies. Ann Rheum Dis. (1981); 49: 632.

Grau G. E., Piguet P. F., Vassalli P., and Lambert P. H., Tumour necrosis factor and other cytokines in cerebral malaria: experimental and clinical data. Immunol. Rev. (1989); 112: 49

Grau G. E., Taylor T. E., Molyneux M. E., Wirima J. J., Vasalli P., Hommel M., Lambert P. H., Tumour necrosis factor and disease severity in children with falciparum malaria, N Engl. J. Med. 320 (1989) 1586-1591.

Grau G., and de Kossodo S., Cerebral malaria: Mediators, mechanical obstruction or more? Parasitol. Today. (1994); 10: 408-409.

Grau G., Fajardo L., Piguet P., Allet B., Lambert P., Vassali P., TNF (cachectin) as an essential mediator in murine cerebral malaria. Science (1987); 237: 1210-1212.

Grau G., Piguet P., Engers H., Louis P., Vassalli., and Lambert P., $L3T4^+$ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. (1986); 137: 2348-2354.

Greenwood B. M., Bradley A. K., Greenwood A. M., *et al.*, Mortality and morbidity from malaria among children in a rural area of the Gambia. Trans. R. Soc. Trop. Med. Hyg. (1987); 81: 478-486.

Hatcher F., Kuhn R., Cerrone M., and Burton R., Increased natural killer cell activity in experimental American trypanasomiasis. J. Immunol. (2001); 127: 1126-1130.

Hawley S. R., Bray P. G., O'Neill P. M., Park B. K., and Ward S. A., The role of drug accumulation in 4-aminoquinoline antimalarial potency. The influence of structural substitution and physicochemical properties. Biochem. Pharmacol. (1996); 52: 723-733.

Hearn J., Rayment N., Landon D., Katz D., and de Souza B., Immunopathology of cerebral malaria: Morphological evidence of parasite sequestration in murine brain microvasculature. Infect. And Immun. (2000); 68(9): 5364-5376.

Hellgren U., Kihamia C., Mahikwano L., Bjorkman A., Eriksson O., Rombo L., Response of Plasmodium falciparum to chloroquine treatment: relation to whole blood concentrations of chloroquine and desethylchloroquine. Bull. WHO. (1989); 67: 197-202.

Heufler C., Koch F., Stanzl U., Topar M., Schuler G., IL-12 is produced by dendritic cells and mediates Th1 development as well as IFN-gamma by Th1 cells. Eur. J. Immunol. (1996); 26: 659-668.

Ho M., Webster H. K., T-cell responses in acute falciparum malaria. Immunol. Lett. (1990); 25: 135-138

Holloway P. A., Krishna S., and White N. J., *Plasmodium berghei*: lactic acidosis and hypoglycaemia in a rodent model of severe malaria; effects of glucose, quinine and dichloroacetate. Exp Parasitol. (1991); 72: 123-133

Homewood C. A., Warhurst D. C., Peters W., Baggaley V. C., Lysosomes, pH and the antimalarial action of chloroquine. Nature. (1972); 235: 50-52.

Hutchings A., Zulu Medicinal Plants. An Inventory, University of Natal Press, Pietermaritzburg. (1996).

Imhof B., Dunon D., Leukocyte migration and adhesion. Adv. Immunol. (1995); 58: 345.

Jacobs P., Radzioch D., and Stevenson M., A Th1-associated increase in tumour necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. Infect. Immun. (1996); 64(2): 535-541.

Jacobs P., Radzioch D., Stevenson M., A Th1-associated increase in TNF-α expression in the spleen correlates with resistance to blood stage malaria in mice. Infect. Immun. (1996); 64: 535-541.

Janota I., Doshi B., Cerebral malaria in the United Kingdom. J. Clin. Pathol. (1979); 32: 769-772.

Jansen F. H., Artesunate and Artemether: Towards the eradication of malaria? Dafra Pharma LTD, (2002).

Jason J., Archibald L. K., Nwanyanwu O. C., et al., Cytokines and malaria parasitaemia. Clin. Immunol. (2001); 100: 208-218.

Jeong J., Choi J., Jeon K., Jue D., Chloroquine decreases cell-surface expression of tumour necrosis factor receptors in human histiocytic U-937 cells. Immunology (2002); 105: 83-91.

Jeong J., Jue D., Chloroquine inhibits processing of TNF in LPS-stimulated RAW 264.7 macrophages. J. Immunol. (1997); 158: 4901-4907.

Jung M., Li X., Bustos D. A., Elsohly H. N., McChesney J. D., and Milhous W. K., J. Med. Chem. (1990); 33: 1516-1518.

Karbwang J., Bunnag D., and Harinasuta T., Comparison of oral artesunate and quinine plus tetracycline in acute uncomplicated falciparum malaria. WHO Bulletin OMS. (1994); 72(2): 233-238.

Karres I., Kremer J., Dietl I., Steckholzer U., Jochum M., Ertel W., Chloroquine inhibits pro-inflammatory cytokine release into human whole blood. Am. J. Physiol. (1998); 274: R1058-1064.

Kawo N. G., Msengi A. E., Swai A. B., Chuwa L. M., Alberti K. G., McLarty D. G., Specificity of hypoglycaemia for cerebral malaria in children. Lancet. (1990); 336: 454-457

Klayman D. Qinghaosu (artemisinin): an antimalarial drug from China. Science. (1985); 228: 1049-1055.

Krogstad D. J., Gluzman I. Y., Kyle D. E., Oduala A. M., Martin S. K., Milhous W. K., and Schlesinger P. H., Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science. (1987); 238: 1283-1285.

Kumaratilake L. M., Ferrante A., IL-4 inhibits macrophage-mediated killing of *Plasmodium falciparum in vitro*. A possible parasite-immune evasion mechanism. J. Immunol. (1992); 149: 194-199.

Kurtzhals J. A., Adabayeri V., Goka B. Q., et al., Low plasma concentrations of IL-10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. Lancet. (1998); 351: 1768-1772.

Kwiatkowski D., Bate C., Inhibition of tumour necrosis factor production by antimalarial drugs used in cerebral malaria. Trans. Roy. Soc. Trop. Med. Hyg. (1995); 89: 215-216.

Kwiatkowski D., Cannon J. G., Manogue K. R., Cerami A., Dinarello C. A., and Greenwood B. M., Tumour necrosis factor production in Falciparum malaria and its association with schizont rupture. Clin. Exp. Immunol. (1989); 77: 361-366

Kwiatkowski D., Cytokines and the anti-disease immunity to malaria. Res Immunol. (1991); 142: 707-712

Kwiatkowski D., Genetic susceptibility to malaria getting complex, Curr. Opin. Genet. Dev. 10 (2000) 320-324.

Kwiatkowski D., Hill A. V., Sambou I., TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet (1990); 336: 1201-1204

Kwiatkowski D., Hill A., Sambou I., TNF concentration in fatal, non-fatal cerebral and uncomplicated *Plasmodium falciparum* malaria. Lancet. (1990); 336: 1201-1204

Kwiatkowski D., Molyneux M. E., Stephens S., Curtis N., Klein N., Pointaire P., Smith M., Allan R., Brewster D. R., Grau G. E., and Greenwood B. M., Anti-TNF therapy inhibits fever in cerebral malaria. Q. J. Med. (1993); 86: 91-98

Landewe R., Miltenburg A., Breedveld F., Daha M., Dijkmans B., Cyclosporine and chloroquine synergistically inhibit the IFN-gamma production by $CD4^+$ and $CD8^+$ T-cell clones derived from a patient with rheumatoid arthritis. J. Rheumatol. (1992); 19: 1353-1357.

Landewe R., Miltenburg A., Verdonk M., Verweij C., Chloroquine inhibits T cell proliferation by interfering with IL-2 production and responsiveness. Clin Exp. Immunol. (1995); 102: 144-151.

Langhorne J., Gillard S., Simon B., Slade S., and Eichmann K., Frequencies of $CD4^+$ T-cells reactive with *Plasmodium chabaudi*: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. Int. Immunol. (1989); 1: 416-424.

Lanhers M., Fleurentin J., Mortier F., Vinache A., and Younos C., Anti-inflammatory and analgesic effects of an aqueous extracts of *Harpagophytum procumbens*. Planta Medica. (1992); 58(2): 117-123.

Lee I. S., and Hufford C. D., Metabolism of antimalarial sequiterpene lactones. Pharmacol. Ther. (1990); 48: 345-355

Lee S., Crocker P and Gordon S., Macrophage plasma membrane and secretory properties in murine malaria. J. Exp. Med. (1986); 163: 54-74.

Li W. H., Shu H., Xu G. Y., and Zeng Y. L., The binding of qinghaosu (artemisinin) and its derivatives to plasma protein. Acta Pharmacol Sin. (1982); 17: 783-786.

Likhitwatayawuid K., Kaewamatawong R., Ruangrungsi N., and Krungkrai J., Antimalarial naphthoquinones from *Nepenthes thorelii*. Planta Medica. (1998); 64: 237-241.

Lin X., Cheng F., Zhang L., Song Q., and Zhang H., Comparison of the immunosuppression of Qinghaosu and its derivatives. Shanghai J. Immunol. (1984); 4: 348-351.

Liu Y., Wei S. H., Ho A. S., de Waal Malefyt R., Moore K. W., Expression cloning and characterization of a human IL-10 receptor. J. Immunol. (1994) Feb 15; 152(4): 1821-1829.

Locksley R. M., Killeen N., Lenardo M. J., The TNF and TNF receptor superfamilies: intergrating mammalian biology. Cell (2001) Feb. 23; 104(4): 487-501.

Looareesuwan S., Chulay J. D., Canfield C. J., and Hutchinson D. B. A., Malarone (atovaquine and proguanil hydrochloride): a review of its clinical development for treatment of malaria. Am. J. Trop. Med. Hyg. (1999); 60: 533-541.

Lou J., Donati Y., Juillard P., Giroud C., Vesin C., Mili., and Grau G., Platelets play an important role in TNF-induced microvascular endothelial cell pathology. Am. J. Pathol. (1997); 151: 1397-1405.

Luty A. J., Lell B., Schmidt-Ott R., *et al.*, Interferon-gamma responses associated with resistance to re-infection with *P. falciparum* in young African children. J. Infect. Dis. (1999); 179: 980-988.

Luty A. J., Perkins D. J., Lell B., et al., Low interleukin-12 activity in severe *Plasmodium falciparum* malaria. Infect. Immun. (2000); 68: 3909-3915.

Luty A., Perkins D. J., Lell B *et al.*, Low interleukin-12 activity in severe *Plasmodium falciparum* malaria. Infection and Immunity. (2000); 68: 3909-3915.

Macatonia S. E., Hosken N., Litton., O'Garra A., Dendritic cells produce IL-12 and direct the development of Th1 cells from naïve $CD4^+$ T-cells. J. Immunol. (1995); 154: 5071-5079.

Maeno Y., Meshnick S., Aikawa M., A nonhuman primate model for human cerebral malaria: effects of artesunate on rhesus monkeys experimentally infected with *Plasmodium coatneyi*. American Journal of Tropical Medicine and Hygiene. (1993); 49: 485-491.

Malaguarnera L and Musumeci S. The immune response to *Plasmodium falciparum* malaria. The Lancet. (2002); 2: 472-478.

Malakoff D., The rise of the mouse, biomedicine mammal. Science. (2000) Apr.14.; 288(5464): 248-253.

Male D, Brostoff J and Roitt I; Immunology, Churchill Livingstone, 1995.

Maneerat Y., *et al.*, Inducible nitric oxide synthase expression is increased in the brain in fatal cerebral malaria. Histopathology. (2000); 37: 269-277.

Mannel D., and Grau G., Role of platelet adhesion in homeostasis and immunopathology. J. Clin. Pathol-Mol. Pathol. (1997); 50: 175-185.

Manez S., Alcaraz M., Paya M., Rios J., Hancke J., Selected extracts from medicinal plants as anti-inflammatory agents. Planta Medica. (1990); 56: 656.

Mantovani A., Bussolono F., Dejana E., Cytokine regulation of endothelial cell function. FASEB J. (1992); 6: 2591-2599.

Marsh K., Forster D., Waruiru C., Mwangi I., Winstanley M., Marsh V., Newton C., Winstanley P., Snow R. W., Indicators of life-threatening malaria in African children. N Engl. J. Med. (1995); 332: 1399-1404.

Matsabisa M., The antimalarial activity of *D. anomala* and the chloroquine resistance reversing effect of *S. birrea* in *Plasmodium falciparum in vitro*. PhD thesis. (2001); University of Cape Town, South Africa.

Matsuda M., Miyagi K., Yanagisawa N., Tsukada N., Nakayama J., Effect of intracerebral injections of tumour necrosis factor and IL-1. Jpn J. Allergy. (1993); 42: 1623-1627.

McGuire W., Hill A. V., Allsopp C. E., Greenwood B. M., Kwiatkowski D., Variation in TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature (1994); 371: 508-510.

McGuire W., Knight J. C., Hill A. V., Allsopp C. E., Greenwood B. M., Kwiatkowski D., Severe malaria anaemia and cerebral malaria are associated with different tumour necrosis factor promoter alleles, J. Infect Dis. 179 (1999) 287-290.

Medana I. M., Chaudhri G., Chan-Ling T and Hunt N. H., Central nervous system in cerebral malaria: 'Innocent bystander' or active participant in the induction of immunopathology? Immunology and Cell Biology, (2001) 79, 101-120.

Medana I., Chan-Ling T., Hunt N., Redistribution and degeneration of retinal astrocytes in experimental murine cerebral malaria: relationship to disruption of the blood-retinal barrier. Glia (1996); 16: 51-64.

Medana I., Hunt N., and Chang-Ling T., Early activation of microglia in the pathogenesis of fatal murine cerebral malaria. Glia. (1997); 19: 91-103.

Meding S., Cheng S., Simon-Haarhaus B., and Langhorne J., Role of gamma interferon during infection with *Plasmodium chabaudi* AS infection. Infect. Immun. (1990); 58: 3671-3678.

Melby P., Tabares A., Restrepo B., Cardona A., McGuff H., and Teale J. *Leishmania donovani*: Evolution and architecture of the splenic cellular immune response related to control of infection. Exp. Parasitol. (2001); 99: 17-25.

Meshnick S. R., and Kamchonwongpaisan S., The mode of action of antimalarial artemisinin and its derivatives. Gen. Pharmac. (1996); 27(4): 587-592.

Meshnick S. R., Chloroquine as intercalator: a hypothesis revived. Parasitology Today. (1990); 6: 77-79.

Meshnick S. R., Taylor T. E. and Kamchonwongpaisan S., Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol. Rev. (1996); 60:301-315.

Meshnick S. R., Taylor T. E., and Kamchonwongpaisan S., Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiol. Rev. (1996); 60: 301-315.

Meshnick S. R., The mode of action of antimalarial endoperoxides. Trans. Roy. Soc. Trop. Med. Hyg. (1994); 1: S31-S32.

Metzger W.G., Mordmuller B.G., Kremsner P.G., Malaria pigment in leukocytes. Trans R Soc Trop Med Hyg. (1995); 89: 637-38.

Milijaona R., Jambou R., Raharimalala L., Ranaivo L., Ranson M. A., and Roux J., Mefloquine-resistant strains of *Plasmodium falciparum* in Madagascar: impact on tourists and public health. Ann. Trop. Med. Parasitol. (2000); 94: 313-317.

Mohan K., and Stevenson M., IL-12 corrects severe anaemia during blood-stage *Plasmodium chabaudi* AS in susceptible A/J mice. Exp Hematol. (1998); 26(1): 45-52.

Mohan K., Moulin P., and Stevenson M., NK cell cytokine production, not cytotoxicity, contributes to resistance against *Plasmodium chabaudi* AS infection. J. Immunol. (1997); 159: 4990-4998.

Mohan K., Sam H., and Stevenson M. M., Therapy with a combination of low doses of interleukin-12 and chloroquine completely cures blood-stage malaria, prevents severe anaemia, ad induces immunity to reinfection. Infection and Immunity. Feb. (1999); 67(2): 513-519.

Molyneux M. E., Taylor T. E., Wirima J. J., Borgstein A., Clinical features and prognostic indicators in paediatric cerebral malaria: A study of 131 comatose Malawian children. Q. J. Med. (1989); 71: 441-459.

Moore K. W., Vieira P., Fiorentino D. F., Trounstine M. L., Khan T. A., Mosmann T. R., Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science. Jun 8 (1990); 248(4960): 1230-4

Mosmann T. R., Coffman R. L., Two types of mouse helper T-cell clone: implication of immune regulation. Immunobiol Today, (1987); 8: 223-227

Mosmann T., Moore K., The role of IL-10 in crossregulation of Th1 and Th2 responses. Immunol. Today. (1991); 12: A49-53

Mshana R. N., Boulandi J., Mshana N. M., Mayombo J., Mendome G., Cytokines in the pathogenesis of malaria: levels of IL-1B, IL-4, IL-6, TNF-a and IFN-g in

plasma of healthy individuals and malaria patients in a holoendemic area. J. Clin. Lab. Immunol. (1991); 34: 131-139.

Mueller M., Karhagomba I., Hirt H., and Wemakor E., The potential of Artemisia annua as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects. J. Ethnopharmacol. (2000); 73: 487-493.

Muniz-Junqueira M. I., dos Santos-Neto L. L., Tosta C. E., Influence of TNF-alpha on the ability of monocytes and lymphocytes to destroy intraerythrocytic *Plasmodium falciparum in vitro*. Cell Immunol. (2001); 208: 73-79.

Newton C. R., Warn P. A., Winstanley P. A., Peshu N., Snow R. W., Pasvol G., Marsh K., Severe anaemia in children living in a malaria endemic area of Kenya. Trop. Med. Int. Health. (1997); 2: 165-178

Newton P., Bates I., and White N., Antimalarial bioavailability and disposition of artesunate in acute falciparum malaria. Antimicrobial Agents and Chemotherapy. (2000); 44(4): 972-977.

Newton P., White N., Malaria: New developments in treatment and prevention, Annu. Rev. Med. 50 (1999) 179-192.

Ockenhouse C.F., Shear H.L., Oxidative killing of the intraerythrocytic malaria parasite *Plasmodium yoelii* by activated macrophages. J. Immunol. (1984); 132: 424-431.

Othoro C., Lal A. A., Nahlen B., Koech D., Orago A. S., Udhayakumar V., A low IL-10 tumour necrosis factor-alpha ratio is associated with malaria anaemia in children residing in a holoendemic malaria region in Western Kenya. J. Infect. Dis. (1999); 179: 279-282.

Patwardhan B., Kalbag D., Patki P., Nagsampagi B., Search for immunomodulatory agents: a review. Indian Drugs. (1990); 28(2): 56-63.

Perez H., Diaz F., and Medina J. D., Chemical investigation and in vitro antimalarial activity of *Tabebuia ochracea* ssp. *neochrysantha*. Int. J. Pharmacogn. (1997); 35: 227-231.

Peyron F., Vuillez J. P., Barbe G., Boudin C., Picot S., Ambroise-Thomas P., Plasma levels of tumour necrosis factor during a longitudinal survey in an endemic area of malaria. Acta Trop. (1990); 47: 47-51.

Phillips J., Wright C., Kirby G., and Warhurst D., Tropical plants as sources of antiprotozoal agents. Recent Advances in Phytochemistry. (1993); 27: 1-40.
Phillips R. E., Soloman T., Cerebral malaria in children. Lancet. (1990); 336: 1355-1359

Pichyangkul S., Saengkrai P., Webster H. K., Plasmodium falciparum pigment induces monocytes to release high levels of tumour necrosis factor alpha and interleukin 1 beta. Am. J. Trop. Med. Hyg. (1994); 51: 430-435.

Pichyangkul S., Saengkrai P., Webster H. K., Plasmodium falciparum pigment induces monocytes to release high levels of TNF-alpha and IL-1 beta. Am. J. Trop. Med. Hyg. (1994); 51: 430-435.

Picot S., Peyron F., Donadille A., Vuillez J., Barbe G., Ambroise-Thomas P., Chloroquine-induced inhibition of the production of TNF, but not IL-6, is affected by disruption of iron metabolism. Immunol. (1993); 80: 127-133.

Plebanski M., and Hill A. V., The immunology of malaria infection. Current Opinion in Immunology. (2000); 12: 437-441.

Prada J., Malinowski J., Muller S., Bienzle U., Kremsner P.G., Effects of *Plasmodium vinckei* haemozoin on the production of oxygen radicals and nitrogen oxides in murine macrophages. Am. J. Trop. Med. Hyg. (1996); 54: 620-624.

Probert L., Akassoglou K., Pasparakis M., Kontogeorgos G., Kollias G., Spontaneous inflammatory demyelinating disease in transgenic mice showing

central nervous system-specific expression of tumour necrosis factor-alpha. Proc. Natl. Acad. Sci. USA. (1995); 92: 11294-11298.

Qian R., Li Z., Xie M., Xu L., Liang M., and Liu X., Influence of Qinghaosu on the phagocytosis function of macrophages. Chin Trad. Herbal Drug. (1987); 18: 14-17.

Qian R., Li Z., Yu J., and Ma D., Immunomodulating action and antivirus effect of Qinghaosu. Trad. Chin. Med. J. (1981); 6: 63-66.

Rabe T and van Staden J., Antibacterial activity of South African plants used for medicinal purposes. J. Ethnopharmacol. (1997); 56: 81-87.

Riley E. M., Is T-cell priming required for initiation of pathology in malaria infections? Immunol Today. (1999); 20: 228-233.

Robert R., Pathology of the mouse. Blackwell Publishers, 1999.

Rodriguez M. et al., $CD8^+$ T-cytolytic T-cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. Int. Immunol. (1991); 3: 579-585.

Roitt I, Essential Immunology, ninth edition, Blackwell Science Ltd, 1997

Roman G. C., Senanayake N., Malaria and the nervous system. Rev. Ecuat. Neurol. (1992); 1: 77-85

Romero P. et al., Cloned cytotoxic T-cells recognize an epitope in the circumsporozoite protein and protect against malaria. Nature. (1989); 341: 323-326.

Sanchez C. P., Wunsch S., and Lanzer M., Identification of chloroquine importer in *Plasmodium falciparum*. Differences in import kinetics are genetically linked with the chloroquine resistant phenotype. J. Biol. Chem. (1997); 272: 2652-2658.

Schofield L., and Hackett F., Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J. Exp. Med. (1993); 177: 145-153.

Shaffer N., Grau G., Hedberg K., et al., Tumour necrosis factor and severe malaria. J. Infect. Dis. (1991); 163: 96-101.

Shear H., Srinivasan R., Nolan T., Ng C., Role of IFN-gamma in lethal and non-lethal malaria in susceptible and resistant murine hosts. J Immunol (1989); 143: 2038-2044.

Song S., Ling-Hu H., Roebuck K., Rabbi M., Donnelly R., Finnegan A., Interleukin-10 inhibits interferon-gamma-induced intercellular adhesion molecule-1 gene transcription in human monocytes. Blood (1997); 89: 4461-4469.

Steinman R. M., The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 9: 271-296.

Stevenson M and Kraal G., Histological changes in the spleen and liver of C57BL/6 and A/J mice during *Plasmodium chabaudi* AS infection. Exp. Mol. Path. (1989); 51: 80-95.

Stevenson M., Tam M., Wolf S., Sher A., IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-γ and TNF-α and occurs via a nitric oxide dependent mechanism. J. Immunol. (1995); 155: 2545-2556.

Sun X., Xie S., Long Z., Zhang W., and Tu Y., Experimental research on the immune suppression of Qinghaosu and its derivatives. Chin. J. Integrated Trad. West. Med. (1991); 11: 37-38.

Taniguchi M., and Kubo I., Ethnobotanical drug discovery based on medicine men's trials in the African savannah: screening of east African plants for antimicrobial activity II. Journal of Natural Products. (1993); 56: 1539-1546.

Taramelli D, Recalcati S, Basilico N, Olliaro P and Cairo G., Macrophage preconditioning with synthetic malaria pigment reduces cytokine production via heme iron-dependent oxidative stress. Lab Invest. (2000) Dec; 80 (12): 1781-8

Taverne J., Sheikh N., de Souza J., Playfair J., Probert L., Kollias G., Anaemia and resistance to malaria in transgenic mice expressing human TNF. Immunol. (1994); 82: 397-403.

Tawfik A., Bishop S., Ayalp A., and El-Feraly F., Effects of artemisinin, dihydroartemisinin and arteether on immune response of normal mice. Int. J. Immunopharmacol. (1990); 12: 385-389.

Taylor A. M. *et al.*, Reactive nitrogen intermediates and outcome in severe adult malaria. Trans. R. Soc. Trop. Med. Hyg. (1998); 92: 170-175

Taylor T. E., Molyneux M. E., Wirima J. J. Fletcher A., Morris K., Blood glucose level in Malawian children before and during the administration of intravenous quinine for severe falciparum malaria. N. Engl. J. Med. (1988); 319: 1040-1047

Taylor-Robinson A. W., Immunoregulation of malarial infection: balancing the vices and virtues. Int. J. Parasitol. (1998); 28: 135-148

Taylor-Robinson A. W., Phillips R., Functional characterization of protective $CD4^+$ T-cell clones reactive to the murine parasite *Plasmodium chabaudi*. Immunol. (1992); 77: 99-105.

Taylor-Robinson A. W., Phillips R., Severn A., Moncada s., Liew F., The role of Th1 and Th2 cells in a rodent malaria infection. Science. (1993); 260: 1931-1934.

Taylor-Robinson A. W., Regulation of immunity to malaria: Valuable lessons learned from murine malaria models, Parasitology Today 11 (1995) 334-342.

Thomson Angus. The cytokine Handbook, Third edition, Academic Press, 1998.

Trigg P. I., Economical medicinal plant research. (WHO Technical report). (1984); 3: 20-54.

Trinchieri G., Interleukin 12: A cytokine at the interface of inflammation and immunity, Adv. Immunol. 70 (1998) 83-243.

Trinchieri G., Interleukin-12: a pro-inflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Ann. Rev. Immunol. (1995); 12: 251-272.

Turner G., Cerebral malaria. Brain Pathol. (1997); 7: 569-582.

Turner G., Morrison H., Jones M., Davis M., Looareesuwan S., Buley I., Gatter K., Newbold C., Pukritayakamee S., and Nagachinta B. An immunohistochemical study of the pathology of fatal malaria: evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am. J. Pathol. (1994); 145: 1057-1069.

Urban B. C., and Roberts D. J. Malaria, monocytes, macrophages and myeloid dendritic cells: sticking of infected erythrocytes switches off host cells. Current Opinion in Immunology, 2002; 14(4): 458-465.

Urban B. C., Ferguson D. J., Pain A., Willcox N., Plebanski M., Austyn J. M., and Roberts D. J. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. Nature (1999); 400: 73-77

van Agtmael M. A., Eggelte T. A., and van Boxtel C. J., Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. Trends Pharmacol. Sci. (1999); 20: 199-204.

van den Borne B., Djikmans B., de Rooij H., le Cessie S., Verweij C., Chloroquine and hydroxychloroquine equally affect TNF-alpha, IL-6, and IFN-gamma production by peripheral blood mononuclear cells. J. Rheumatol. (1997); 24: 55-60.

van Wyk B. E., van Oudtshoorn B., and Gericke N., Medicinal plants of South Africa. Briza Publications, Pretoria. (1997).

Verdier F., Le Bras J., Clavier F., Hatin I., and Blayo M. C., Chloroquine uptake by *Plasmodium falciparum*- infected human erythrocytes during *in vitro* culture and its relationship to chloroquine resistance. Antimicrobial Agents and Chemotherapy. (1985); 27: 561-564.

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

von Stebut E., Belkaid Y., Jakob T., Sacks D., and Udey M., Uptake of *Leishmania major* amastigotes results in activation of IL-12 release from murine skin-derived dendritic cells: Implications for the initiation of anti-*Leishmania* immunity. J.Exp. Med. (1998); 188: 1547-1552.

Wagner H., and Proksch A., Immunostimulatory drugs of fungi and higher plants in economic and medicinal plant research. Academic Press, London. (1983); 1: 113-153.

Waki S., Uehara S., Kanbe K., Ono K., Suzuki M., Nariuchi H., The role of T-cells in pathogenesis and protective immunity to murine malaria. Immunol. (1992); 75: 646-651.

Waller D., Krishna S., Greenwood B., White N. J., Clinical features and outcome of severe malaria in Gambian children. Clin. Infect. Dis. (1995); 21: 577-587.

Wang T., and Xu R.,. Clinical studies of treatment of falciparum malaria with artemether, a derivative of qinghaosu. J Tradit Chin Med (1985);5: 240-242

Warhurst D. C., Antimalarial schizontocides: why a permease is necessary. Parasitol. Today. (1986); 4: 211-213.

Watt J., and Breyer-Brandwijk M., The Medicinal and Poisonous Plants of Southern Africa. E & S Livingstone Ltd., London.

Wegener T., Devil's Claw: From African traditional remedy to modern analgesic and anti-inflammatory. Herbalgram- The Journal of the American Botanical Council and Herb Research Foundation. (2000); 50: 47-54.

Weiss W. R., Sedegah M., Berzofsky J. A., Hoffman S. L., The role of $CD4^+$ T-cells in immunity to malaria sporozoites. J. Immunol. (1993); 151: 2690-2698

Weiss W. R. et al., A T cell clone directed at the circumsporozoite protein, which protects mice against both *Plasmodium yoelii* and *Plasmodium berghei*. J. Immunol. (1992); 149: 2103-2109.

Wenisch C., Parschalk B., Narzt E., Looareesuwan S., and Graninger W., Elevated levels of IL-10 and IFN-γ in patients with acute *Plasmodium falciparum* malaria. Clin. Immunol. Immunopathol. (1995); 74: 115-117

Wenish C., Parschalk B., Narzt E., Looareesuwan S., Graninger W., Elevated levels of IL-10 and IFN-gamma in patients with acute *Plasmodium falciparum* malaria. Clin. Immunol. Immunopathology. (1995); 74: 115-117

White N. J., Miller K. D., Marsh K., Berry C. D., Turner R. C., Williamson D. H., Brown J., Hypoglycaemia in African children with severe malaria. Lancet. (1987); i: 708-711.

WHO (1995). Traditional practitioners as primary health care workers. WHO/SHS/DHS/TRM/95.6; Geneva.

WHO (2000). "General guidelines for methodologies on research and evaluation of Traditional Medicine" WHO/EDM/TRM/2000.1; Geneva.

World Health Organisation. Malaria Fact Sheet. World Health Organisation Fact Sheet: Number 94. (1998).

Winkler S., Willheim M., Baier K *et al.*, Frequency of cytokine-producing T-cells in patients of different age groups with *Plasmodium falciparum* malaria. J. Infect. Dis. (1999); 179: 209-216.

Xuan W, Zhao Y, Li A, Xie P and Cai Y,. Experimental studies on antimalarial activity estimation by artesunate and artemether preparations per skin absorption. J. Trad. Chin. Med. (1988); 8: 282-284.

Yadava A., Kumar S., Dvorak J., Milon G., and Miller L., Trafficking of *Plasmodium chabaudi adami*-infected erythrocytes within the mouse spleen. Proc. Natl. Acad. Sci. (1996); 93: 4595-4599.

Yamada-Tanaka M. S., Ferreira-da-Cruz M. F., Alecrim M. G., Mascarenhas L.A., and Daniel-Ribeiro C.T. Tumour necrosis factor-alpha, interferon-gamma and macrophages stimulating factor in relation to the severity of *Plasmodium falciparum* malaria in the Brazilian Amazon. Trop. Geogr. Med. (1995); 47: 282-285.

Yap G. S., Jacobs P., Stevenson M. M., Th cell regulation of host resistance to blood-stage *Plasmodium chabaudi* AS, Res. Immunol. 145 (1994) 419-423.

Yayon A., Timberg R., Friedman S., and Ginsburg H., Effects of chloroquine on the feeding mechanism of the intraerythrocytic human malarial parasite *Plasmodium falciparum*. J. Protozoal. (1984); 31: 367-372.

Ye X., Cheng D., and Wang Y., Effect of Qinghaosu on phagocytosis of peritoneal macrophages in mice. Acta Beijing Med. Coll. (1982); 14: 141-142.

Zhang F., Gosser D. K., and Meshnick S. R., Hemin-catalyzed decomposition of artemisinin. Biochem. Pharmacol. (1992); 43: 1805-1809.