The role of cytokines and inflammatory mediators in immunity to *Plasmodium chabaudi chabaudi* AS infection

by

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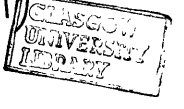

For all my family, Kirsteen and my wee Gran

Table of Contents

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Declaration

I declare that this thesis is of my own composition and that the research described herein was performed entirely by myself except where expressely stated.

Paul Balmer
1997

Abbreviations

APC	Antigen presenting cell
B cell	B lymphocyte
BSA	Bovine serum albumin
CD	Cluster designation
Ci	Curie
CMI	Cell-mediated immunity
Con A	Concanavalin A
cpm	Counts (of radioactivity) per minute
CRP	C-Reactive protein
CSP	Circumsporozoite protein
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HLA	Histocompatibility leukocyte antigens
ICAM-1	Intracellular adhesion molecule-1
IFAT	Indirect fluorescent antibody test
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneally
i.u.	International unit(s)
i.v.	Intravenously
kDa	Kilodalton
L-NMMA	L-N^G-monomethyl arginine
LPS	Lipopolysaccharide
M	Molar
MHC	Major Histocompatibility complex

mg	Milligramme(s)
µg	Microgramme(s)
ml	Millilitre(s)
µl	Microlitre(s)
mm	Millimetre(s)
µM	Micromolar
MSP	Merozoite surface protein
NK cell	Natural Killer cell
NO	Nitric oxide
nRBC	Normal red blood cell
PBS	Phosphate buffered saline
PBMN cells	Peripheral blood mononuclear cells
pRBC	Parasitised red blood cell
SAP	Serum amyloid P
SNAP	S-nitroso-acetyl-penicillamine
SD	Standard deviation
T cell	T lymphocyte
Th	T helper lymphocyte
TGF	Transforming growth factor
TNF	Tumour necrosis factor
<	Less than
>	More than
%	Percentage point(s)

Summary

Immunity to an asexual erythrocytic *Plasmodium chabaudi chabaudi* AS infection in NIH mice is mediated sequentially by Th1 and Th2 cells. The predominantly Th1 mediated response is responsible for the control of the acute phase of infection and there is then a switch to predominantly Th2 mediated response(s). Cytokines and inflammatory mediators are important molecules involved in the parasiticidal mechanisms induced by Th1 and Th2 cells during the course of a *P. chabaudi* infection. IFNγ (Th1 associated cytokine) and IL-4 (Th2 associated cytokine) production during a *P. chabaudi* infection reflects the sequential involvement of Th1 and Th2 cells. IL-6 is a cytokine which is involved in Th2 mediated responses but can also stimulate the production of inflammatory mediators such as acute phase proteins. The actual role of individual cytokines during the course of experimental malaria infection can be investigated by depletion of the cytokine by antibody treatment or addition of exogenous cytokine and observing the outcome of the infection. Studies were performed, utilising cytokine or cytokine receptor gene deficient mice to investigate the role of individual cytokines during the course of *P. chabaudi* infection.

The induction of inflammatory mediators, such as nitric oxide (NO) and acute phase proteins, by cytokines is an important aspect of the protective immune response to *P. chabaudi* AS infection but it is unclear where and how these molecules can mediate a protective response. During *P. chabaudi* infection, mature asexual erythrocytic stage parasites sequester to the liver, making this non-lymphoid organ a potential site of a protective immune response. Serum amyloid P (SAP) and NO are two inflammatory mediators that are synthesised in the liver and may participate in parasiticidal mechanisms.

IFNγ receptor (IFNγR) deficient mice are more susceptible to a *P. chabaudi* infection than intact mice. A high mortality rate was observed in the IFNγR deficient mice whereas none of the control mice died. The IFNγR deficient mice consistently had a

higher peak primary parasitaemia compared to the control mice but this was never statistically significant. Total IgG2a and parasite-specific IgG responses in the serum of IFNγR deficient mice were reduced compared with control mice, whereas both groups had similar total IgG1 levels in their serum. Interestingly, a large total IgE response was observed in the serum of the IFNγR deficient mice. Control mice had negligible levels of total IgE in their serum, which is the normal IgE response during a primary *P. chabaudi* infection. Analysis of leukocytes present in the spleen and liver during the course of infection revealed that there was a reduction in the numbers of lymphoid cells in the spleen at peak parasitaemia and a reduction of lymphoid cell, monocyte and polymorphonuclear (PMN) cell numbers present in the liver of *P. chabaudi* infected mice during the acute phase of the infection. This study demonstrated the importance of IFNγ mediated responses during a primary erythrocytic *P. chabaudi* infection.

The role of the Th2 associated cytokines during a *P. chabaudi* infection was investigated by infecting, in separate experiments, IL-4 and IL-6 deficient mice. *P. chabaudi* infection of IL-4 deficient mice consistently resulted in an exacerbation of the peak of the primary parasitaemia compared to that of intact control mice. This observation was repeated in *P. chabaudi* infection of IL-4 deficient mice on three different genetic backgrounds. There was a reduction in the total IgG1 response in the serum of the IL-4 deficient mice during the acute phase of *P. chabaudi* infection compared to that of control mice but both groups had similar total IgG2a and parasite-specific IgG responses. Analysis of leukocytes present in the spleen and liver during the course of a *P. chabaudi* infection revealed a reduction in the number of lymphoid cells in the spleen and a reduction in the number of lymphoid cells, monocytes and PMN cells in the liver of IL-4 deficient mice compared with intact controls. These studies confirmed previous reports that IL-4 is not essential for the control of a primary *P. chabaudi* infection.

Infection of IL-6 deficient mice with $1x10^5$ parasitised erythrocytes of *P. chabaudi* consistently resulted in an exacerbated peak of the primary parasitaemia (not statistically

significant) and a significantly reduced rate of parasite clearance compared to intact control mice. There was no exacerbation of the peak of the primary parasitaemia in IL-6 deficient mice compared to control mice following infection with $2x10^6$ parasitised erythrocytes of *P. chabaudi*. The reduced rate of parasite clearance was, however, still evident in the IL-6 deficient mice. Total IgG1 and total IgG2a responses in the serum of the IL-6 deficient mice (for both challenge doses) were reduced compared to the intact control mice and there was no significant difference observed in the production of parasite specific IgG. Hence, IL-6 deficient mice have a slower rate of parasite clearance than control mice and also demonstrated dose-dependent immune responses illustrated by the loss of the exacerbation in the peak parasitaemia observed in IL-6 deficient mice infected with $1x10^5$ pRBCs upon infection with $2x10^6$ pRBCs.

To examine if the liver was involved in a protective immune response to the asexual erythrocytic stage of a *P. chabaudi* infection, lymphomyeloid (LM) cells were isolated from the livers of *P. chabaudi* infected mice. An increase in the numbers of LM cells was observed during the acute phase of a primary *P. chabaudi* infection with peak numbers of LM cells present in the liver one or two days after the peak of the primary parasitaemia. Adoptive transfer of LM cells isolated at this time of the infection, significantly protected irradiated recipient mice against a *P. chabaudi* challenge compared to leukocytes isolated from spleens and peripheral blood of *P. chabaudi* infected mice at the same time in the course of infection and leukocytes isolated from the spleens of naive mice. Preliminary data was also obtained on the role of the resident liver macrophages, Kupffer cells, during *P. chabaudi* infection. Depletion of Kupffer cells in mice resulted in an exacerbation of the peak of the primary parasitaemia of a *P. chabaudi* infection. These experiments suggest that the liver may be a site of a protective immune response involving the recruitment of effector cells, and Kupffer cells.

P. chabaudi infection of mice was shown to induce the production of SAP, the major acute phase protein in mice. SAP levels were elevated during the course of a *P. chabaudi*

infection compared to non-infected mice. *In vitro* studies using SAP isolated from serum taken from *P. chabaudi* infected mice at day 11 post infection, demonstrated that SAP may have a direct effect on the growth of erythrocytic stage malaria parasites and is potentially an important immunomodulatory molecule. The liver is a site which has the potential to produce a high local concentration of NO, an inflammatory molecule which has been shown to have parasiticidal activity. *In vitro* studies were performed, using a NO donor, to investigate if NO had any effect on the development of asexual erythrocytic stage parasites. Late stage parasites were found to be more susceptible to NO and the effect of NO may be cytostatic rather than cytotoxic.

Chapter One
General Introduction

Background

Malaria remains one of the most prevalent diseases in man today. Approximately 40% of the world's population are at risk of infection and 2-3 million people are killed by malaria each year, most of these being children in sub-Saharan Africa (WHO 1993) and there are between 200-300 million clinical cases of malaria each year. Eradication of malaria appears to be a more difficult objective to achieve now than at any other time. Several different problems are compounding the difficulties faced by clinicians and researchers. The increasing incidence of drug resistance in the parasite and insecticide resistance in the vector mosquitoes are allowing the disease to spread, which could result in malaria returning to regions where it has been eradicated for years. Reduced funding for control programmes, political unrest and the problem of refugees in the Third World are all adding to the biological problems already faced. The prospects for an effective malarial vaccine in the immediate future have diminished slightly. The much heralded SPf66, produced by Manuel Patarroyo and colleagues has disappointed in recent trials in Africa and Thailand (Nosten *et al.,* 1996, Alonso *et al.,* 1994) following promising results initially (Valero *et al.,* 1993). SPf66 has shown that partial immunity to malaria can be generated by a peptide polymer and the controversy surrounding the SPf66 vaccine trials has led to better definition of the conduct for future trials of a malaria vaccine.

The Parasite

There are four species of malaria parasite which infect humans, *Plasmodium falciparum, P. vivax, P. malariae and P. ovale. P. falciparum* is responsible for the majority of deaths attributed to malaria infection and hence is regarded clinically as the most important species. A diagrammatic summary of the malaria life cycle is illustrated in figure 1.

Infection is initiated in humans when a female *Anopheles* mosquito takes a blood meal. During this process, anticoagulant from the salivary glands is released and introduces the infective sporozoites (of malaria) into the bloodstream. These pass rapidly to the liver and penetrate hepatocytes, initiating the pre- or exo-erythrocytic stage (Fairley, 1947, Sinden and Smith, 1982). Following growth and replication within the hepatocyte, the liver schizont ruptures releasing thousands of merozoites into the circulation (Garnham, 1966). The duration of this stage of the life cycle depends upon the species of the parasite. The exo-erythrocytic stage of *P. falciparum* infection lasts approximately 5-6 days. In *P. vivax* and *P. ovale* dormant exo-erythrocytic forms, known as hypnozoites, can remain in the liver for several months (Krotoski *et al.*, 1982). The merozoites, released from the ruptured liver schizont, quickly attach to and invade circulating erythrocytes where they undergo asexual replication repeatedly. It is this phase of infection, the asexual erythrocytic stage that causes the morbidity and mortality associated with disease. The parasite develops through ring stages to trophozoite and then schizonts which rupture releasing merozoites into the bloodstream which repeat the cycle by invading new erythrocytes (Dvorak *et al.*, 1975). Each asexual erythrocytic cycle can be 24, 48 or 72 hours depending upon the species of malaria.

In the final third of the asexual cycle of *P. falciparum* and a few other species, the infected erythrocytes classified as late trophozoite/schizont stages, stop circulating and adhere to the endothelial cells of post capillary venules. This process is known as sequestration and the infected erythrocytes can cytoadhere to endothelial cells in post capillary venules of several organs including the brain, heart, kidneys, gut and liver.

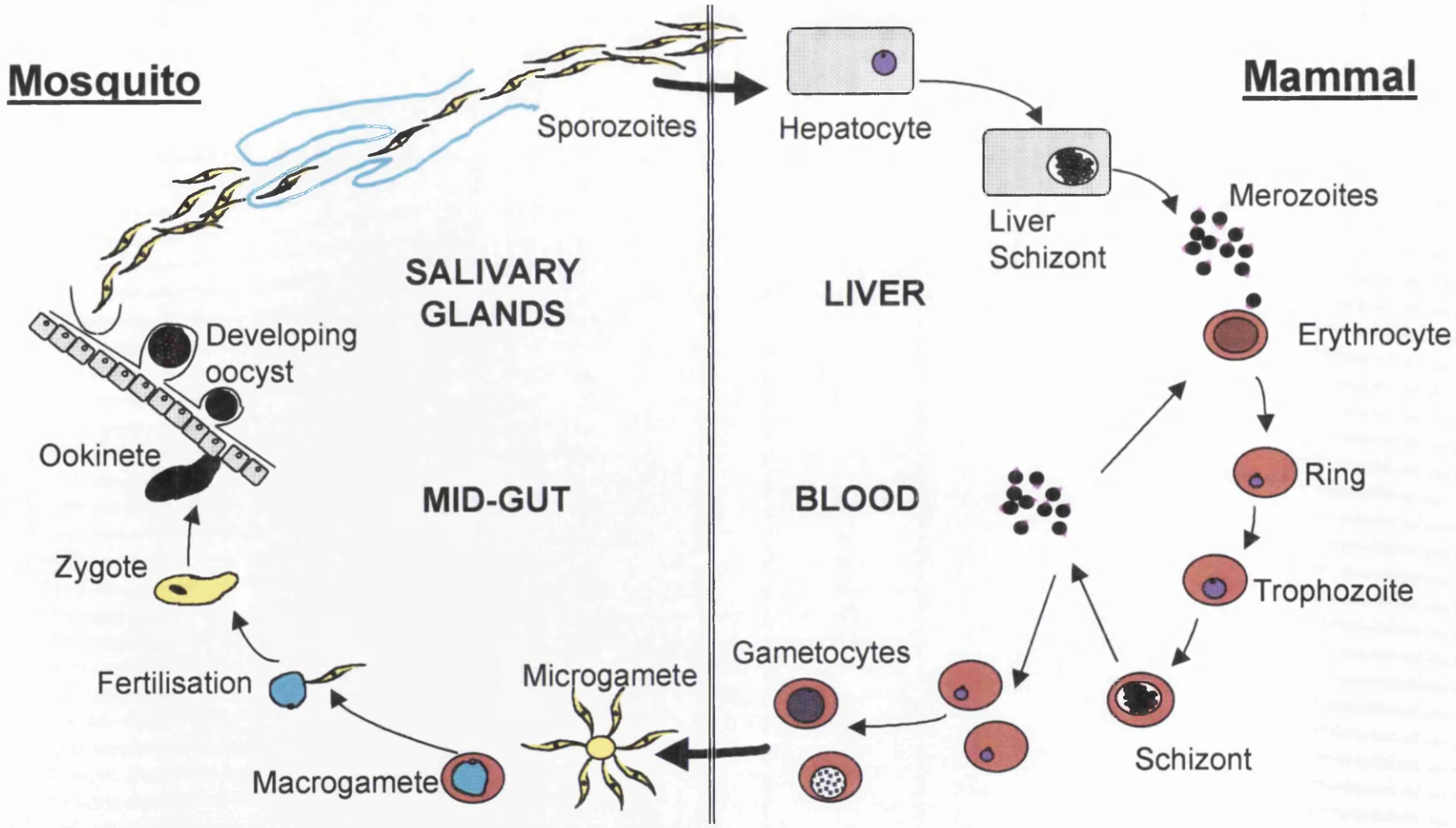

Diagram 1. The life cycle of *Plasmodium* species.

Merozoites, following invasion of an erythrocyte, can differentiate into gametocytes which are the sexual forms and infective to the mosquito during a blood meal. These can then mature in the mid-gut of the mosquito to the extracellular gametes. Fertilisation produces a zygote, the only stage of the life cycle where the parasite is in a diploid form, which develops into a motile ookinete. This burrows into the mid-gut wall where it encysts on the outer surface. The oocyst, develops and grows to release sporozoites which migrate to the salivary glands of the mosquito ready to infect at the next blood meal (Vanderberg, 1975).

The *P. chabaudi* Laboratory Model

In order to produce a successful malaria vaccine, it is logical that a comprehensive understanding of immunity to malarial infection will facilitate the achievement of such a project. The definition of immune responses induced by malarial antigens will aid the decision of what type of antigens are to be included in a vaccine and the identification of the type of immune response which will be required to be induced by the prospective vaccine. To achieve this aim, studies are required to elucidate the mechanisms of protective immune responses induced by either specific malarial antigens or malaria infection. It is possible to culture human malaria parasites *in vitro* but there are limitations to any *in vitro* system making it difficult to extrapolate any observations made to an *in vivo* situation. Human *Plasmodium* species are host specific restricting infection studies of animal models with human malaria parasites. Furthermore, experimental infections of volunteers with human *Plasmodium* species, are strictly controlled with the welfare of the volunteer of the utmost importance. For these reasons, researchers have turned to animal models which allow examination of malaria infections *in vivo*. Rodent models are mainly used because of the availability of the mammalian hosts, rats or mice, and due to the fact that it is relatively easy to maintain a supply of the respective rodent

parasite species. Animal models have supplied researchers with a convenient method of studying immunity to malaria infections.

During the studies undertaken in this thesis, the parasite species used was *P. chabaudi chabaudi* AS. This species represents a reasonable model of *P. falciparum* infection because it shares several important features with *P. falciparum*. Both species invade normocytes, sequester from circulation during the latter stages of their asexual cycle (although *P. chabaudi* has a 24 hour asexual cycle compared with *P. falciparum*'s 48 hours) and undergo antigenic variation. Hence *P. chabaudi* infection in mice provides a relevant model which can be utilised to analyse immunity to malaria infection.

Clinical manifestations of malaria

Malaria infection can result in a spectrum of disease manifestations, from asymptomatic to fatal infections. The parasite species can determine the outcome of infection. *P. falciparum* is responsible for the majority of deaths associated with human malaria infections. Transmission intensity, anti-malarial drug use, the drug resistance of parasites and host resistance are all known to contribute to the severity of malaria infection. The ability of different parasite strains to induce cytokine production and their rosetting or cytoadherent properties may also influence the nature of malaria infection (Molyneux, 1995).

Fever, headaches, chills, rigors and sweating are common symptoms of all human malarias (Warrell, 1993) although the intensity of individual symptoms varies depending upon the infecting *Plasmodium* species (Shute, 1952, Covell and Nicol, 1951). These symptoms are associated with uncomplicated malaria infection and anaemia may develop if infections are prolonged (Molyneux, 1995). Malaria infection is considered severe when it is life threatening. For research purposes severe malaria is defined by the presence of any of the following complications: coma, renal failure, severe anaemia,

acidosis, respiratory distress syndrome, hypoglycaemia, bleeding, shock or intravascular haemolysis. Other characteristic symptoms include altered consciousness, weakness, convulsions, jaundice and hyperparasitaemia. It is thought that approximately one infection in a hundred of children with severe malaria may be fatal in the absence of drug treatment (Greenwood *et al.*, 1987). Children in intense transmission areas (subSaharan Africa) are at greatest risk of developing severe malaria. Severe anaemia, cerebral malaria, acidosis, hypoglycaemia and respiratory distress are the five main symptoms in African children with severe falciparum malaria (Molyneux, 1995). Cerebral malaria, characterised by altered consciousness and convulsions is the main cause of death due to malaria in children (Molyneux, 1995). In adults, severe malaria infection differs from that in children because complications such as acute renal failure and respiratory distress syndrome are more common.

Host resistance to malaria

Natural resistance

Susceptibility and resistance to malaria infection are influenced by several factors which are not immunologically mediated responses. Erythrocytic stage malaria parasites gain entry into the erythrocyte through a complex process following interaction between molecules on the parasite surface and receptors on the erythrocyte membrane (Butcher, Mitchell and Cohen, 1973, Miller *et al.*, 1973). If the erythrocyte lacks this receptor then the merozoite is unable to penetrate the cell. *P. knowlesi* merozoites are unable to enter cells lacking the Duffy blood group antigens (Miller *et al.*, 1975). This observation is extended to the human malaria *P. vivax* and it is possible that the absence of *P. vivax* in areas of West Africa can be explained by the incidence of Duffy negative individuals (Miller *et al.*, 1976).

The intracellular environment of the erythrocyte can also influence the growth of malaria parasites. Alteration in the haemoglobin constitution due to the genetic disorder, sickle-cell anaemia, may have an inhibitory effect on the development of malaria parasites. Sickle-cell haemoglobin has a single amino acid mutation in the β chain, which results in the erythrocyte adopting a sickle-like shape due to the precipitation of sickle-cell haemoglobin (HbS) when the oxygen concentration is reduced. An inhibitory effect of HbS on the growth of *P. falciparum* blood-stage parasites was suggested by the high frequency of the sickle-cell gene in areas of hyperendemic malaria transmission (Allison, 1954). Homozygotes for the sickle cell gene tend to die young often as a result of infection, renal failure, cardiac failure or thrombosis. Recently, a report has demonstrated a higher resistance to *P. falciparum* infection in patients homozygous for sickle-cell in Western Kenya (Aluoch, 1997). Furthermore, transgenic mice expressing high levels of HbS are protected against a lethal infection of *P. yoelii* (Hood *et al.*, 1996). The growth rate of *P. falciparum in vitro* in homozygous or heterozygous erythrocytes for the sickle-cell trait is normal but as the oxygen level is reduced, development of the parasite is inhibited (Friedman, 1978). It is thought that the mechanism of growth inhibition may be due to a loss of potassium under hypoxic conditions (Friedman *et al.*, 1979).

A number of other haemoglobin mutations and erythrocyte abnormalities are thought to be positively selected for by malaria infection in populations within areas of high transmission. Thalassaemias, genetic disorders of haemoglobin synthesis are thought to confer protection against malaria infection (Williams *et al.*, 1996, Senok *et al.*, 1997). Deficiencies in red cell enzymes, such as glucose-6-phosphate dehydrogenase, are also thought to influence resistance to malaria infection. A double genetic defect of thalassaemia trait and severe glucose-6-phosphate dehydrogenase deficiency has been correlated with enhanced protection against malaria infection (Oo *et al.*, 1995). Individuals carrying the sickle-cell trait have a significant retardation in the switch from foetal to adult haemoglobin during the first five years of life (Giardina *et al.*, 1995).

Persistence of foetal haemoglobin may contribute to host resistance because *P. falciparum* growth is retarded in cord blood cells containing approximately 85% foetal haemoglobin, in erythrocytes that contain 20% foetal haemoglobin from infants and in erythrocytes containing foetal haemoglobin from adult homozygotes for hereditary persistence of foetal haemoglobin (Giardina *et al.*, 1995). It is thought the retardation of *P. falciparum* growth in all these cells illustrates a role for foetal haemoglobin in mediating an increase in oxidative stress which may lead to early elimination of parasitised erythrocytes (Nagel and Roth, 1989).

Dietary intake is another factor which may influence host resistance to malaria infection. Variation in diet has been shown to alter the outcome of experimental malaria infection and has been proposed to have a role in host resistance to human malaria infection. *P. berghei* infection in rats can be suppressed by a milk diet (Maegraith, Deegan and Sherwood Jones, 1952). It was shown that milk is deficient in ρ-aminobenzoic acid (PABA) and that PABA supplementation could reverse the suppression of *P. berghei* infection in rats and *P. knowlesi* in rhesus monkeys (Hawking, 1954). Mice fed a diet containing cod liver oil survive for a significantly longer period following infection with lethal *P. berghei* infection (Godfrey, 1957). The protective effect was reversed by the addition of vitamin E and subsequently a vitamin E-deficient diet has been shown to suppress lethal *P. yoelii* infection in mice (Taylor *et al.*, 1997). Restriction of the dietary intake of mice (food was restricted to produce a body weight loss of 1-2%) during a *P. berghei* ANKA infection reduced mortality compared to mice fed *ad libitum* (Hunt, Manduci and Thumwood, 1993). The reduction in the level of protein intake in mice and rats (fed on a cassava meal which has a low protein content compared to controls which were given a protein-rich diet) has been shown to reduce the severity of *P. yoelii*, *P. berghei* and *P. vinckei* infections (Ibekwe and Ugwunna, 1990, Edirisinghe, Fern and Targett, 1981). The addition of vitamin E free fish oil to antimalarial chemotherapy treatment of Chinese patients with *P. falciparum*, enhanced the rate of clinical cure (Levander *et al.*, 1994). The development of *P. falciparum in vitro* can be inhibited by

the addition of omega-3-fatty acids derived from fish oil to culture medium (Fevang, Bjorkman and Hostmerk, 1992, Kumaratilake *et al.*, 1992).

Acquired immunity to malaria

The complexity of the life cycle of the *Plasmodium* species in the mammalian host, the large number of different parasite antigens presented to the host and the ability to evade the immune response through mechanisms such as antigen diversity results in the slow development of immunological memory in humans to malaria infection. Effective levels of natural immunity to infection develop slowly and only upon repeated exposure. Infants up to the age of 6 months are protected from infection possibly because of the presence of maternal antibodies (Bruce-Chwatt, 1952), or the lack of PABA in the milk diet of the infants. The persistence of foetal haemoglobin may also contribute to the low frequency of malaria during the first 6 months of life. Children from 6 months to 5 years are most at risk from *P. falciparum* infection (McGregor, 1964) but the development of immunity is largely determined by the level of malarial transmission. Clinical malaria in children less than 1 year of age occurs in hyperendemic areas with the major manifestation being anaemia (Brewster, Kwiatkowski and White, 1990, Snow *et al.*, 1994). Seasonal or less intense transmission of malaria results in levels of exposure which are insufficient to induce significant protecive immunity. Hence individuals of all ages are susceptible to severe infection. Anaemia is the main clinical symptom in children of under 1 year, while cerebral complications manifest in the older groups. Recently, it has been suggested that naturally acquired immunity in adults develops after only 1-2 years of exposure to hyperendemic malaria and that this is an age-dependent phenomenon (Baird *et al.*, 1991, Baird *et al.*, 1993). An age-dependent decrease in susceptibility to high-grade and frequent parasitaemia was observed in transmigrants with limited history of exposure to endemic malaria (Baird *et al.*, 1993). The authors suggest that a protective immune response to an endemic falciparum malaria is governed by a relatively brief heavy exposure and an unknown intrinsic immune factor(s) which

are associated with the host. The brief period of heavy exposure to malaria infection conferred greater protective advantage to adults than children. However, one parameter that is ignored is the greater antigen load that adults will have compared to children and this may be a factor in the development of the age-dependent, naturally acquired immunity.

Immunity to malaria is traditionally regarded as stage-specific but the potential exists for effector mechanisms generated in response to one stage of the life cycle to act against another stage which is present in the host at that time.

Immunity to Pre-erythrocytic Stage

Information on immunity to sporozoites and intra-hepatic stages comes mainly from experimental immunisation of volunteers or animals with attenuated (u.v., x-ray or γ-ray irradiated) sporozoites which are able to invade and partially develop in hepatocytes (Ramsey, Beaudoin and Hollingdale, 1982). Irradiated sporozoites of *P. falciparum* and *P. vivax* induced protective immunity in a high proportion of immunised volunteers (Clyde *et al.*, 1973, Clyde *et al.*, 1975).

The major immunodominant protein found during the sporozoite stage of the parasite's life cycle is the circumsporozoite protein (CSP). The CSP consists of a central region which has immunodominant, multiple repeat sequences and two flanking regions, Region I and Region II. The repeat region is unique for each species of parasite but Region I and II are highly conserved and found in the CSP of all species (Phillips 1994a). The motility of the sporozoites is linked with the secretion of the CSP from the apical end of the parasite and its translocation posteriorly (Stewart and Vanderberg, 1988 and 1991). Region II of the CSP has a relatively high degree of homology to thrombospondin, (Cerami *et al.*, 1992) an adhesion molecule and hence it has been suggested that the CSP is involved, via Region II, in the binding of the sporozoite to the

hepatocyte prior to invasion. When the sporozoite enters the hepatocyte, substantial quantities of the CSP are shed into the hepatocyte cytoplasm (Khan, Ng and Vanderberg, 1992). As the intrahepatic parasite developed, the CSP was localised around the periphery of the parasite but the function of this distribution of the CSP remains unclear. The demonstration that sporozoites release quantities of CSP suggests a possible mechanism for the presentation of fragments of the CSP to the immune system which may induce a CSP specific response to the intrahepatic parasite. This protein has been demonstrated to induce immune responses mediated by both antibody dependent and independent mechanisms (Mazier *et al.*, 1988).

Antibody-dependent protective mechanisms against the pre-erythrocytic stage

Evidence that protective immunity to pre-erythrocytic stages of malaria, is mediated by antibody-dependent mechanisms is mainly provided by immunisation experiments. Serum from mice immunised with irradiated *P. bergehi* sporozoites inhibited sporozoite invasion and development of exo-erythrocytic forms *in vitro* (Chatterjee *et al.*, 1996). The antibody recognised both Region II sequences of *P. falciparum* CSP and Liver Stage Antigen-1 (LSA-1) based repeat sequences. Antibodies from mice immunised with irradiated *P. bergehi* or *P. yoelii* sporozoites, protected recipient mice against a viable homologous sporozoite challenge (Potocnjak *et al.*, 1980, Charoenvit *et al.*, 1991). The repeat region of the CS protein was the epitope recognised by these protective antibodies.

A monoclonal antibody which recognises a 17kDa protein found on the parasitophorous vacuole membrane of hepatocytes or erythrocytes infected with *P. yoelii*, can inhibit the development of intrahepatic forms of *P. yoelii in vitro* (Charoenvit *et al.*, 1995). Cross-species protection to the pre-erythrocytic stage has been reported (Sina *et al.*, 1995). Following immunisation with *P. falciparum* sporozoites, mice were protected against a

P. berghei sporozoite challenge (Sina *et al.,* 1995). Serum and monoclonal antibodies derived from these mice recognise a novel 42/54 kDa antigen designated circumsporozoite protein 2 (CSP 2), in both *P. falciparum* and *P. berghei* and can inhibit *P. falciparum* and *P. berghei* sporozoite invasion of hepatoma cells *in vitro* (Sina *et al.,* 1995). Anti-CSP 2 monoclonal antibody was also shown to protect mice from a *P. berghei* sporozoite challenge (Sina *et al.,* 1995).

The mechanism by which an antibody protects or prevents the development of the parasite at the pre-erythrocytic stage of the parasite's life cycle remains unclear. Anti-CSP antibodies have been shown to inhibit sporozoite penetration into hepatocytes (Mazier *et al.,* 1987, Mazier *et al.,* 1986, Nudelmann *et al.,* 1988) but there have also been reports of 'protective' antibodies failing to block the penetration of sporozoites (Mellouk *et al.,* 1986a, Mellouk *et al.,* 1986b).

Involvement of anti-CSP antibodies in post-penetration inhibitory events was observed with *P. falciparum* (Mazier *et al.,* 1988) where it was noted that attachment of the sporozoite to the hepatocyte membrane and subsequent intrahepatocytic development of the parasite were interfered with by the anti-CSP antibodies. It has been suggested that the antibodies may destroy the intracellular parasite (through their presence) in the parasitophorous vacuole. Antibodies have been observed in the parasitophorous vacuole following staining with fluorescent labelled anti-IgG (Nudelmann *et al.,* 1988). Anti-CSP antibodies have also been reported to enhance the penetration of the sporozoite. Low titres of antibodies against the CS protein of *P. falciparum* (Hollingdale *et al.,* 1988) and *P. yoelii* (Nudelmann *et al.,* 1988) show an increase in the number of liver parasites when compared with controls. Enhancement was observed with both polyclonal and monoclonal antibodies.

Most of the reports regarding the activity of protective antibodies against the exo-erythrocytic stage, the majority of these being anti-CSP antibodies, are experiments

performed *in vitro*. Hence the results should be interpreted with caution because the results obtained in an *in vitro* system may not define the role of these protective antibodies *in vivo*. Indeed, although repeated exposure of humans to sporozoites may result in detectable antibody levels in serum (Nardin *et al.*, 1979) there is still a lack of strong evidence that this antibody is actually protective (Hoffman *et al.*, 1987).

The assumption that the protection observed in immunised volunteers (Clyde *et al.*, 1973, Clyde *et al.*, 1975) was mediated by a protective antibody, led to the design of the first *P. falciparum* vaccines based on the immunodominant B cell epitope, (NANP)n from the conserved central region of the CS protein. These vaccines had very little success (Ballou *et al.*, 1987, Herrington *et al.*, 1987, Fries *et al.*, 1992) and indicated that there were antibody independent mechanisms which were important in the protective response against the exo-erythrocytic stages. However, recently an immunogenic recombinant CSP vaccine has been developed which protects adults, with no previous exposure to malaria, against experimental challenge with *P. falciparum* sporozoites (Stoute *et al.*, 1997). The vaccine contains CSP central tandem-repeat epitopes and carboxy-terminal epitopes which are fused to hepatits B surface antigen (HBsAg) and is expressed together with unfused HBsAg. The vaccine is given in conjunction with a potent adjuvant and can induce large titres of antibodies against the CSP repeat epitopes (Stoute *et al.*, 1997).

Protective immunity to pre-erythrocytic stages mediated by antibody-independent mechanisms

The demonstration that it was possible to induce protection in B cell deficient mice following immunisation with irradiated sporozoites (Chen, Tigelaar and Weinbaum, 1977) gave an early indication that protective responses induced may involve antibody-independent mechanisms. Similar to the experiments demonstrating antibody-dependent

protective immunity, the understanding of the nature of antibody-independent mediated responses in immunisation experiments comes from *in vitro* observations.

Cytotoxic CD8$^+$ T cells

CD8$^+$ cytotoxic T lymphocytes (CTL) recognise antigens which have been processed by antigen presenting cells and are presented in association with MHC Class I (Townsend and Bodmer 1989). A series of *in vivo* depletion experiments (Schofield *et al.*, 1987b, Weiss *et al.*, 1988) and adoptive transfer of T cell clones (Egan *et al.*, 1987) has demonstrated that CTL are protective in some rodent models following immunisation with irradiated sporozoites. Since CTL function is MHC restricted, the target of their cytotoxic function would appear to be the intracellular form residing within the hepatocyte because this cell is capable of expressing functional MHC class I peptides. CTL taken from mice immunised with irradiated sporozoites have been shown to inhibit the development of exo-erythrocytic parasites cultured in mouse hepatocytes (Hoffman *et al.*, 1989). The need for parasite derived liver stage antigens presented in association with MHC class I to CD8$^+$ CTL in order to induce effective protection has been illustrated by studies using beta2-microglobulin (beta2m) knockout mice. Beta2m knockout mice are resistant to *T. gondii* (Denkers *et al.*, 1993) and *T. cruzi* (Tarleton *et al.*, 1992) infections but failed to develop protective immunity against a *P. berghei* sporozoite challenge following immunisation with attenuated *P. berghei* sporozoites (White, Synder and Krzych, 1996).

Fragments or epitopes of the CSP have been shown to be one of the main targets for CTL (Kumar *et al.*, 1988, Weiss *et al.*, 1990). *In vivo* depletion of CD8$^+$ CTL abrogated protection following vaccination with attenuated vaccinia virus (NYVAC) recombinants expressing the CSP (Lanar *et al.*, 1996). MHC class I restricted CD8$^+$ CTL against the CSP protect mice from a *P. yoelii* challenge (Malik *et al.*, 1995). Immunisation with synthetic peptides, derived from the CSP of *P. berghei*, failed to

protect mice against a subsequent challenge (Renggli *et al.*, 1995). CTL clones, harvested from mice immunised with synthetic peptides derived from the CSP of *P. berghei*, were expanded *in vitro* (long term culture, re-stimulated every 7-10 days with a specific synthetic peptide), and were able to protect naive, recipient mice upon adoptive transfer against a *P. berghei* sporozoite challenge (Renggli *et al.*, 1995). No protection against a *P. berghei* sporozoite challenge was observed in the mice immunised with the peptides. It is thought that CTL-dependent protection may require the migration of CTL to the liver and that this is achieved when the CTL are adoptively transferred but does not seem to occur efficiently in peptide immunised mice (Renggli *et al.*, 1995). Immunisation with irradiated sporozoites produces a significant increase in $CD8^+$ CTL in the livers of challenged mice (Faure *et al.*, 1995). *In vivo*, the effector function of CSP specific $CD8^+$ CTL clones, appears to be related to the expression of CD44 and VLA-4 (Rodrigues *et al.*, 1992). These adhesion molecules have important roles in cell trafficking and their expression may be a critical step in the migration of $CD8^+$ CTL to the liver. A CTL clone which recognises an epitope within the *P. berghei* CSP, in a MHC class 1 restricted manner, can protect mice against a homolougous sporozoite challenge if administered in conjunction with IL-2 (Romero *et al.*, 1989). *Salmonella typhimurium* recombinants expressing the *P. berghei* CSP gene have been used as a delivery vehicle to induce protection mediated by specific $CD8^+$ CTL (Aggarwal *et al.*, 1990).

The sporozoite surface protein 2 (SSP2) is another protein which has been shown to be a target of $CD8^+$ CTL (Khusmith *et al.*, 1994, Wizel *et al.*, 1995). Mice immunised with irradiated *P. yoelii* sporozoites produce CTL against CSP and *P. yoelii* SSP2 (PySSP2). *In vivo* depletion of these $CD8^+$ CTL abrogated the protection (Khusmith *et al.*, 1994). $CD8^+$ CTL clones specific for PySSP2 were able to protect naive recipient mice against *P. yoelii* following adoptive transfer (Khusmith *et al.*, 1994). This illustrates that $CD8^+$ CTL against PySSP2 could protect mice against a *P. yoelii* sporozoite challenge in the absence of other parasite specific immune responses. The clones appear to recognise and

eliminate infected hepatocytes because protection was still mediated even when the $CD8^+$ CTL clones were transferred to recipients three hours after sporozoite inoculation which is a time when the sporozoite will have entered the hepatocyte (Khusmith *et al.*, 1994).

As already stated the evidence demonstrating a protective role for $CD8^+$ CTL comes from either immunisation or *in vitro* experiments. The majority of experiments involve the rodent parasites *P. berghei* and *P. yoelii*. These parasites can infect mice but their natural hosts tend not to be used in laboratory models. Indeed, if correctly matched host-parasite combinations are used, they are often not as effective as using *P. berghei* sporozoite infection in mice. However, protective immunity induced by irradiated sporozoites is not observed in all mouse strain-*P. berghei* or *P. yoelii* combinations (Suhrbier 1991). In some examples of parasite-mouse strain experiments protection is $CD8^+$ CTL independent.

A second point to consider when analysing results from sporozoite infection of mice is the infectivity of the sporozoite itself. If non-irradiated sporozoites are highly infective to the mouse strain, often the protection induced by immunisation with irradiated sporozoites from the same parasite species is weak and correlates with a poor $CD8^+$ CTL response (Suhrbier, 1991). Poor infectivity of sporozoites in some models, such as *P. berghei* sporozoite infections of mice, induce a strong $CD8^+$ CTL response. Large numbers of *P. berghei* sporozoites are thought to perish shortly after invasion of the hepatocyte *in vitro* (Suhrbier, 1991), releasing sporozoite antigens into the cytoplasm of the hepatocyte. The sporozoite antigens are then processed and associate with MHC Class I molecules, resulting in the induction of a strong $CD8^+$ CTL response (Suhrbier *et al.*, 1990). Hence, the infectivity of the sporozoite, which is dependent upon the host-parasite combination, will influence the level of the $CD8^+$ CTL response. Release of CSP by *P. berghei* sporozoites has been described *in vivo* (Khan, Ng and Vanderberg, 1992) and, therefore, the strong $CD8^+$ CTL response induced by immunisation with

irradiated sporozoites may be related to the poor infectivity of the sporozoites. Poor infectivity is not believed to occur in human malarias (Rosenberg *et al.*, 1990). Hence the question remains as to which immune responses observed in animal models actually occur during natural infections in humans.

Immunisation of volunteers, with irradiated sporozoites of *P. falciparum*, induced a $CD8^+$ CTL response, which recognised a region of the CSP (Malik *et al.*, 1991). Adult Kenyans (Sedegah *et al.*, 1992) and Australians (Doolan *et al.*, 1991) who lived in a malarious area were found to have $CD8^+$ CTL circulating, which recognised the same region of the CSP as the $CD8^+$ CTL found in the immunised volunteers. Studies in the Gambia have provided indirect evidence that $CD8^+$ CTL play a protective role against *P. falciparum* (Aidoo *et al.*, 1995). Further studies in the Gambia (Hill *et al.*, 1991, Hill *et al.*, 1992) have demonstrated an association between individuals expressing HLA-Bw53, a gene of the MHC, and a reduced incidence of severe *P. falciparum.* A small number of these individuals were found to have a $CD8^+$ CTL which recognised a peptide from the LSA-1 (Hill *et al.*, 1992).

$CD4^+$ T cell mediated immunity to pre-erythrocytic stages

The importance of $CD4^+$ T cells in the protective immune response against the exo-erythrocytic stage has slowly emerged. A $CD4^+$ T cell clone, which has cytotoxic activity *in vitro*, was shown to mediate protection against a *P. berghei* challenge (Tsuji *et al.*, 1990). Several $CD4^+$ T cell determinants have been identified in the CSP (Zvering *et al.*, 1992) but it is unclear which epitopes can induce protective immunity. $CD4^+$ T cells which recognise epitopes from the CSP can eliminate infected hepatocytes *in vitro* (Renia *et al.*, 1993) and following adoptive transfer can protect naive mice against a sporozoite challenge, apparently by direct killing (Renia *et al.*, 1993). Activation of $CD4^+$ T cells in response to the *P. falciparum* CSP in individuals from Papa New Guinea has been observed (Doolan *et al.*, 1994). As with the evidence demonstrating

MHC Class I restricted $CD8^+$ CTL activity, it appears that processed fragments of exo-erythrocytic parasite derived proteins can be expressed in association with MHC Class II. Hepatocytes do not express MHC Class II antigens constitutively but its expression can be upregulated during infection (Franco *et al.*, 1988).

Mice primed intrasplenically with exo-erythrocytic forms of *P. yoelii* were protected against a sporozoite challenge but this protection was abrogated following *in vivo* depletion of $CD4^+$ T cells prior to the sporozoite challenge (Renia, Rodrigues and Nussenzweig, 1994). Numbers of $CD4^+$ T cells in the extravascular hepatic compartment of the liver, following priming with live *P. yoelii* sporozoites were raised when compared with controls (Faure *et al.*, 1995).

The division of murine $CD4^+$ T helper cells into T helper 1 (Th1) or Th2 $CD4^+$ T cells (Mosmann and Coffmann 1987) according to their cytokine profiles, led to a change in the approach for studying the role of $CD4^+$ T cells in the immune response to the exo-erythrocytic stage of malaria. Each subset of T helper cell can influence the immune system through their cytokine production. Th1 cells produce IL-2 and IFNγ, and IL-4, IL-6 and IL-10 are produced by Th2 cells (Mosmann and Coffmann 1987). One of the most studied cytokines in this area is the Th1 associated IFNγ. *In vivo* and *in vitro* studies have demonstrated that IFNγ can inhibit the development of rodent and human pre-erythrocytic stage malaria parasites (Ferreira *et al.*, 1986, Mellouk *et al.*, 1987, Maheshwari *et al.*, 1986). Administration of IFNγ systemically will protect rhesus monkeys against a *P. cynomolgi* B sporozoite challenge (Maheshwari *et al.*, 1986). The sterile immunity observed in mice immunised with irradiated sporozoites is abrogated following anti-IFNγ neutralising antibody treatment (Schofield *et al.*, 1987b). Immunisation of mice with *P. berghei* sporozoites induces IFNγ production (White, Jarboe and Krzych, 1994). IFNγ does not exert an inhibitory effect on free sporozoites but on the intracellular development of liver stage parasites (Mazier *et al.*, 1988). Optimal inhibition of *P. berghei* sporozoite development was observed when the primary

hepatocyte culture was treated with IFNγ 6 hours prior to the addition of the sporozoites (Schofield *et al.*, 1987a). Addition of IFNγ to developed intracellular liver stages, resulted in the lysis of parasites (Mellouk *et al.*, 1987). This demonstrates that IFNγ can have a cytotoxic effect on liver stage parasites as well as the cytostatic role defined by the inhibition of development of parasites intracellularly (Mazier *et al.*, 1988). There are several mechanisms through which IFNγ can mediate inhibition of growth. IFNγ receptors are found on the hepatocyte (Schofield *et al.*, 1987a) and it is thought that the binding of IFNγ to its specific receptor induces intracellular alterations which result in an unsuitable environment for parasite development (Schofield *et al.*, 1987a). However, IFNγ induced tryptophan starvation, a mechanism which can kill the intracellular pathogen *Toxoplasma gondii*, does not appear to participate in the inhibitory effect on malaria parasite development mediated by IFNγ (Schofield *et al.*, 1987a). The density of IFNγ receptors present on hepatocytes may explain the observations that *in vitro* (Mellouk *et al.*, 1987) and *in vivo* (Vergara *et al.*, 1987) some parasites are able to evade the inhibitory action of IFNγ. A lower density of receptors may result in a failure to reach a threshold level of IFNγ stimulation of the hepatocytes and consequently the parasite is able to develop within the cell.

IFNγ may mediate anti-parasite immunity by stimulating resident liver macrophages, Kupffer cells, to phagocytose sporozoites (Seguin *et al.*, 1989). Another function of IFNγ during this phase of infection may be to stimulate an increase in antigen presentation by hepatocytes or Kupffer cells through the upregulation of MHC Class II expression. Subsequently this would increase the presentation of parasite antigens to $CD4^+$ T cells and result in an amplification of IFNγ production and other T cell products. Hence, IFNγ can upregulate its own production via the stimulation of antigen presenting cells ensuring that enough is present locally in the liver to mediate protective immunity. Initially it was thought that $CD8^+$ CTL were the source of IFNγ in the liver (Schofield, 1989) but the demonstration of $CD4^+$ T cell presence in the liver (Faure *et al.*, 1995) provides another source of IFNγ. Indeed it has been shown that during *P. yoelii*

infection, IFN γ can also be produced by a non-T cell source presumably natural killer (NK) cells which require IL-12 and TNF stimulation (Sedegah, Finklemann and Hoffman, 1994).

The only direct role that Th2 $CD4^+$ T cells appear to have in the immune response to the exo-erythrocytic stages is as a possible source of IL-6 which has been shown to be an important mediator in the protective response (Pied *et al.,* 1992). An important indirect mechanism, which Th2 cells are vital for is the production of a humoral response. Th2 cells supply crucial "helper" signals which are involved in the stimulation, differentiation and proliferation of B cells. This results in plasma cells producing an antibody response which, as already discussed, can prevent the development of the parasite by various means. This "help" is usually the production of cytokines which are classically Th2 associated. There is however, very little evidence for a large production of IL-4 during this phase of the infection. No IL-4 production was observed during *in vitro* proliferation of splenocytes following immunisation of mice with *P. berghei* sporozoites (White, Jarboe and Krzych, 1994). However, a systemic increase in IL-4 production was noted following immunisation with *P. yoelii* CSP plasmid DNA (Mor *et al.,* 1995). The increase in IL-4 was associated with a primary humoral response following the first immunisation but this switched to an IFNγ mediated response after the booster of *P. yoelii* CSP plasmid DNA. IL-4 production was found not only in the lymph nodes but in several lymphoid organs. This experimental immunisation model illustrates a mechanism which may explain the absence of evidence for an IL-4 mediated response. In this model there are low levels of parasite derived antigen and under these conditions B cells are involved in antigen presentation which results in a preferential activation of Th2 cells (Mamula and Janeway, 1993, Ron and Sprent, 1987) and consequently an increase in IL-4 production. The levels of parasite derived antigen in other experimental immunisation models is probably higher and would result in activation of Th1 $CD4^+$ T cells and an increase in IFNγ production.

The Th2 associated cytokine, IL-6, has been extensively studied in conjuction with IL-1 and TNFα. They are known as endogenous pyrogens and are important mediators of the acute inflammatory response to various stimuli. TNF has been shown to inhibit the development of malarial exo-erythrocytic forms (Schofield *et al.,* 1988). No inhibitory effect mediated by TNF was observed when using a primary culture of hepatocytes *in vitro* (Mazier *et al.,* 1990). The addition of non-parenchymal cells, such as lymphocytes, NK cells and Kupffer cells, restored the inhibitory effect of TNF (Mazier *et al.,* 1990). Elevated IL-6 concentrations were observed in these *in vitro* cultures. Addition of anti-IL-6 monoclonal antibodies resulted in a decrease in the TNF inhibition suggesting that IL-6 is a crucial mediator in the protective mechanism (Mazier *et al.,* 1990). IL-6 was observed in the sera of mice infected with *P. yoelii* sporozoites (Pied *et al.,* 1992). Intraperitoneal administration of IL-6 significantly reduced the development of *P. berghei,* liver schizonts in rats (Vreden *et al.,* 1992). Administration of anti-IL-6 resulted in a 40% increase of liver schizonts (Vreden *et al.,* 1992). IL-6, IL-1 and TNF mediate their protective response via their role as inducers of the acute phase response. They participate in an intricate network which results in the stimulation of hepatocytes and non-parenchymal cells. These endogenous pryogens can act in synergy (Vreden *et al.,* 1992, Mazier *et al.,* 1990) and can also induce the production of each other (Pied *et al.,* 1992). This inflammatory network can induce the production of a series of proteins known as acute phase reactants (Ramadori *et al.,* 1985). Acute phase proteins are synthesised mainly by the hepatocyte and include C-Reactive protein (CRP), α2-macroglobulin, serum amyloid A and serum amyloid P. Rapid increase in systemic levels of these proteins is observed following various stimuli. CRP has been shown to prevent sporozoite penetration into the hepatocyte and can block parasite division through an antibody-like effect (Pied *et al.,* 1991). Acute phase reactants represent a non-specific response stimulated by inflammatory cytokines. Another non-specific response, which is an effector mechanism stimulated by IL-6 and TNF is the production of nitric oxide (NO). NO is derived from L-arginine and can inhibit the development of *Toxoplasma* (Adams *et al.,* 1990) and *Leishmania* (Green *et al.,* 1990). Macrophages,

Kupffer cells and hepatocytes have all been shown to produce NO upon stimulation (Nussler *et al.*, 1991a, Billiar *et al.*, 1989, Klotz *et al.*, 1995). Inhibition of NO production by inhibitors of NO synthesis resulted in the abrogation of the protective effect mediated by TNF and IL-6 (Nussler *et al.*, 1991a). *In vitro* experiments have demonstrated that the protective effect observed with IFNγ is also mediated via NO (Mellouk *et al.*, 1991). The production of NO is now regarded as one of the major protective mechanisms against the exo-erythrocytic stages of malaria infection.

Immunity to the asexual erythrocytic stages of malaria

Naturally acquired immunity to the asexual erythrocytic stages of malaria is manifested in individuals who harbour a low grade parasitaemia with no clinical symptoms. As the asexual blood-stage parasites are responsible for the morbidity of the disease, it has been hypothesised that immunity is partly anti-parasitic and partly anti-toxic (Sinton, Harbhag and Singh, 1931), a theory which has been redefined recently as anti-parasite and anti-disease immunity (Playfair *et al.*, 1990). Humoral responses may be sufficient for the anti-disease immunity but it is thought that a combination of humoral and cellular mechanisms is required for the development of effective anti-parasitic immunity, however, the exact nature of the anti-malarial mechanisms are unclear.

There are difficulties in investigating the immune response in humans and these studies usually involve a serological approach or analysis of peripheral blood mononuclear cells usually by restimulation *in vitro*. Often the history of exposure to malaria for an individual is unknown and concurrent infections other than malaria may influence the serological profile or the composition of the cells in the peripheral blood. Hence, much of our understanding of the immune mechanisms to the blood-stage of infection comes from experimental models. The mechanism of protective immunity in these models often depends upon the host-parasite combination. However, this does reflect the situation in human malaria infection because the genetic background of the host and the strain of the

infecting *Plasmodium* species will influence the development of the protective immune response and ultimately the outcome of the infection.

Humoral Immunity

Transfusion of γ-globulin or purified umbilical cord IgG isolated from immune adults, to non-immune children during falciparum malaria infection results in a decrease in parasitaemia (Cohen, McGregor and Carrington, 1961, Sabchareon *et al.*, 1991). Passive transfer of IgG from immune donors protects Thai patients against *P. falciparum* infection (Bouharoun-Tayoun *et al.*, 1990) and the antibodies which demonstrated this clinical effect were able to mediate inhibition of *P. falciparum* development *in vitro* by a process termed antibody-dependent cellular inhibition (ADCI) (Bouharoun-Tayoun *et al.*, 1990). Antibodies that proved clinically ineffective in the same individuals did not promote ADCI. The mechanism of ADCI is dependent upon IgG binding to monocytes via their FcγRII receptors which induces the release of soluble factors, thought to include TNF, which act upon young intraerythrocytic parasites (Bouharoun-Tayoun *et al.*, 1995). A correlation between the presence of cytophilic or non-cytophilic antibodies and the clinical status of protection has been observed (Bouharoun-Tayoun and Druilhe, 1992). An increase in IgG1 and IgG3 correlated with the development of clinical immunity whereas a susceptible group of the population in an endemic area produced the non-cytophilic antibodies, IgM and IgG2 in response to infection (Bouharoun-Tayoun and Druilhe, 1992). It has been suggested that anti-malarial antibody may block invasion of host erythrocytes by merozoites (Cohen and Butcher, 1970, Phillips *et al.*, 1972) and may also prevent cytoadherence (David *et al.*, 1983). However, the interaction of malarial antibody with cellular mechanisms was demonstrated by the requirement for the presence of antibody to enable PBMN cells to reduce the growth of *P. falciparum* (Brown and Smalley, 1980).

Mice immunised with a recombinant fusion protein incorporating the carboxy-terminal region of *P. yoelii* MSP, are resistant to a homologous challenge (Daly and Long, 1995). Furthermore, resistance was passively transferred by immune serum or purified Ig from the immunised mice to recipient mice challenged with *P. yoelii* (Daly and Long, 1995). It has been reported that during a primary infection with *P. chabaudi* there is a polyclonal activation of B cells resulting predominantly in the production of IgG2a (Langhorne, Kim and Asofky, 1985, Falanga *et al.*, 1987). Protection against re-infection appears to involve IgG1 (D'Imperio *et al.*, 1996). IgG1 has also been implicated in the control of the recrudescence parasitaemia of a primary *P. chabaudi* infection following treatment of mice with recombinant IL-6 (Akanmori, Kawai and Suzuki, 1996), a cytokine known to stimulate B cells to secrete immunoglobulins (Teranishi *et al.*, 1982, Muraguchi *et al.*, 1988, Takatsuki *et al.*, 1988). Both IgG1 and IgG3 have been proposed to confer protection against human malaria (Groux and Gysin, 1990).

Cell mediated immunity - rodent malaria

Immunity to the asexual blood-stages of malaria in rodent models can be transferred with immune spleen and lymph node cells (Stechschulte, 1969, Roberts and Tracey-Patte, 1969, Phillips, 1970, Kasper and Alger, 1973). Rats infected with the blood-stages of *P. berghei* rapidly control the infection after receiving immune spleen cells (Phillips and Jones, 1972). Thymectomized mice are more susceptible to *P. chabaudi* infection than controls (McDonald and Phillips, 1978b). Adoptive transfer of immune spleen cells protects irradiated mice against a *P. chabaudi* challenge (McDonald and Phillips, 1978b). Unfractionated spleen cells were observed to confer better protection than enriched T cell populations demonstrating the requirement for both T cells and B cells. The same observation was made by Jayawardena *et al.*, (1982) where both T cells and B cells recovered from *P. yoelii* infected mice, conferred protection to non-immune irradiated recipients to a homologous challenge. However, it was noted that optimal protection was

obtained by the adoptive transfer of B cells and CD4+ T cells (Jayawardena *et al.*, 1982). B cell depleted mice are able to control an infection with *P. chabaudi adami* or *P. vinckei petteri* (Grun and Weidanz, 1981, Grun and Weidanz, 1983, Clark, 1987, Kumar *et al.*, 1989, Cavacini, Parke and Weidanz, 1990) demonstrating the importance of T cell regulated cell-mediated immunity. The role of CD4+ T cells in protection against the asexual erythrocytic stage of infection in mice has been the focus of extensive research. Depletion of CD4+ T cell function by a monoclonal antibody during the primary parasitaemia of a *P. chabaudi* infection confirmed the essential role which CD4+ T cells have in mediating the control of this acute erythrocytic stage (Langhorne, 1989). Analysis of splenic lymphocytes from mice during a primary infection of *P. chabaudi*, showed a greater frequency of Th1 CD4+ T cells characterised by the production of IFNγ and IL-2 (Langhorne, 1989). As the infection progressed, the frequency of these cells decreased, replaced by Th2 cells characterised by the production of IL-4. Hence, it appears that Th1 cells mediate the acquired immunity to the acute phase of a *P. chabaudi* infection but there is a switch to Th2 mediated immunity (see Diagram 2). This implies that early protective mechanisms are antibody-independent with a switch to antibody-dependent mechanisms during the later stages of infection. This was clearly demonstrated when SCID mice, reconstituted with CD4+ T cells from naive animals or infected mice, survived a *P. chabaudi* infection but a persistent recrudescence parasitaemia was observed (Meding and Langhorne, 1991). However, the transfer of B cells in addition to the CD4+ T cells, resulted in the SCID mice eliminating the parasite.

Characterisation of CD4+ T cell clones from *P. chabaudi* infected mice recovering from a primary parasitaemia or from mice which had cleared a secondary infection, confirmed the polarisation of the CD4+ T cell response during *P. chabaudi* infection (Taylor-Robinson and Phillips, 1992). The clones derived from the period when the acute primary parasitaemia was in decline, were of the Th1 phenotype whereas the clones derived from mice which had cleared a secondary infection were characteristic of Th2 cells. Adoptive transfer of both the Th1 and Th2 clones conferred protection against a *P.*

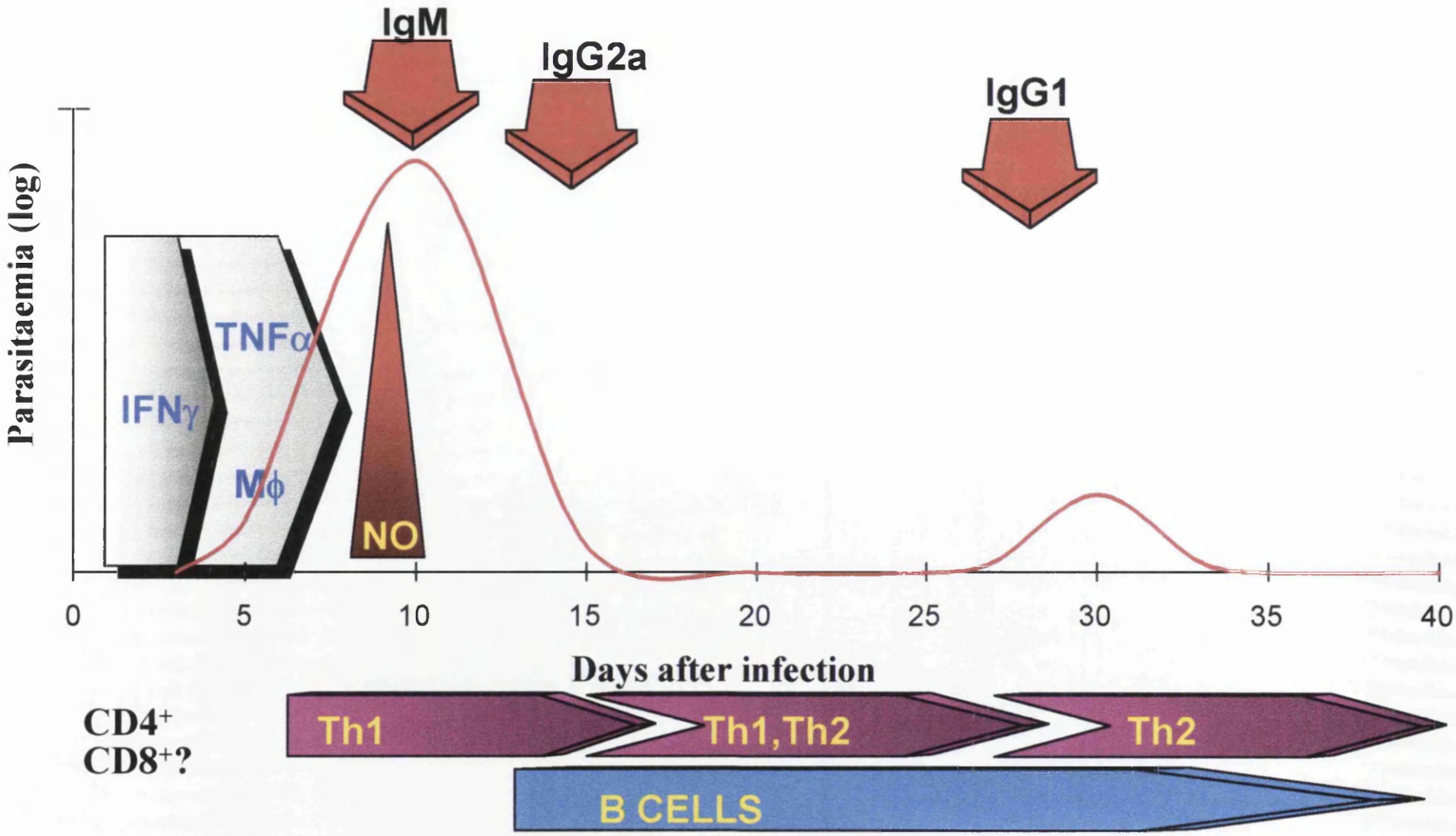

Diagram 2. Immunity to *P. chabaudi* AS infection

chabaudi challenge in immunocompromised hosts (Taylor-Robinson and Phillips, 1993). However, the transfer of the Th2 clone also required the transfer of splenic B cells to be fully protected in immunocompromised hosts. The Th1 clone is thought to regulate the development of protective immunity through the production of IL-2 and IFNγ possibly via a NO dependent mechanism and the Th2 clone mediates protection by promoting the production of a specific antibody via the secretion of IL-4 (Taylor-Robinson and Phillips, 1993, Taylor-Robinson *et al.*, 1993).

B cells appear to have an important role in the development of the Th2 responses during *P. chabaudi* infection, thus extending the function of B cells beyond that of specific antibody production. As already mentioned, SCID mice require the transfer of both $CD4^+$ T cells and B cells to resolve a *P. chabaudi* infection efficiently (Meding and Langhorne, 1991). Passive transfer of immune IgG did not confer protection to *P. chabaudi* challenged SCID mice (von der Weid *et al.*, 1994), demonstrating that the presence of B cells is required for efficient elimination of the parasite. The B cells, most likely act as antigen presenting cells and produce cytokines or display important co-stimulatory molecules which promote Th2 cell development (Troye-Blomberg, Berzins and Perlmann, 1994).

The interaction between Th1 and Th2 cells and their respective responses is a crucial factor in the development of a protective immune response to not only *P. chabaudi* infection in mice but several other infectious diseases. Inappropriate responses can often contribute to the pathology of a disease. As already described $CD4^+$ T cells are subdivided into two distinct subsets by the pattern of cytokine secretion. Th1 cells produce IL-2 and IFNγ whereas Th2 cells characteristically produce IL-4, IL-5, IL-6 and IL-10 (Mosmann and Coffman, 1989). Both Th1 and Th2 cells provide help for B cell function through the secretion of IFNγ and IL-4 or IL-5 (Mosmann and Coffman, 1989). Th1 cells mediate delayed type hypersensitivity inflammatory reactions (Cher and Mosmann, 1987) whereas Th2 cells are associated with allergic responses (Mosmann

and Coffman, 1989). IFNγ can inhibit the proliferation of Th2 cells (Gajewski and Fitch, 1988) but does not affect proliferation or lymphokine production by Th1 cells (Fernandez-Botran *et al.*, 1988). IL-10 can inhibit antigen presenting cell induced cytokine production by Th1 cells (Fiorentino *et al.*, 1991). Hence, Th1 and Th2 cells can regulate the proliferation and expansion of the other subset which has important consequences on the response to a pathogen.

During *P. chabaudi* infection in mice, the sequential involvement of Th1 and then Th2 mediated responses results in efficient control of the infection. The regulation and differentiation mechanisms of the $CD4^+$ T cell response are unclear. Naive $CD4^+$ T cells, when first stimulated produce IL-2 and then differentiate into either the Th1 or Th2 phenotype (Mosmann and Sad, 1996). T cells expressing the cytokines from both Th1 and Th2 phenotypes have been designated Th0 (Romagnani, 1996). The differentiation of effector Th1 or Th2 cells proceeds through this Th0 phase of expressing multiple cytokines (Mosmann and Sad, 1996).

There are several factors which can influence the process of Th1/Th2 differentiation. One of the most important factors is the microenvironment of the responding Th cell. The presence of IL-4 is a strong stimulus for Th2 differentiation whereas IL-12 is regarded as the main stimulus for Th1 development (Seder and Paul, 1994). During an initial response, antigen specific T cells are present at a low frequency and hence, other cells can produce the cytokines necessary for the induction of the required Th response. Mast cells, basophils and a subpopulation of T cells, $NK1.1^+$ cells may be a source of early IL-4 production and macrophages are major producers of IL-12 (Mosmann and Sad, 1996). Naive Th0 cells have recently been shown to produce IL-4 in small quantities upon initial activation and it is thought that this may promote differentiation from naive Th0 cell to a Th2 cell (Romagnani, 1996). Early IFNγ production, by NK cells for example, may inhibit the development of Th2 cells (Seder and Paul, 1994) and hence promote Th1 expansion.

It was initially thought that antigen presenting cells (APC) may be able to influence the Th1/Th2 decision. Th1 cells respond optimally to purified adherent cells from spleen cells, while Th2 cells had optimal proliferative responses when splenic B cells were used as APC (Gajewski *et al.*, 1991). However, it is now known that dendritic cells, macrophages and B cells are all capable of inducing differentiation of Th1 or Th2 cells in the presence of the appropriate cytokines (Mosmann and Sad, 1996). Recently, IL-6 derived from APC was shown to be capable of inducing polarisation of naive Th cells to Th2 cells by stimulating IL-4 production in $CD4^+$ T cells (Rincon *et al.*, 1997). Hence, a role for APC in the induction of Th1 or Th2 cells cannot be excluded because they may exert an influence via the cytokines they secrete or as has been recently suggested, the expression of co-stimulatory molecules on APC may selectively influence the Th1/Th2 decision.

B7-1 and B7-2 molecules are expressed on activated B cells, dendritic cells, activated T cells and monocytes (Constant and Bottomly, 1997). They bind to CD28 on T cells and CTLA-4 on activated T cells (Lenschow, Walunas and Bluestone, 1996). The presence of either B7 molecule is essential to induce naive $CD4^+$ T cells to produce IL-2 and proliferate (Chen and Nabavi, 1994). B7-2 expression is induced more rapidly than B7-1 (Hathcock *et al.*, 1994) and a 100 fold higher level of B7-2 expression than B7-1 was shown on dendritic cells (Inaba *et al.*, 1994). Both B7-1 and B7-2 have been shown to induce Th1 or Th2 differentiation (Seder and Paul, 1994) but B7-2 appears to be the dominant costimulatory molecule during primary responses whereas B7-1 may be important in maintaining primary and secondary responses (Mosmann and Sad, 1996).

Antigen dose is another factor which may influence the Th1/Th2 decision. Early *in vivo* studies suggested that low doses of antigen, either *Leishmania* or *Trichuris muris* infection, induced a Th1 response, but an increase in antigen dose induced a Th2 response (Bretscher, Wei and Menon, 1992, Bancroft, Else and Grencis, 1994).

However, conflicting reports indicated that using various soluble proteins as antigens, an increase in antigen dose skewed the response to a Th1 phenotype (HayGlass *et al.*, 1986, Chaturvedi *et al.*, 1996). Hence, the type of antigen may account for the discrepancies in the studies with Th1 responses induced by low doses of parasites as immunogens whereas Th2 responses were observed following administration of low doses of soluble proteins. A further explanation may be that at high doses of antigen, Th1 cells are more susceptible to activation-induced cell death (apoptosis), and therefore, high doses of parasite antigen may promote the expansion of Th2 cells because the negative cross-regulation of Th1 cells is eliminated (Constant and Bottomly, 1997). The regulation of Th1/Th2 differentiation by antigen dose may also involve the interaction of the T cell receptor (TCR) with the antigen. The strength of this ligation and the role of various co-receptors will determine if there is a sustained pattern of signal transduction. It has been suggested that for a naive Th cell to produce IFNγ (Th1 phenotype) a threshold of activation is required (Constant and Bottomly, 1997). If the interaction between the TCR and antigen is strong, this will lead to constant signal transduction, sustained activation and IFNγ production. A weak interaction between the TCR and antigen may result in a transient pattern of activation, resulting in IL-4 production (Th2 phenotype). The role of antigen dose in the induction of Th1 or Th2 responses is complex and the studies performed have to be interpreted with caution because there are many variable factors involved and hence, it is difficult to construct a clear hypothesis.

The induction of either Th1 or Th2 responses during *P. chabaudi* infection is an important step in the development of protective immunity. The sequential involvement of Th1 mediated responses followed by Th2 induced effector mechanism is required for the efficient elimination of the parasite. The mechanisms of induction of the initial Th1 differentiation and secondly a switch to Th2 mediated responses during *P. chabaudi* remain unclear. The cytokine environment, various APC and the level of antigen dose have all been proposed to induce the appropriate type of Th cell response and effector functions. Investigation into these mechanisms will have important consequences on the

research for a vaccine targeted to the asexual blood stages because the demonstration that human Th1 and Th2 cells exist (Romagnani, 1996) illustrates that the vaccine will need to induce the appropriate $CD4^+$ T cell response.

$CD8^+$ T cells do not appear to have a significant role in the protective immunity to the asexual blood-stage of malaria infection in rodents. Adoptive transfer of $CD8^+$ T cells was shown to confer a degree of protection against a *P. yoelii* infection (Mogil, Patton and Green, 1987) but this was contradictory to reports demonstrating that only $CD4^+$ T cells were required for protection against *P. yoelii* (Vinetz *et al.*, 1990). However, mice immunised with a crude *P. falciparum* antigen were partially protected against a *P. yoelii* challenge and this protection was apparently $CD8^+$ T cell mediated (Lucas *et al.*, 1993). $CD8^+$ T cells were also proposed to participate in the resolution of the later phases of *P. chabaudi adami* and *P. chabaudi* AS infection (Weidanz, Melancon-Kaplan and Cavacini, 1990, Podoba and Stevenson, 1991). Infection of β_2-microglobulin deficient mice, which lack surface expression of the MHC class I molecule on nucleated cells and are, therefore, essentially devoid of a functional $CD8^+$ T cell response, with either *P. chabaudi* AS, *P. chabaudi adami* or *P. yoelii* resulted in a similar resolution of infection as in the intact control mice (van der Heyde *et al.*, 1993). This observation suggests that the role of $CD8^+$ T cells during a primary malaria infection in mice is minimal, although the possibility of compensatory mechanisms, such as increased production of IFNγ by another source cannot be ignored.

Cell mediated immunity - human malaria

Investigations of the role of cell-mediated immunity in human malaria infection has mainly consisted of studies examining the *in vitro* response of PBMN cells from infected patients or malaria-immune donors. However, these studies have to be interpreted with

caution because they are influenced by various factors. Details of an individual's parasitological history and current parasitological and immune status are sometimes unknown. Non-malaria related illness and pregnancy can also influence the parameters measured in these studies (Riley and Greenwood, 1990). The proliferative response and the ability to produce cytokines in response to stimulation with malarial antigen are the main parameters analysed in the studies of human T cell responses during malaria infection. As in mice, human $CD4^+$ T cells can be separated into distinct subsets by cytokine profiles (Troye-Blomberg and Perlmann, 1988). $CD4^+$ T cell derived IL-4 production can be induced by stimulation of PBMN cells from immune donors *in vitro* with malarial antigens (Troye-Blomberg *et al.*, 1990a). Clinically immune adults were shown to give strong proliferative and IFNγ responses to a soluble malarial antigen (Riley *et al.*, 1988). Both IFNγ and IL-4 have been implicated in protective immune mechanisms during blood-stage malaria infection (see later). IFNγ, a potent activator of effector cells (Boehm *et al.*, 1997), is thought to be involved in the killing of blood-stage parasites (Ockenhouse, Schulman and Shear, 1984, Shear *et al.*, 1989). Increased IL-4 levels in the serum of aparasitaemic individuals from an area of perennial and holoendemic *P. falciparum* transmission, suggests this cytokine may be involved in an anti-parasitic response (Mshana, Boulandi and Mshana, 1991).

The role of $CD8^+$ T cells in immunity to the asexual blood-stage of human malaria infection is thought to be a minor one. There are several reports of increased numbers of $CD8^+$ T cells in individuals who have recently recovered from an attack of malaria (Hoffman *et al.*, 1984, Troye-Blomberg *et al.*, 1984). Malaria-specific $CD8^+$ T cell clones have been isolated from the peripheral blood of recently infected individuals (Sinigaglia, Matile and Pink, 1987). However, it is thought that the role of $CD8^+$ T cells may be immunoregulatory and indeed may be immunosuppressive (Troye-Blomberg, Berzins and Perlmann, 1994). Removal of $CD8^+$ cells from isolated PBMN cells by anti-$CD8^+$ treatment, enhanced the proliferative and IFNγ responses of cells which were normally low responders to *in vitro* stimulation (Riley, Jobe and Whittle, 1989). As

already described, $CD8^+$ T cells have an important role in the protective immune response to the intra-hepatic stage of malaria infection and that this mechanism is partly IFNγ dependent. The production of high levels of IFNγ by $CD8^+$ T cells in response to the pre-erythrocytic stage of infection could influence the immune response of an individual to a concurrent blood-stage infection. Furthermore the demonstration that $CD8^+$ T cells (Mosmann, Li and Sad, 1997), as with $CD4^+$ T cells, can be separated into different subsets, characterised by their cytokine secretion profiles (Mosmann and Coffman, 1987) demonstrates the potential immunoregulatory role of $CD8^+$ T cells during human malaria infection.

A characteristic of the T cell response of patients with acute falciparum malaria is an antigen-specific immunodepression. This unresponsiveness may be due to an absence of antigen-specific T cells in the peripheral blood possibly due to a sequestration of the appropriate T cells to sites from the peripheral blood (Hviid *et al.*, 1991, Hviid *et al.*, 1993). Defective IL-2 production may contribute to the immunosuppression observed (Troye-Blomberg *et al.*, 1985, Ho *et al.*, 1988). PBMN cells from patients with acute falciparum malaria fail to produce IL-2 or express IL-2 receptors upon stimulation with malaria specific antigen (Ho *et al.*, 1988). Serum from malaria patients can suppress *in vitro* cellular responses of PBMN cells from children with acute malaria to restimulation with malaria antigens, non-malaria antigens and mitogens (Riley *et al.*, 1988). It has been shown that prostaglandin E, which can inhibit antigen and mitogen-induced blastogenesis (Leung and Mihich, 1980), is in part responsible for the suppression of the *in vitro* cellular responses of PBMN cells from malaria patients (Riley *et al.*, 1989). However, Ho and Webster (1990) in contrast, report that they have been unable to consistently demonstrate suppressor serum factors including prostaglandin E. It has been suggested that the counter regulatory roles of $CD4^+$ T cell subsets may contribute to the immunosupression through the production of inhibitory cytokines such as IFNγ or IL-4 (Ho and Webster, 1990). The induction of NO production by Th1 mediated

inflammatory responses may also contribute to the immunosuppression observed (Rockett *et al.,* 1994).

Role of γδ T cells in immunity to the asexual erythrocytic stages

Recent evidence has suggested that T cells expressing the γδ T cell receptor may be involved in immunity to the blood-stages of malaria infection. γδ T cells constitute only a small minority of peripheral T cells in man and mouse (Falini *et al.*, 1989, Haas, Pereira and Tonegawa, 1993, Allison, 1993). These cells are characteristically $CD3^+$ but in the majority of cases do not express CD4 or CD8 (Haas, Pereira and Tonegawa, 1993). A protective role for γδ T cells in various infectious and parasitic diseases has been proposed (Modlin *et al.*, 1989, Minoprio *et al.*, 1989, Hiramatsu *et al.*, 1992, Russo *et al.*, 1993). Elevated numbers of γδ T cells in the peripheral blood of patients during acute *P. falciparum* infection or convalescence has been reported (Ho *et al.*, 1990, Roussilhon *et al.*, 1990). Recently, two studies have demonstrated the activation of γδ T cells during human malaria infection (Rzepczyk *et al.*, 1996, Worku *et al.*, 1997), confirming the possible protective and/or immunomodulatory role for γδ T cells in malaria. Preliminary reports have suggested that human γδ T cells can inhibit the growth of *P. falciparum in vitro* (Elloso *et al.*, 1994).

These observations in human malaria are confirmed in experimental malaria infections. γδ T cell numbers increase in the spleen of mice resolving a *P. chabaudi* infection (Langhorne, Pells and Eichmann, 1993, van der Heyde *et al.*, 1993b). There is a pronounced increase in the γδ T cell number in β_2-microglobulin deficient mice and B cell deficient mice during experimental malaria infection (van der Heyde *et al.*, 1993b, Langhorne, Pells and Eichmann, 1993). Mice lacking T cells with the γδ TCR were able to control a *P. chabaudi* infection with a slight delay in the time of clearance of the acute phase of infection and higher recrudescent parasitaemias were observed compared to controls (Langhorne, Mombaerts and Tonegawa, 1995). It was observed that the

increase in the splenic γδ T cells in *P. chabaudi* infected mice was localised in the red pulp areas of the spleen (Langhorne, Pells and Eichmann, 1993). This occurs during human malaria infection as well (Bordessoule, Gaulard and Mason, 1990). The location of γδ T cells in the red pulp of the spleen would allow these cells to be in close contact with parasitised erythrocytes and hence, γδ T cells may have a role in parasite destruction. γδ T cells are known to produce IFNγ and TNFβ (Patel, Wacholtz and Duby, 1989, Kabelitz, Pechold and Bender, 1991, Ferrini, Prigione and Bottino, 1989) and resemble NK cells, lymphokine activated cells and αβ T cells in that they can lyse target cells (Borst *et al.*, 1987, Lanier, Ruitenberg and Phillips, 1986). Therefore they have the potential to mediate or perform parasiticidal activity. However, it must be noted that αβ T cell deficient mice were unable to control a *P. chabaudi* infection despite a functional γδ T cell response (Langhorne, Mombaerts and Tonegawa, 1995). It is possible that in the absence of αβ T cells, γδ T cells are not able to provide the required help for antibody production which is necessary for parasite elimination. Furthermore, γδ T cell deficient mice have been shown to be able to control a *P. chabaudi* infection with a similar efficiency to that of control mice (Taylor-Robinson *et al.*, 1994b) suggesting that γδ T cells do not play a significant role in control of blood-stage malaria infection.

Role of cytokines in immunity to asexual erythrocytic stages

The interactions of cytokines clearly influence the development of immunity to the asexual blood stage of malaria infection. IFNγ, produced by $CD4^+$ Th1 cells, $CD8^+$ T cells and NK cells, has been shown to activate macrophages to kill intracellular malaria parasites (Ockenhouse, Schulman and Shear, 1984). T cell clones derived from PBMN cells from individuals living in an endemic region, immune to *P. falciparum* infection, produce IFNγ upon re-stimulation with malarial antigen *in vitro* (Sinigaglia and Pink, 1985). T cells isolated from patients acutely infected with *P. falciparum* produce IFNγ *in vitro* following antigen specific stimulation (Troye-Blomberg *et al.*, 1985). IFNγ has

been detected in the sera of patients with either *P. falciparum* or *P. vivax* (Rhodes-Feuilletee *et al.*, 1985, Kern *et al.*, 1989). *In vivo* treatment of *P. chabaudi* infected mice with recombinant IFNγ enhanced protection (Clark *et al.*, 1987) and mice treated with anti-IFNγ antibodies during *P. chabaudi* infection were found to have an exacerbated parasitaemia (Stevenson *et al.*, 1990). The role of IFNγ during *P. chabaudi* infection appears to be restricted to the primary parasitaemia with peak production occurring 2 or 3 days prior to the peak of the primary parasitaemia (Slade and Langhorne, 1989, Stevenson *et al.*, 1990). IFNγ can activate macrophages to secrete inflammatory molecules such as NO and oxygen radicals, IL-1, TNFα and IL-6 (Phillips, 1994a, Taylor-Robinson, 1995) which have all been proposed to be involved in parasiticidal mechanisms (Phillips, 1994a, Taylor-Robinson, 1995). The macrophage has a pivotal role during the development of immunity to the asexual erythrocytic stages because it is involved in the induction of the immune response via antigen presentation and is also an effector cell.

TNF is an important macrophage product which is induced by IFNγ stimulation (Langhorne, 1989, Stevenson *et al.*, 1990, Taylor-Robinson and Phillips, 1992, Waki *et al.*, 1992) or directly by stimulation with parasite derived toxins (Kwiatkowski *et al.*, 1989, Schofield and Hackett, 1993, Bate *et al.*, 1990, Bate *et al.*, 1992). Mononuclear cells isolated from the peripheral blood of patients with acute malaria are primed to secrete enhanced levels of TNF (Bate *et al.*, 1990). Macrophages from the spleens and livers of mice infected with rodent malarial parasites have also been shown to have an enhanced capacity to secrete TNF (Bate *et al.*, 1990). High levels of TNF have been detected in the sera of patients infected with *P. falciparum* (Scuderi *et al.*, 1986, Kern *et al.*, 1989, Grau *et al.*, 1989b, Kwiatkowski *et al.*, 1990). Recombinant TNF treatment of *P. chabaudi adami* infected mice resulted in an earlier resolution of the parasitaemia and an earlier appearance of crisis forms (Clark *et al.*, 1987). Human recombinant TNFα treatment protects susceptible A/J mice against a lethal *P. chabaudi* AS infection (Stevenson and Ghadirian, 1989). An increase in TNF expression in the spleen

correlates with resistance to a *P. chabaudi* AS infection (Jacobs, Radzioch and Stevenson, 1996). TNF may not have a direct cytotoxic effect on parasites because the recombinant molecule had no cytotoxic effect on *P. yoelii* (Taverne *et al.*, 1987) or *P. falciparum* (Hviid *et al.*, 1988). Hence, TNF mediated parasite destruction may be through the activation of various cells such as macrophages, neutrophils, lymphocytes and endothelial cells (Stevenson, Nowotarski and Yap, 1990).

Raised levels of IL-1 and IL-6, both macrophage products, have been observed in the serum of patients infected with *P. falciparum* (Jakobsen *et al.*, 1994) and both IL-1 and IL-6 can synergise with TNF in mediating the characteristic malarial fever (Kwiatkowski, 1995). IL-12 has been recently shown to mediate protection to *P. chabaudi* infection via IFNγ and TNF induction of NO secretion (Stevenson *et al.*, 1995). IL-12 promotes the development of Th1 mediated immunity and the production of IFNγ by both NK cells and T cells (Trinchieri, 1995) and hence is an important mediator of cell mediated immunity. There is no direct evidence that IL-12 has a role during human malaria infection but it is proposed that it is important in the induction of cell mediated immunity during the acute phase of infection.

The Th2 associated cytokines IL-4 and IL-10 are important for the development of the humoral immunity to the asexual blood stages of malaria infection. Th2 clones derived from *P. chabaudi* infected mice protected recipient mice in an IL-4 and B cell dependent manner (Taylor-Robinson and Phillips, 1993). IL-4 is primarily responsible for the production of IgE (Finkelman *et al.*, 1990) and hence, elevated concentrations of IgE in the serum of *P. falciparum* infected individuals may reflect elevated IL-4 levels (Helmby *et al.*, 1996, Perlmann *et al.*, 1997). Elevated levels of IL-10 have been detected in the sera of patients infected with *P. falciparum* (Peyron *et al.*, 1994) and in mice challenged with a lethal *P. yoelii* infection (Kobayashi *et al.*, 1996). However, it is unclear if the elevated levels of IL-10 are detrimental because they are capable of inhibiting cellular immune responses or beneficial by reducing parasite-induced inflammatory responses.

Malarial Pathology

The erythrocytic stage of malaria causes the pathology associated with the disease. Symptoms can range from mild fever and oedema to cerebral malaria. Malaria pathology depends upon the strain of parasite and the immune status of the host (White and Ho, 1992). Non-immune individuals in endemic areas are at the most risk of developing severe pathology but this is rarely observed in individuals older than 7 years of age.

Although T cells are regarded as being crucial for the host response to malaria infection, they were first examined in the pathology of the disease. Wright (1968) proposed that thymic atrophy found in children suffering from Kwashiorker (a protein deficiency disease) explained the low incidence of cerebral manifestations in these children when they became infected. Neonatally thymectomised hamsters survived *P. berghei* infection much longer than control animals (Wright, 1968). *P. berghei* ANKA strain is regarded as a reasonable laboratory model for examining the pathology of malaria infection. *P. berghei* infection in some strains of mice, leads to the development of cerebral malaria. *In vivo* depletion of $CD4^+$ T cells in *P. berghei* infected mice with an anti-$CD4^+$ monoclonal antibody prevented the development of cerebral malaria without any direct effect on the parasitaemia (Hermsen *et al.*, 1997). Adoptive transfer of $CD4^+$ $CD8^-$ T cells from mice with cerebral malaria exacerbated mortality in infected euthymic mice (Grau and Behr, 1994).

TNF has emerged as the crucial mediator in the development of cerebral malaria. Injection of anti-TNF antibodies prevented the onset of cerebral pathology in mice infected with *P. berghei* ANKA (Grau *et al.*, 1987). Overproduction of TNF appears to be mediated by $CD4^+$ T cells (Grau *et al.*, 1987). IFNγ is another T cell product which has been implicated in the pathology of malaria. Administration of neutralising anti-IFNγ antibody reduced the incidence of cerebral symptoms in infected mice with *P. berghei*

ANKA (Grau *et al.*, 1989a). Susceptibility to cerebral malaria is accompanied by the up-regulation of IFNγ gene (Grau and Behr, 1994) and the expression of IL-4 and TGFβ, both antagonists of IFNγ/TNF mediated responses, are significantly down-regulated in susceptible mice. Therefore the balance of Th1 and Th2 cytokine production appears to be important in determining resistance or susceptibility. IFNγ is capable of activating macrophages to produce TNF and can induce the up-regulation of TNF receptors (Pandita *et al.*, 1992). Thus overproduction of IFNγ and TNF leads to the activation of endothelial cells resulting in the up-regulation of many adhesion molecules such as ICAM-1 which have been implicated in the pathogenesis of cerebral malaria. Two recent studies have demonstrated the importance of IFNγ, TNF and ICAM-1 in the development of pathology. IFNγ receptor deficient mice following *P. berghei* ANKA infection did not develop cerebral malaria (Rudin *et al.*, 1997a). TNF α/β deficient mice were completely resistant to *P. berghei* ANKA induced cerebral malaria (Rudin *et al.*, 1997b). In both studies the normal up-regulation of ICAM-1 observed during *P. berghei* ANKA infection of normal mice is reduced in IFNγ receptor deficient mice and TNF α/β deficient mice, illustrating the importance of IFNγ or TNF induced up-regulation of ICAM-1 expression to the development of cerebral malaria in this model.

It is well established that IFNγ and TNF can synergise to stimulate the production of NO by macrophages (Drapier, Wietzerbin and Hibbs, 1988) but the role of NO in cerebral malaria remains unclear. Sequestered parasitised red blood cells which adhere to endothelial cells can induce the release of cytokines locally at high concentrations which will stimulate the production of NO. It has been proposed that NO could alter brain functions and possibly lead to coma (Clark, Rockett and Cowden, 1991). Another effector mechanism in the development of cerebral malaria could involve platelets (Grau *et al.*, 1991). Treatment of *P. berghei* ANKA infected mice with anti-LFA-1 monoclonal antibodies, even when mice developed neurological symptoms prevented cerebral lesions and death ensuing (Grau *et al.*, 1991, Falanga and Butcher, 1991). Platelets express LFA-1, the ligand for ICAM-1 expressed on endothelial cells and hence the

adherence between platelets and endothelial cells may have an important role in mediating the microvascular damage observed in cerebral malaria.

P. berghei ANKA infection in mice has provided evidence that a Th1 mediated response may be responsible for the development of cerebral malaria. Indeed, it has recently been proposed that Th1 $CD4^+$ T cells may initiate the inflammatory response to malaria infection in non-immune individuals and contribute to the pathology of the disease (Dick *et al.*, 1996). However, the same T cell subset has been shown to be critical in the control of *P. chabaudi* erythrocytic stages (Langhorne *et al.*, 1989, Taylor-Robinson and Phillips, 1992). Therefore, it appears that the balance of Th1 versus Th2 mediated responses is vital for the protection against malaria but overproduction of Th1 mediated inflammatory responses is involved in the development of pathology.

Immunity to sexual stages

Immunity to the sexual stages of malaria infection is referred to as transmission blocking immunity because it will reduce the infectivity of the parasites to mosquitoes and hence, reduce transmission. There are two main mechanisms involved in immunity to the sexual stages. The first is mediated by non-specific factors such as TNF and IFNγ which reduces the infectivity of gametocytes to mosquitoes (De Naotunne *et al.*, 1991). T cells isolated from individuals previously infected with *P. falciparum* produce IFNγ *in vitro* following stimulation with a gamete antigen (Good *et al.*, 1987). PBMN cells from semi-immune individuals can be stimulated *in vitro* by a *P. vivax* antigen to produce TNF (Karunaweera *et al.*, 1992). It is thought that NO may be the effector molecule stimulated by TNF and IFNγ to reduce the infectivity of malaria sexual stages (Motard *et al.*, 1993).

The second mechanism of transmission blocking immunity is mediated by a humoral response. Antibodies directed against surface antigens of gametes or gametocytes of

malaria parasites can modify the infectivity to mosquitoes (Carter *et al.*, 1988). There are various target antigens on the surface of the sexual stages which elicit a humoral response. Antibodies to the glutamate-rich repeat region of Pf11.1 in the presence of complement were found to suppress transmission (Feng *et al.*, 1993) and it has been suggested that these antibodies can penetrate the membrane of a gametocyte infected erythrocyte because Pf11.1 (also known as Pfs2400) is confined to the parasitophorous vacuolar membrane (Scherf *et al.*, 1992). Complement mediated lysis of gametes has also been shown with antibodies to Pfs230, a gamete surface antigen (Quakyi *et al.*, 1987, Healer *et al.*, 1997). Pfs 48 and Pfs45 are an antigenically similar doublet, present in gametocytes and on gamete surfaces (Carter et al., 1990). The Pfs48/45 doub;et forms a non-covalent comples with Pfs230 (Kumar and Wizel, 1992). Antibodies to Pfs48/45 have transmission blocking activity and bind to known B cell epitopes present in the Pfs48/45 doublet (Kaslow, 1993). The binding of one monoclonal antibody to one epitope on Pfs48/45 appears to enhance the binding of monoclonal antibodies to other epitopes, perhaps explaining why some monoclonal antibodies do not suppress transmission when tested alone but do block transmission when mixed together (Rener *et al.*, 1983). Pfs40 is another possible antigen which may be a target of antibodies which mediate transmission blocking immunity. (Rawlings and Kaslow, 1992). Pfs25 is a zygote/early ookinete target antigen which in a recombinant form can induce antibodies which block transmission (Kaslow *et al.*, 1991, Barr *et al.*, 1991). The humoral response to sexual stage antigens appears to require the presence of complement and hence, for a successful transmission blocking vaccine it must induce antibody isotypes which fix complement efficiently such as IgG1 or IgG3 (Healer *et al.*, 1997).

The strategy of developing a vaccine designed to prevent transmission is an attractive one. A transmission-blocking vaccine could be used to control or prevent the re-introduction of malaria in areas where malaria is currently absent. The transmission-blocking vaccine may be included in a multi-component malaria vaccine which would target several stages of the malaria life cycle. The problems of antigenic diversity and

poor immunogenecity of gametocyte target antigens will have to be overcome to ensure the vaccine will induce the correct immune mechanisms which mediate transmission-blocking immunity.

Project aims

The development of a protective immune response to the asexual erythrocytic stage of *P. chabaudi* AS infection in mice has been the focus of extensive research within Professor Phillips' laboratory and others. It is well established that immunity to *P. chabaudi* infection in mice is mediated by a sequential Th1/Th2 response (Langhorne *et al.*, 1989, Taylor-Robinson and Phillips, 1992). The cytokines secreted by each of these $CD4^+$ T cell subsets are important mediators of the effector mechanism induced by either Th1 or Th2 cells. Experiments were performed to define further the role of individual cytokines during a primary *P. chabaudi* infection using cytokine or cytokine receptor gene deficient mice. IFNγ deficient, IL-4 deficient and IL-6 deficient mice were used in these studies which allowed the comparison of responses to *P. chabaudi* infection in mice with dysfunctional Th1 responses (IFNγR deficient mice) or Th2 responses (IL-4 and IL-6 deficient mice).

One characteristic of *P. chabaudi* is that it undergoes sequestration, similar to *P. falciparum*. The liver is a main site of sequestration during *P. chabaudi* infection in mice. It has been proposed that the liver may be a site of a protective immune response during experimental blood-stage malaria infection in mice (Dockrell, DeSouza and Playfair, 1980). Extensive research has been performed on the immune response present in the liver against the intrahepatic stage of malaria following experimental infection initiated with sporozoites (reviewed in Suhrbier, 1991). Experiments were designed to investigate if there was a protective immune response present in the livers of mice during *P. chabaudi* infection. Lymphomyeloid cells were isolated from the liver of *P. chabaudi* infected mice during the course of infection and adoptive transfer studies were

performed to see if the lymphomyeloid cells could protect recipient mice from a homologous challenge. The role of Kupffer cells (tissue macrophages resident in the liver) during a blood-stage infection was also investigated by depletion of the Kupffer cells in mice before and during a *P. chabaudi* infection.

The liver is the main site of acute phase protein synthesis and C-reactive protein has been shown to inhibit pre-erythrocytic stage parasite development (Pied *et al.*, 1989). Studies were performed to determine if infection of mice with the erythrocytic stages of *P. chabaudi* induced an acute phase response, indicated by the production of SAP (the main acute phase protein of mice) and to determine the role, if any, of SAP during the course of a *P. chabaudi* infection.

During the process of sequestration, the parasite progresses from schizont to merozoite, the two stages of the parasites' life cycle which are probably the most vulnerable to immune attack from cytotoxic molecules such as NO. The liver is a site where potentially a high local concentration of NO may be produced. *In vitro* studies were performed, using a NO donor to determine if NO has a direct effect on the development of asexual erythrocytic stage malaria parasites.

The aim of the final section of studies presented in this thesis was to continue ongoing studies within the laboratory of Professor Phillips investigating the interaction of chemotherapy with protective immune mechanisms. Chloroquine has been previously shown to inhibit TNFα and IL-6 production by macrophages (Picot *et al.*, 1993). *In vitro* studies were designed to determine if chloroquine could also inhibit NO production by macrophages.

Chapter Two
Materials and Methods

Mice

Inbred female NIH mice were used routinely for experimental infections. These mice were purchased from Harlan Oak (Bicester, UK) and kept in the University of Glasgow Joint Animal Facility at 22°C and 50-60% humidity. They were maintained in 12 hours artificial light from 0700 to 1900 and given pelleted CRM breeder diet (Labsure Ltd). Both food and water were given *ad libitum.*

Male IL-6 -/- (129SVJ)F_2, IL-4 -/- (129SV x C57BL/6)F_2, IL-4 -/- (B6 x 129SV)F_2, IL-4 -/- (Balb/c)F_2, IFNγ receptor -/- (129 SVEV)F_2 were generated as described (Kopf *et al.*, 1994, Kopf *et al.*, 1993, Huang *et al.*, 1993). These and wild-type control animals of the same strain combination were obtained from Professor J. Alexander, University of Strathclyde. The original breeding pairs were obtained from H. Bluethmann, Basel. The mice were bred and maintained in the animal facility at the University of Strathclyde in 12 hours artifical light (0700 to 1900) and given pelleted CRM breeder diet (Labsure Ltd.). Food and water were given *ad libitum.*

All mice used for experimental purposes were aged 6-12 weeks old and weighed approximately 25 grams.

Parasites

Plasmodium chabaudi chabaudi AS was originally isolated from adult thicket rats (*Thamnomys rutilans*) from La Maboke, Central African Republic, in 1969 by Professor David Walliker (University of Edinburgh). The strain was established in laboratory mice and cloned by limiting dilution (Walliker, Carter and Morgan, 1971). Stabilates of parasites, derived from the original AS parent parasite clone, were maintained by frequent cryopreservation and subpassage through mice. *P. berghei* KSP11 parasites were maintained in a similar fashion to the *P. chabaudi* AS stabilates.

Maintenance of *P. chabaudi* AS

For long-term preservation, vials of blood infected with *P. chabaudi* AS parent are kept in liquid N_2 (-196°C). Infected blood was recovered for experimental use by immersion of the vial in a 37°C waterbath (Gallencamp). Once defrosted, an equal amount of a solution of 15% glucose added slowly dropwise with frequent mixing. The diluted blood was administered by i.p. or i.v. injection into one or two recipient naive mice from which the experimental groups were infected.

Maintenance of *P. falciparum*

P. falciparum erythrocytic stage parasites were cultured routinely by the *in vitro* culture method developed by Trager and Jenson (1976). The strain of *P. falciparum* used was the JS strain which was culture adapted in Professor Phillips' laboratory in 1994 and originally derived from Malawi. Briefly, RPMI 1640 medium was prepared with 25 mM HEPES buffer at pH 7.2. Sodium bicarbonate was added to a final concentration of 0.2% to form incomplete medium. Human serum, normally AB (heat-inactivated at 60°C for 30 minutes) was added to a final concentration of 10%, forming complete malaria medium. *P. falciparum* stabilates were stored in liquid N_2 until required. The stabilate was thawed and transferred to a 20 ml universal (Greiner). 0.5 ml of 4.5 % saline was added dropwise, followed by the addition of 3.5% saline dropwise in order to prevent lysis of the erythrocytes. The suspension was centrifuged for 5 minutes at 250g and the supernatant discarded. The pellet was resuspended in incomplete malaria medium and washed twice (250g for 5 minutes). The final pellet was resuspended in 1.5 ml of complete malaria medium and transferred to a sterile petri dish (35x10mm, Falcon). A few drops of washed, packed O group erythrocytes were added to the culture. The petri dish was placed in a candle jar and incubated at 37°C, 5% CO_2, 5-18% O_2. The medium was changed on a daily basis and the growth of the parasites was

monitored by daily preparation of thin blood smears fixed with methanol and stained with Giemsa's stain. The cultures were diluted on a regular basis by the addition of fresh erythrocytes and complete malaria medium.

Cryopreservation of blood

Infected blood at a parasitaemia of 5-10% containing ring stage parasites was collected by cardiac puncture into a syringe containing sodium heparin (1000 i.u./ml) in phosphate buffered saline (PBS, pH 7.2) as an anticoagulent at 10 i.u. heparin per ml of blood from mice, sacrificed in a CO_2 chamber. The infected blood was diluted 1:1 with a solution of sorbitol-glycerol (38% glycerol, 2.9% sorbitol, 0.63% NaCl). 200ml aliquots were dispensed into 1.2 ml cryopreservation vials and labelled with the species of parasite and a code. The vials were snap frozen by immersing in liquid N_2 and stored.

Challenge infections

The infected blood was obtained by cardiac puncture as described. The parasitaemia was determined from Giemsa's stained thin blood smears. The blood was diluted in RPMI 1640 medium to the required concentration. The mice were placed in a warm box at 32°C for 10 minutes to allow vasodilation. Mice were infected with $1x10^5$ pRBCs (except where stated) administered i.v. as a 200µl inoculum using a 1ml syringe fitted with a 26 gauge needle. Experimental groups consisted of 5 or 6 mice.

Determination of parasitaemia

The parasitaemia of infected mice was determined by daily examination of Giemsa's stained thin blood smears from peripheral blood. Samples were taken between 0900-1030 each day by piercing the tip of the tail with a lancet. A new lancet was used for each experimental group. A drop of blood was placed at one end of a glass microscope

slide (BDH Ltd), smeared and allowed to dry at room temperature. The smear was fixed in 100% methanol (Analar, BDH Ltd) and then stained in Giemsa's stain (1:10 of Giemsa's stain in Giemsa's phosphate buffer, pH 7.2, see appendix). The slide was then rinsed in tap water, air dried and examined under oil immersion using x100 objective and x10 eyepiece lenses on a Leitz S.M. Lux binocular microscope. Parasitaemias were obtained by calculating the number of pRBCs from a total number of RBCs. At least 20 fields containing at least 200 erythrocytes were examined. Infections were considered sub-patent when no parasites were observed in 50 fields. The day of infection was termed day 0 and smears were normally taken from day 3 post infection until at least day 50 post infection.

Presentation of parasitaemic data

The course of infection of a group of mice was represented by plotting the geometric mean of the parasitaemia (mean $\log_{10}$ of the number of pRBC in 10^5 RBC) or the mean percentage parasitaemia against time. For clarity's sake the standard deviation for each data point has been omitted but significant differences at specific timepoints are noted in the text.

Collection of serum

Mice were sacrificed as described and blood was obtained by cardiac puncture if large volumes of sera were required. The blood was allowed to clot, loosened from the edges of the container and allowed to contract overnight at room temperature. The serum was harvested and contaminating RBC were removed by centrifugation for 5 minutes at 9000g. The sample was then labelled and stored at -20°C until required. Smaller samples were collected from tailbleeds of mice during the course of the infection and processed as for larger volumes. Immune serum from infected mice was collected from individual mice in different groups from day 0 onwards. Each mouse within an

experimental group was bled on an equal number of occasions to ensure anaemia did not result from repeated bleeding of an individual mouse.

Irradiation of mice

Mice were irradiated with whole body gamma irradiation from a ^{60}Co source chamber (Nuclear Engineering) in the Department of Veterinary Physiology, University of Glasgow. The mice were exposed to sub-lethal irradiation (400 rads). Irradiation of recipient mice prior to adoptive transfer and challenge infection, occurred no earlier than 24 hours previously.

Analysis of anti-malarial antibody production during infection

The slide IFAT procedure of Van Meirvenne *et al.*, (1975) modified by McLean, Pearson and Phillips, (1982) was used to determine total anti-malarial antibody levels in the serum of infected mice. The procedure is an adaptation of the indirect fluorescent antibody method described by Voller (1964) and O'Neill and Johnson (1970).

To prepare malarial antigen slides, trophozoite/schizont stage parasites were harvested from infected mice as the source of antigen. Mice with a parasitaemia of 5-15% were bled and the infected blood was mixed with 10 i.u./ml heparinised PBS. The pRBCs were washed three times in 20 ml PBS (pH 7.2) by centrifugation (250g for 5 minutes) and resuspension. The pellet was then resuspended in PBS to a volume approximately equal to the original blood volume and used to prepare thin blood smears covering a microscope slide. The slides, following overnight dehydration in a desiccator, were wrapped in tissue, packed with silica gel and stored at -20°C until required (Manawadu and Voller, 1978).

For use in IFAT, the antigen slides were brought up to room temperature in a desiccator for 1-2 hours. The slides were fixed in absolute acetone (Rhone-Poulenc Ltd.) for 5 minutes and air dried. An H series texpen (Deacon Laboratories) was then used to mark out reaction zones on the antigen slides which were allowed to dry for 1 hour in a dessicator. The slides were washed in successive coplin jars of PBS (pH 7.2), drained and rehydrated in a third jar of PBS (pH 7.2) for 15 minutes. 20µl of sample were applied to each reaction zone. Each slide contained a PBS sample, a negative serum and a hyperimmune serum as controls. Test samples were assayed at an initial dilution of 1:20 and diluted until the sample became negative (see later). The slide was then incubated in a humid chamber at room temperature for 15 minutes. Excess sera was poured off and washed in PBS (pH 7.2) as described previously. The edges of the slide were dried and 1 ml of FITC-conjugated sheep anti-mouse IgG (SAPU) diluted 1:200 in PBS (pH 7.2) containing Evans Blue (1:10,000 w/v) (Merck) and incubated for 15 minutes in the humid chamber. The Evans Blue acts as a counter-stain (El Nahel and Bray, 1963). The excess conjugate-PBS solution was poured off and the slide washed as above. A coverslip (22x55mm, Chance Propper Ltd) was mounted with a 1:1 solution of non-fluorescent PBS:glycerol (Merck).

The slide was examined for fluorescence by a Leitz Ortholux linked to an Epson PX4 computer through a Leitz MPV Compact 2 microscope photometer. The overhead u.v. source used was a Wotan HBO-50 mercury lamp with 2 x kP490 exciting filters and a TK 510 dichroic beam-splitting mirror and a k515 suppression filter. The slide was examined by a x600 water immersion objective. The endpoint titre of the sample was considered to be the last dilution of serum with which specific fluorescence was observed. The control zones of hyperimmune and normal serum and PBS (pH 7.2) were examined for comparison.

Determination of total antibody production

Sera were collected as described previously and assayed for production of total IgG1, IgG2a or IgE antibodies. Capture anti-antibody monoclonals were diluted to the required concentration (anti-IgG1 at 4μg/ml and both anti-IgG2a and anti-IgE at 2μg/ml) in coating buffer (bicarbonate buffer, pH 8.2). 50μl of capture antibody were aliquoted per well on an Immunlon 4 ELISA plate (Dynatech) and incubated overnight at 4°C. The plate was washed twice with PBS/Tween (0.05%). For each wash, wells were filled with PBS/Tween and allowed to stand for at least one minute. The PBS/Tween was discarded and the plate was pounded onto paper towels after the final wash. The wells were then blocked by the addition of 200μl/well of 10% FCS/PBS for one hour at 37°C. The plate was then washed twice as described above. Standards and samples were then added at 50μl/well in the required concentration. Standard IgG1 was added at 1μg/ml, IgG2a at 0.4μg/ml and IgE at 1μg/ml. The serum samples were diluted 1:100 for both IgG1 and IgE assays and 1:500 for the IgG2a assay. The standards and samples were diluted in 10% FCS/PBS. The plate was washed four times as before. Detecting monoclonal antibodies were diluted in 10% FCS/PBS to the required concentration. Biotinylated anti-IgG2a and IgE were both used at 2μg/ml. Biotinylated anti-IgG1 k and l chains were added in a 1:1 mixture at 2μg/ml each. 50μl of the respective detecting antibody were added per well and incubated at 37°C for one hour. The plate was washed six times in PBS/Tween and 100μl of streptavidin-peroxidase (Sigma) at 2μg/ml were added per well. The plate was incubated at 37°C for one hour and then washed 8 times as before. 100μl/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KPL) was added and colour (blue) was allowed to develop (5-30 minutes). The plate was read at 630 nm with a reference filter at 405 nm on a MRX plate reader (Dynatech). The results of the unknown samples were calculated against a standard curve of known concentrations plotted using Biolinx software (Dynatech).

Preparation of spleen cell suspensions

Mice were sacrificed and the spleens removed aseptically. The spleens were disrupted mechanically using the plunger from a sterile syringe to disrupt the tissue through a sterile stainless steel sieve (mesh size 0.025 mm^2) and collected in a 9 cm Petri dish (Sterlin) containing incomplete RPMI 1640 medium. The supernatant was harvested using a sterile Pasteur pipette after allowing sedimentation of tissue debris and clumps of cells. The spleen cells were washed in 10% Foetal Calf Serum (FCS) RPMI 1640 medium (250g for 5 minutes) and then resuspended in 1 ml of 10% FCS RPMI 1640 medium. Contaminating erythrocytes were lysed by incubating 1 ml of spleen cell suspension in 9 ml 0.83% Tris-ammonium chloride (Tris-NH_4Cl, pH 7.4) for 5 minutes at room temperature. The spleen cell suspension was washed twice with 10% FCS RPMI 1640 medium at 250g for 5 minutes. The pellet was resuspended in 1 ml of 10% FCS RPMI medium for determination of cell viability and number.

Preparation of liver lymphomyeloid cells

Livers were excised from mice aseptically, cut into small pieces and digested in a warmed (37°C) solution of RPMI 1640 medium containing 50 U/ml collagenase (Sigma) for 30 minutes under constant agitation at 37°C. The suspension was washed in 10% FCS RPMI 1640 medium (250g for 5 minutes). The remaining tissue was mechanically disrupted as previously described and washed in 10% FCS RPMI 1640 medium (250g for 5 minutes). The cell suspension was then passed through a packed glass wool column to remove tissue debris and clumps of cells. After a further wash in 10% FCS RPMI 1640 medium (250g for 5 minutes), the cell suspension was washed in 100% FCS for 3 minutes at 100g and then washed in 10% FCS RPMI 1640 medium as described above. The cell suspension was pipetted onto a petri dish and incubated at 37°C, 5% CO_2 for one hour to allow adherent cells to stick to the plastic. The non-adherent cells were harvested and washed in 10% FCS RPMI 1640 medium.

Contaminating erythrocytes were lysed by treating with Tris-NH_4Cl and then washed in 10% FCS RPMI 1640 medium. 6ml of the cell suspension were layered onto 3 ml NycoPrep™ in a 15 ml centrifuge tube and centrifuged at 600g for 15 minutes. The mononuclear cells formed a layer at the interface between the NycoPrep™ and the medium with the hepatocytes found at the bottom of the centrifuge tube. The lymphomyeloid cells were harvested by a pasteur pipette and washed in 10% FCS RPMI 1640 medium. The final pellet of lymphomyeloid cells was resuspended in 1 ml of 10% FCS RPMI 1640 medium for determination of cell number and viability.

Preparation of peripheral blood mononuclear cells

Mice were sacrificed and bled as described previously. The whole blood was diluted by the addition of an equal volume of 0.9% sodium chloride and then separated by density centrifugation. 6 ml of the diluted blood were layered over 3 ml NycoPrep™ (Nycomed) in a 15 ml centrifuge tube and centrifuged at 600g for 15 minutes. The peripheral blood mononuclear (PBMN) cells were harvested from the interface between the plasma layer and the NycoPrep™ solution using a sterile pasteur pipette. The PBMN cells were washed twice in 10% FCS RPMI 1640 medium and then resuspended in 1 ml of 10% FCS RPMI 1640 medium for determination of cell number and viability.

Preparation of murine splenic macrophages

Mice were sacrificed and the spleens removed aseptically. The spleens were mechanically disrupted as described previously and harvested in a Petri dish containing 10% FCS RPMI 1640 medium. The spleen cell suspension was incubated at 37°C, 5% CO_2 for one hour to allow the macrophages to adhere to the plastic of the petri dish. Non-adherent cells were discarded and the adherent cell population was harvested using a pasteur pipette and washed three times with 10% FCS RPMI 1640 medium (250g for minutes). Contaminating erythrocytes were lysed with Tris-NH_4Cl as described

previously. The adherent cell suspension was washed twice with 10% FCS RPMI 1640 medium as described above and then resuspended in 1 ml of 10% FCS RPMI 1640 medium for determination of cell number and viability.

Preparation of peritoneal wash cell suspensions

Mice were sacrificed and 5 ml of sterile incomplete RPMI 1640 medium were injected into the peritoneal cavity. The abdomen of the mouse was gently massaged and the medium plus the peritoneal wash cells were harvested. The cell suspension was washed twice with 10% FCS RPMI 1640 medium and treated with Tris-NH_4Cl if necessary. The final pellet of peritoneal wash cells were resuspended in 1 ml of 10% FCS RPMI 1640 medium for determination of cell number and viability.

Determination of cell number and viability

The viability of cell preparations was measured by the trypan blue dye exclusion test. A dilution of 1:10 or 1:100 of cells in PBS (pH 7.2) was prepared and further diluted 1:1 with a solution of 0.2% w/v trypan blue (Gurr, BDH Ltd) in PBS (pH 7.2). The suspension was mixed and incubated at room temperature for 2-3 minutes and then examined by phase contrast under oil immersion (x1000) on a light microscope. Viable cells remained clear, whereas dead cells were unable to exclude the dye and stained blue. The proportion of live to dead cells gave the percentage viability. At least 100 cells were counted.

To determine cell numbers, a 20µl aliquot of the 1:10 or 1:100 dilution of the cell suspension was pipetted into a haemocytometer (ARH) and the cells were counted under phase contrast (x400) on a light microscope. The calculation gave a total number cells per ml and from this the correct number of cells required for the experimental procedure was determined.

In vivo depletion of Kupffer cells

Kupffer cells were depleted in mice by adaptation of the method described by Claasen and van Rooijen (1986). The liposomes were prepared by David Taylor (Department of Biochemistry, University of Glasgow). Briefly, 45mg phosphatidyl choline, 5 mg cholesterol, 10mg dicetyl phosphate and 5mg galactose ceramide (all from Sigma) were dissolved in 5ml chloroform (BDH) and 5ml methanol (Fisons Ltd). The solvent was removed by rotary evaporation under low vacuum at 37°C. The lipid film formed was once more removed by rotary evaporation under low vacuum at 37°C. The lipid film was then resuspended in 5ml PBS containing 945mg dichloromethylene diphosphonate (clodronate, Boehringer Mannheim) and rotated gently for 2 hours at 37°C. The suspension was then sonicated in a water bath sonicator (Ultrasonics Ltd) for 3 minutes and left at room temperature for 2 hours. This allowed liposome formation. The liposomes were washed by diluting to 36ml with PBS, centrifuged at 100,000g at 16°C for 30 minutes. The supernatant was discarded and the liposomes were resuspended in 20ml PBS. The concentration of liposome-entrapped clodronate was determined by the protocol described by Claasen and van Rooijen (1986). It was determined by David Taylor, that a regime of 5μg total lipid per gram body mass in a 200μl total volume given intravenously every 7 days, depleted the phagocytic activity of the liver most efficiently whilst the spleen remained unaffected.

Proliferative assays

Cell suspensions were prepared as described and adjusted to a final concentration of $1x10^6$/ml in 10% FCS RPMI 1640 medium. 100μl of cells/well were aliquoted into 96 well flat bottomed plate (Nunc) with 100μl of either a crude *P. chabaudi* antigen (250μg/ml), a normal RBC antigen (250μg/ml) and Con A (1μg/ml, from stock solution of 1mg/ml, Sigma). Control cultures were unstimulated. The plate was

incubated at 37°C, 5% CO_2 for 72 hours. Each well was pulsed with 1μCi (37kBq) of ^{3}H-methyl-thymidine (1mCi/ml, specific activity 5Ci/mmol, Amersham) in a 10μl volume of 10% FCS RPMI 1640 medium and the plate was incubated for a further 18 hours at 37°C. The wells were harvested with a semi-automatic cell harvester (Titertek, Flow) onto glass fibre filter paper (FG/A Whatman) and air-dried. Each filter disc, corresponding to a specific well, was transferred to plastic beta vials (LKB) and 2 ml non-aqueous scintillation fluid (Optiscint safe, LKB) added. Lids were placed on the insert tubes which were placed inside outer plastic scintillation vials. The beta activity present in each sample was detected during 1 minute using a scintillation counter (LKB Wallac 1219 Rackbeta) and the counts per minute (cpm) calculated using the Ultroterm III software package. All samples were set up in triplicate to enable an arithmetic mean of the cpm, representative of the proliferative response, to be calculated.

Preparation of parasitised and normal red blood cell lysates

For *in vitro* stimulation of cell suspensions soluble crude *P. chabaudi* antigens were prepared. *P. chabaudi* infected mice were kept under a reversed light-dark cycle (12hours of light between 1900-0700) conditions, and therefore schizogony, which under normal light condition would occur at around 0100 hour, occurred between 1100-1300. Mice at peak parasitaemia were sacrificed and bled as described before schizogony occurred. Erythrocytes containing mainly late ring stages were washed twice in 5% FCS RPMI 1640 medium at 250g for 5 minutes. The erythrocytes were resuspended to a 10% haematocrit and cultured in a petri dish containing complete malaria medium with the human serum replaced by FCS to at a final concentration of 10%. Once the parasites had reached the schizont stage, monitored every 30 minutes by examination of Giemsa's stained thin blood smears, the cells were washed in 5% FCS RPMI 1640 medium at 250g for 5 minutes and resuspended to the original volume in sterile PBS (pH 7.2). The suspension was filtered through a sterile Whatman CF11 powdered cellulose paper column to remove leukocytes (Beutler *et al.*, 1976). The

filtrate was washed in 5% FCS RPMI 1640 medium at 250g for 5 minutes and then resuspended in PBS (pH 7.2). 1ml of cell suspension was mixed with 1 ml of NaH_2PO_4 and centrifuged at 9000g for 2 minutes. The supernatant was discarded and the process was repeated with 2 volumes of parasitised erythrocytes to 10 volumes NaH_2PO_4 and centrifuged at 9000g for 5 minutes. The supernatant was discarded and the pellet was diluted in PBS. The cell suspension was freeze-thawed three times. Each cycle of freeze-thawing entailed snap freezing the preparation by plunging into liquid N_2 then immediately defrosting the suspension in a 37°C waterbath (Gallencamp). The disrupted parasitised erythrocytes were centrifuged at 1500g for 10 minutes and the supernatant was collected. This was termed the pRBC lysate antigen and was stored at -20°C until required.

Non-infected blood was used to prepare the normal RBC (nRBC) lysate antigen. The procedure was as for the preparation of the pRBC lysate excluding the culturing of the parasitised erythrocytes.

Determination of total protein concentration

The total protein concentration of both the pRBC and nRBC lysate was determined by an adaptation of the method described by Smith *et al.*, (1985). Standards of known protein concentrations were prepared by diluting 2 mg/ml stock BSA standard (Pierce Chemical Co.) in PBS to give a range from 1-25 µg/ml. Dilutions of the unknown protein lysates were prepared in PBS (1:100,1:1000, 1:10000). 100µl/well of standards and samples were pipetted into a 96 well plate. 100µl of Coomassie Protein Assay Reagent (Pierce) was added to each well. The plate was read at 570 nm on a MRX plate reader and the protein concentration was determined by plotting the results against the curve of the protein standards using Biolinx software (Dynatech).

Cytological analysis of cell suspensions

Cell suspensions were prepared as described and adjusted to a final volume of 10^5 cells/ml in 10% FCS RPMI 1640 medium. 500µl of the cell suspension was aliquoted into the sample chamber. A microscope slide with a filter card covering the slide except for two circular areas (6 mm diameter, Shandon) was placed against the sample chamber and placed in a Shandon cytofuge. The samples were centrifuged at 4000g for 10 minutes. A smear of the cells was formed on the microscope slide in the uncovered areas. The slide was air-dried, fixed in methanol and stained with Giemsa's stain. The cells were counted under oil immersion (x1000). At least 500 cells per slide were counted.

Determination of serum amyloid P production

Sera was harvested as described and assayed for the production of serum amyloid P (SAP). SAP standards were prepared in 1% bovine serum albumin (BSA) in PBS to cover a range of 1.5-50µg/ml (original stock SAP was 100µg/ml, Calbiochem) and stored at -20°C until required. The negative control was 1% BSA/PBS alone. Samples and standards were diluted 1:100 in coating buffer (pH 9.6) and 100µl/well was aliquoted onto an Immunlon 2 96 well plate (Dynatech). The plate was covered and incubated overnight at room temperature with continuous gentle shaking. The excess samples and standards were decanted and the non-specific binding sites were blocked with 10% Marvel/PBS and incubated at room temperature for one hour under constant agitation. The excess blocking agent was discarded and the plate was washed three times with PBS/Tween (0.05%). For each wash, wells were filled with PBS/Tween and allowed to stand for 3 minutes prior to the PBS/Tween being discarded. After the final wash, the plate was pounded onto paper towels. 100µl of a 1:4000 dilution of rabbit anti-murine SAP antibody (Calbiochem) in 1% BSA/PBS/Tween were added to each well and incubated at room temperature for 90 minutes. Excess antibody was decanted

and the plate was washed 3 times as before. 150µl of TMB substrate (KPL) was added to each well and the plate was covered in tinfoil and incubated for 15 minutes at room temperature. The reaction was then stopped by the addition of 50µl of sulphuric acid (H_2SO_4) to each well. The absorbance was read at 450 nm on a MRX plate reader (Dynatech) and concentrations of unknown samples were calculated against a plot of the standard curve using Biolinx software (Dynatech).

Isolation of SAP from immune sera

SAP was isolated from immune sera by an adaptation of the technique used by Siripont, Tebo and Mortensen, (1988). Immune sera were pooled from *P. chabaudi* infected mice at day 11 post infection (approximately when peak production of SAP occurred). The pooled serum was passed through a column containing a 5 ml packed gel of o-phosphorylethanolamine-agarose (Sigma) which was equilibrated with 0.02M Tris-buffered (pH 7.4) saline containing 5 mM Ca^{2+}. Bound SAP was eluted with Tris-buffered saline containing 5mM EDTA. The fractions collected were analysed for SAP content by the direct quantitative ELISA utilised to monitor SAP levels in serum. The fraction containing the eluted SAP was dialysed against PBS to ensure it was suitable for *in vitro* use. The sample containing the eluted SAP was aliquoted into standard dialysis tubing and placed in a beaker containing a 1:10 dilution of PBS (pH 7.2). The PBS was changed after several hours and the beaker was covered and incubated at 4°C overnight under constant stirring. The sample was then assayed for SAP content by the direct quantitative ELISA described.

The effect of SAP on the growth of malaria parasites *in vitro*

To determine if SAP had direct activity against malaria parasites, *P. falciparum* and *P. chabaudi* were cultured in the presence of SAP (100-5000 µg/ml) isolated from immune serum by the technique described and chloroquine (0.1-10 µg/ml) was used as a control.

The effect on the growth of either parasite was determined by the uptake of tritiated hypoxanthine. For *P. falciparum*, the cultures were incubated for 48 hours at 37°C under the conditions previously described and the radioactive tracer was added (1μCi/well). The plate was incubated for a further 18 hours and harvested as previously described. The effect on growth was determined as before and expressed as counts per minute.

The effect of SAP on the proliferative response of splenocytes

To determine if SAP had an effect on the proliferative response of splenocytes, isolated SAP (1000μg/ml) was added to cultures of splenocytes ($5x10^6$ cells/ml), prepared as before from a naive mouse, at the same time as stimulation with Con A or LPS (both at 5μg/ml). The cultures were incubated at 37°C for 72 hours and tritiated thymidine (1μCi/well) was added. The cultures were incubated for a further 18 hours and harvested as before. The effect on growth was determined as described previously and plotted as counts per minute.

The effect of NO on the growth of malaria parasites *in vitro*

The effect of NO on the development of malaria parasites *in vitro* was determined using s-nitroso-acetyl penicillamine (SNAP) (Calbiochem), a NO donor. *P. falciparum* was obtained from the continuous cultures previously described. The parasitaemia used was 0.5% and 100μl of parasites at 5% haematocrit was pipetted into each well of a sterile 96 well plate (Costar). SNAP was freshly prepared in complete malaria medium to give a range of concentrations from 45-363μM. Controls included complete malaria medium, D-penicillamine (to control for SNAP) and sodium nitrite (to control for nitrite production), both at the same range of concentrations as SNAP. The cultures were incubated at 37°C under the conditions already described for the maintenance of *P. falciparum* cultures. Growth of the parasite was measured by the uptake of tritiated

hypoxanthine (specific activity 28 Ci/mmol, stock 1mCi/ml, Amersham) added at 1μCi/well in a 10μl volume (in complete malaria medium). The wells were harvested, at the indicated time after the addition of the radioactive tracer (see Chapter 8), using the semi-automatic cell harvester (Titertek, Flow) previously described. The cpm were determined as before and an arithmetic mean growth calculated.

For the experiments using the murine malarias *P. chabaudi* or *P. berghei*, the parasites were obtained from infected mice as described and diluted to the required parasitaemia using normal mouse red blood cells. The assay was performed as described for *P. falciparum* except that the duration of the experiment was for 24 hours.

Determination of the effect of chloroquine on NO production by macrophages *in vitro*

An *in vitro* assay was designed to examine the effect of chloroquine on nitric oxide (NO) production by macrophages *in vitro*. Three types of macrophages were used. The macrophage-like cell line J774 was continuously cultured under standard cell culture conditions (5% CO_2, 37°C) in 10% FCS RPMI 1640 medium until required. The splenic macrophages and peritoneal wash cells were obtained from naive mice as described previously. Chloroquine (Chloroquine diphosphate salt, Sigma) standard solutions containing 1, 10, 100 or 1000μM chloroquine in 10% FCS RPMI 1640 medium were prepared and stored at -20°C until required. The cells were aliquoted into 24 well plates (Greiner) at a concentration of $5x10^5$ cells/ml in 1 ml of 10% FCS RPMI 1640 medium. The cells were treated with chloroquine at the concentrations indicated at -24, -2, 0, +2 and +6 (or +24) hours with respect to stimulation of the cells with IFNg (100 U/ml, stock: 200000 U/ml , Sigma) and LPS (25 ng/ml, stock: 1mg/ml, Sigma). The cultures were incubated for 48 hours at 5% CO_2, 37°C and then the culture supernatants were assayed for nitrite concentration as an indication of NO production.

Determination of NO production

The level of NO production *in vitro* was determined by the measurement of the end product nitrite using the Griess reaction described by Migliorini *et al.*, (1991). Nitrite standards were prepared by diluting a 10^{-2} mM stock solution of sodium nitrite in 10% FCS RPMI 1640 medium to give a range of 5-300μM nitrite. 60 μl of culture supernatant or standard was added to 60 μl of Griess solution (1:1 mix of 0.1% a-napthylamine, Sigma and 1% sulphanilamide in 5% phosphoric acid, Sigma) in a individual well of a 96 well plate (Corning). The plate was incubated for 10 minutes at room temperature and read at 540 nm on a MRX plate reader. The concentration of nitrite for the unkown samples was calculated against a plot of the standard curve using Biolinx software (Dynatech).

Statistical analysis

Results are expressed as means±1SDM and group data were compared using a Student's *t* -test. A significant result was considered to be when the value of $p< 0.05$.

Chapter Three

The liver: a possible site of a protective immune response against the asexual erythrocytic stage of *P. chabaudi* infection.

Introduction

The presence of immunocompetent cells in the liver has been reported in parasitic, viral and bacterial infections (Khan and Vanderberg, 1992, Koziel *et al.*, 1992, Goossens, Jouin and Milon, 1991). Recruitment of peripheral blood mononuclear phagocytes to the liver during *Listeria monocytogenes* infection (North, 1970) has shown the importance of this non-lymphoid organ. This process of recruitment during *L. monocytogenes* infection has now been shown to fall into three distinct phases (Goossens, Jouin and Milon, 1991). There is an initial recruitment of lymphomeyloid cells which is followed by recruitment of myelomonocytic cells and then $CD8^+$ T cells. The hypothesis that the liver is an important organ of the immune system, is gathering momentum. The anatomical position of the liver means it is in contact with several different immunological environments including the spleen, gut and lymphatic system and may be involved in a protective immune response to several different pathogens. The presence of immunocompetent cells in the liver may not only be due to recruitment from the peripheral blood, spleen or bone marrow, but may also be due to expansion of intrahepatic lymphomyeloid cells. Recently, the liver has been described as a meeting place for thymus-dependent circulating T cells and thymus-independent T cells which express NK cell markers (Crispe and Mehal, 1996). The two sets of cells are differentiated by expression markers. One subpopulation believed to derive from the circulating T cell pool is a mixture of $CD8^+$, $CD4^+$ and $CD4^-$ $CD8^-$ (double negative) cells while the other subpopulation of cells express an intermediate level of TCR$\alpha\beta$ and also NK1.1. The population of T cells expressing NK1.1 is not only restricted to the liver but is also found in the bone marrow and thymus (Watanabe *et al.*, 1995), and the numbers of these cells have been shown to increase in response to IL-12 and TNFα (Crispe and Mehal, 1996). Therefore, an active immune response in the liver may involve not only recruitment of cells but expansion of intrahepatic lymphomyeloid cells.

Immunity to the intrahepatic stages of malaria provides one of the best examples of a protective immune response in the liver to a pathogen (refer to Introduction for a review of immunity to pre-erythrocytic stages of malaria). Both $CD4^+$ and $CD8^+$ T cells have been shown to mediate the immune response to pre-erythrocytic stages of various malaria species (Renia *et al.*, 1993, Suhrbier, 1991). The intrahepatic form of the parasite can be a target of antibodies, cytokines, phagocytic and cytotoxic cells (Mazier *et al.*, 1988) and non-specific factors such as acute phase proteins (Mazier *et al.*, 1988). Strong immune responses can be induced to the pre-erythrocytic stages of malaria by vaccination with irradiated sporozoites (Hoffman and Franke, 1994). Cytological analysis of leukocytes isolated from livers following a sporozoite-induced *P. yoelii* malaria infection in mice revealed that after the clearance of the subsequent erythrocytic stage, $CD4^+$ and $CD8^+$ T cells and B cells were the (pre)dominant cells present during a primary infection (Faure *et al.*, 1994). Following challenge of mice with a second sporozoite inoculation one week after clearing the erythrocytic stage of infection, the composition of the leukocytes changed to $CD3^+CD4^-CD8^-$ cells and polymorphonuclear cells (Faure *et al.*, 1994).

The role of the liver in the immune response to the erythrocytic stage of malaria infection has largely been ignored. However, the extensively characterised response to the exo-erythrocytic stage has demonstrated there are several immune effector mechanisms present in the liver which have the potential to be directed against erythrocytic stage parasites. Dockrell and colleagues proposed an important role for the liver in the recovery from blood stage infection (Dockrell, De Souza and Playfair, 1980). Mice vaccinated with fixed parasitised RBCs of *P. yoelii* and *Bordetella pertussis* can clear a normally lethal challenge of *P. yoelii* and this is associated with an increase in the number of cells present in the liver and an increase in the uptake of parasitised erythrocytes by the liver (Dockrell, De Souza and Playfair, 1980, Playfair and De Souza, 1982). Activation of Kupffer cells has also been reported to occur during blood-stage infection (Dockrell, De Souza and Playfair, 1980) and these cells are involved in

phagocytosis of parasitised erythrocytes but also may act as antigen presenting cells (Suss *et al.*, 1988). Increased retention or trapping of lymphocytes in the liver has been demonstrated around peak parasitaemia during *P. chabaudi* infection in mice (Kumararatne *et al.*, 1987) and increased migration of leukocytes to the livers of mice vaccinated against *P. yoelii* with a crude *P. yoelii* parasitised erythrocyte lysate and *Bordetella pertussis* has been observed (Playfair *et al.*, 1979). Concomitantly, there is a decrease in the retention of lymphocytes by spleens of *P. chabaudi* infected mice (Kumararatne *et al.*, 1987) suggesting a redistribution of lymphocytes occurs at peak parasitaemia which results in an exclusion of lymphocytes from the spleen.

One characteristic of the *P. chabaudi* model is that mature parasitised erythrocytes stop circulating in the peripheral blood and cytoadhere to the endothelial linings of the sinusoids in the liver and also endothelial linings of the spleen (Cox, Semoff and Hommel, 1987). Sequestration is traditionally thought of as being a mechanism which the parasite undergoes to avoid splenic filtration which has been demonstrated during both lethal and non-lethal *P. yoelii* infection (Weiss, 1990) and involves splenic barrier cells. Therefore, it is unclear why late stage *P. chabaudi* parasitised erythrocytes would sequester to the spleen. It is unknown where in the spleen the parasitised erythrocyte cytoadheres to and hence the parasitised erythrocyte may avoid splenic clearance by undergoing schizogony before reaching the splenic barrier cells. However, cytoadherence of the parasitised erythrocyte to endothelial cells may induce a localised immune response, with the presence of parasite-derived antigens on the surface of the parasitised erythrocyte being a possible target. During schizogony, merozoites, toxins and cell debris are released into the bloodstream. The merozoite form of the parasite may be the most vulnerable to immune attack because it is extracellular. The release of parasite derived toxins and cellular debris following schizogony may provide a strong antigenic stimulus thereby inducing an intense localised response. The observation that there is an increase in the numbers of lymphomyeloid cells present in the liver around peak parasitaemia compared with a reduction in the numbers present in the spleen

(Kumararatne *et al.*, 1987) suggests different immune responses may be compartmentalised to each of these sites. This was investigated by firstly isolating lymphomyeloid cells from livers of mice during the acute phase of *P. chabaudi* infection. Adoptive transfer studies were performed to determine if the lymphomyeloid cells isolated from the liver could confer protection to a *P. chabaudi* challenge. The degree of protection mediated by these cells was compared with splenocytes isolated at the same time in the acute phase of infection. Preliminary investigations into the role of Kupffer cells by depletion *in vivo* using liposome-encapsulated clodronate further defined the importance of the liver during the asexual blood-stage of a malaria infection.

Results

Isolation of lymphomyeloid cells from livers of *P. chabaudi* infected mice

Lymphomyeloid (LM) cells were isolated from livers of *P. chabaudi* infected mice by the protocol described in Materials and Methods. NIH mice were infected with $1x10^6$ pRBC of *P. chabaudi* and two mice were killed at each of the time points indicated. The number of LM cells harvested from each liver was determined and a mean value was calculated. Peak numbers of LM cells were isolated at day 10 post infection with a mean value of $20x10^6$ cells per liver (Figure 3.1): this was two days after the peak of the parasitaemia.

Adoptive transfer of isolated LM cells

Adoptive transfer studies were performed to determine if LM cells, isolated from the livers of infected mice could confer protection against a *P. chabaudi* challenge. LM cells were isolated, as before, from donor NIH mice infected with $1x10^5$ pRBCs of *P. chabaudi*, at day 11 post infection. Simultaneously, non-adherent splenocytes and

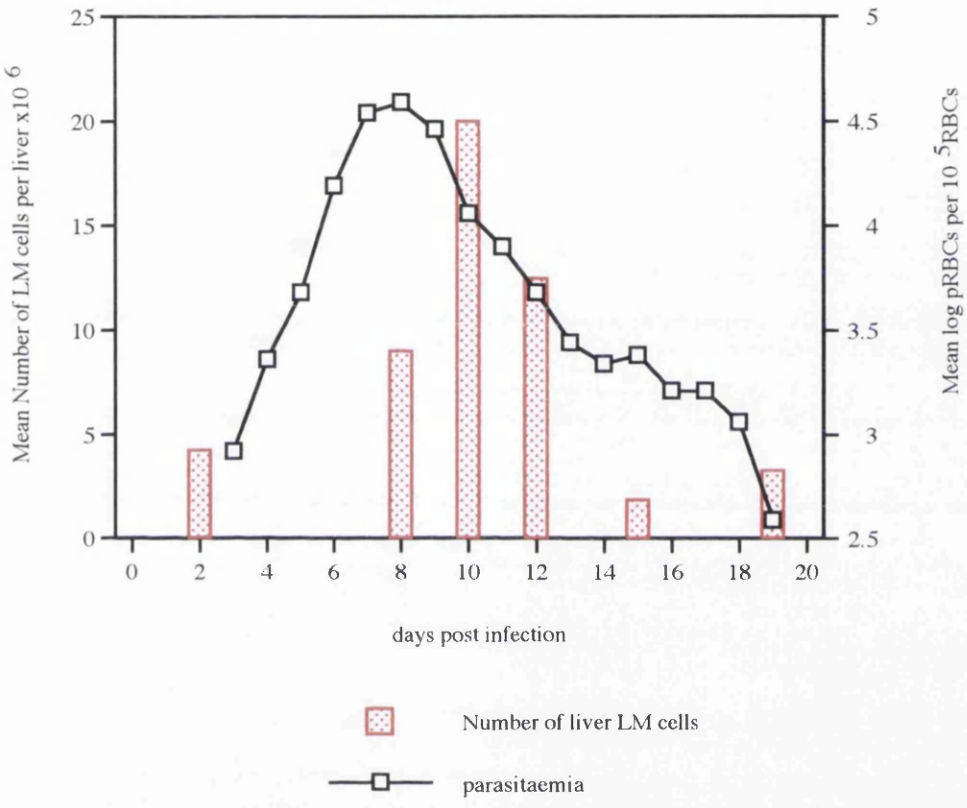

Figure 3.1. Extraction of lymphomyeloid cells (LM) from the livers of *P. chabaudi* infected mice. Mice were infected with $1x10^6$ pRBCs of *P. chabaudi*. Two mice were sacrificed at each timepoint and the mean number of cells per liver ($x10^6$) calculated.

peripheral blood mononuclear (PBMN) cells were harvested by standard isolation techniques (described in Material and Methods) from the same donor mice. Splenocytes were also harvested from naive NIH mice, to enable the study to include cells which had no prior exposure to malaria infection. Recipient NIH mice were sub-lethally irradiated (400 Rads) 24 hours before the transfer of cells. Groups of six mice received either $3x10^6$ LM cells, $3x10^6$ PBMN cells, $1x10^7$ immune splenocytes or $1x10^7$ naive splenocytes. The mice were subsequently infected with $1x10^5$ pRBCs of *P. chabaudi*. The group of mice receiving the LM cells were significantly protected at peak parasitaemia when compared with control groups (Figure 3.2). No mortality was observed in any of the recipient groups. At peak parasitaemia, the control groups had similar values although the mice receiving the PBMN cells had an earlier peak of parasitaemia and subsequently cleared the primary parasitaemia first. No differences were observed between the groups after the primary parasitaemia, with the parasitaemia remaining sub-patent during the time observed (Figure 3.2). Attempts were made to expand the adoptive transfer studies to include LM cells from the livers of non-infected mice but repeat experiments failed to demonstrate any protective effect mediated by the adoptive transfer of, not only the immune LM cells but the immune splenic cells. On two occasions the challenge infection did not establish within the recipient mice. Hence, the data obtained for the adoptive transfer of immune LM cells isolated form the livers of *P. chabaudi* infected mice has to be considered as preliminary data. Further repeats of the adoptive transfer studies are planned.

The effect of Kupffer cell depletion on the course of *P. chabaudi* infection

A preliminary experiment was performed to investigate the role of the Kupffer cell during the asexual-blood stage of *P. chabaudi* infection. Kupffer cells were depleted by treatment of NIH mice with liposome-encapsulated clodronate before infection with $1x10^5$ pRBCs of *P. chabaudi* and once every 7 days thereafter for the duration of the

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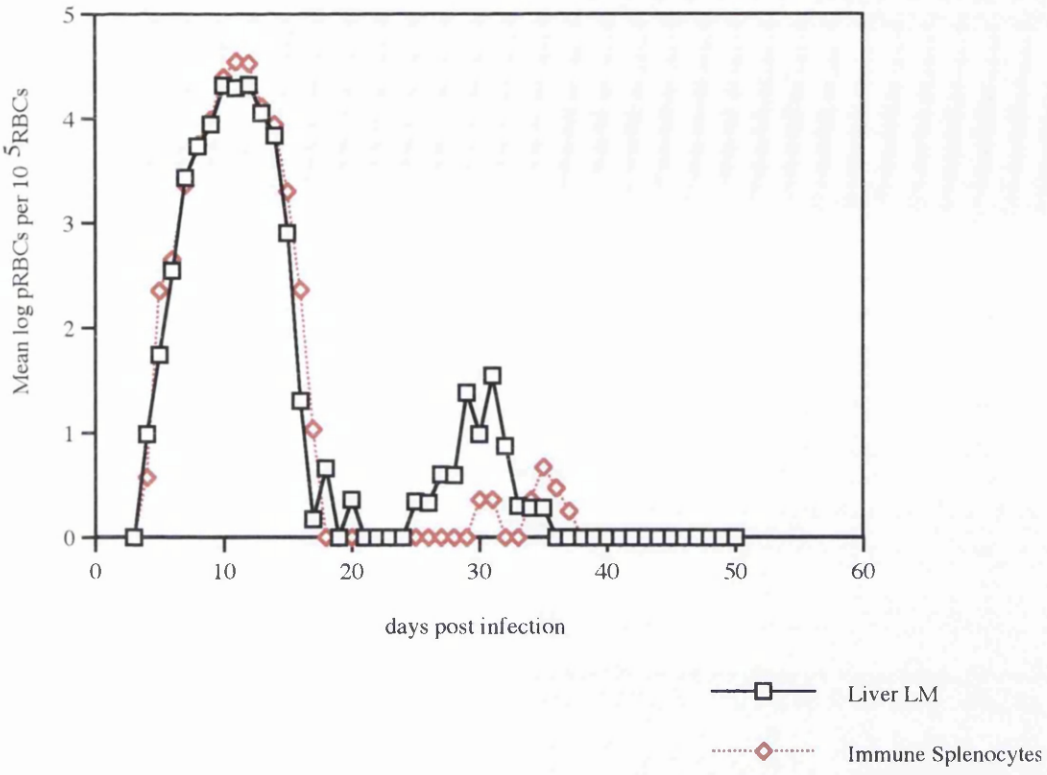

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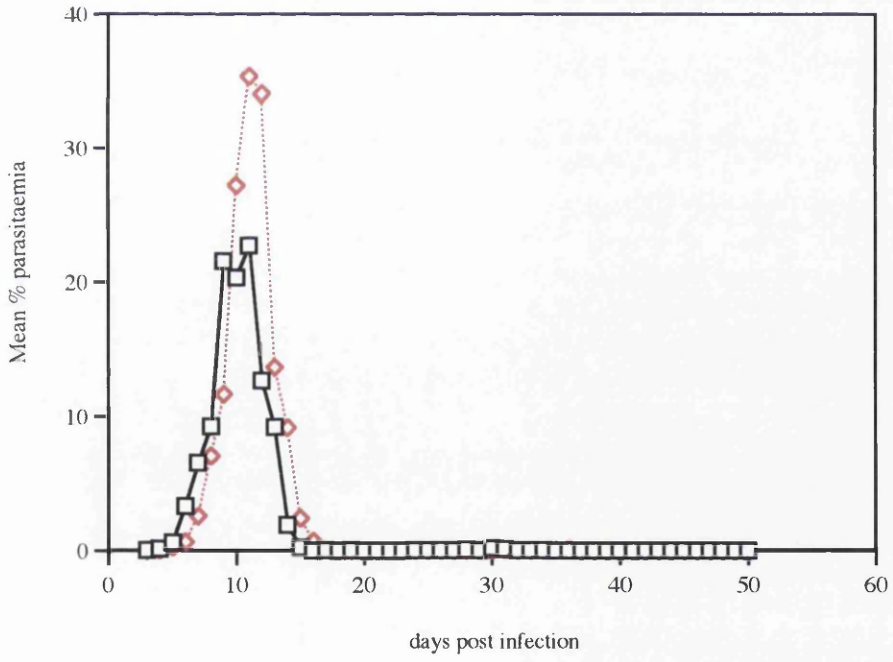

Figure 3.2.1. Adoptive transfer of liver LM cells, isolated at day 11 of a *P. chabaudi* infection, to irradiated recipients, significantly reduced the peak of the primary parasitaemia ($p<0.04$) to an homologous challenge compared with the transfer of immune splenocytes isolated concomitantly. Each group of recipients consisted of six mice and were challenged with $1x10^5$ pRBCs of *P. chabaudi*. The mean log parasitaemia A) and mean percentage parasitaemia B) are presented.

infection to prevent Kupffer cell replenishment (Refer to Materials and Methods). Control NIH mice were infected with 10^5 pRBCS and given no treatment. The mice depleted of Kupffer cells had a significantly exacerbated peak of parasitaemia compared with the control group (p values for day 9, 10 11 and 12 post infection were all < 0.03). No significant differences were observed during the recrudescence parasitaemia between the two groups (Figure 3.3).

Discussion

The demonstration that there is an increase in the number of LM cells present in the liver at a point where the primary parasitaemia of a *P. chabaudi* infection is going into remission, confirms earlier observations (Kumararatne *et al.*, 1987). The present study, unlike others (Playfair and De Souza, 1982, Kumararatne *et al.*, 1987) does not involve administration of radiolabelled cells and tracking of the homing patterns of these cells during infection and hence, identifies that the liver is a site of a possible immune response to the acute phase of a *P. chabaudi* infection. It is not clear if the increase in the number of cells isolated from the liver is a consequence of increased recruitment or an expansion of an intrahepatic LM cell population. Peak numbers of cells were isolated one or two days after the peak of the primary parasitaemia suggesting that these cells might be involved in the immune response at this time of infection. Adoptive transfer of LM cells isolated from the liver at this time conferred protection to irradiated, naive recipient mice against an homologous challenge. The protection observed was significantly greater than that mediated by splenocytes from the same time of infection (day 11 post infection). The LM cells significantly reduced the peak of the primary parasitaemia compared to the control groups indicating that the protection conferred resulted in an increase in anti-parasite effector mechanisms. During a preliminary experiment, LM cells isolated from livers at day 11 post infection were able, upon adoptive transfer, to delay mortality observed in irradiated, naive recipient mice, following a *P. chabaudi* challenge (data not shown). The cause of death in all groups of

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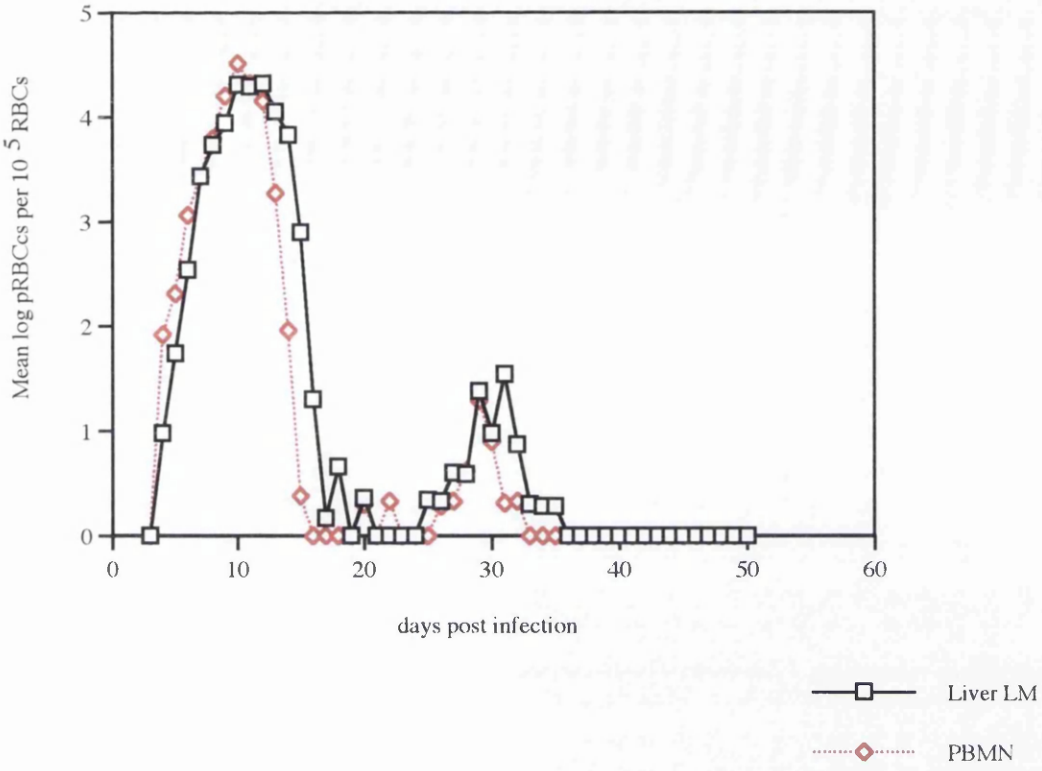

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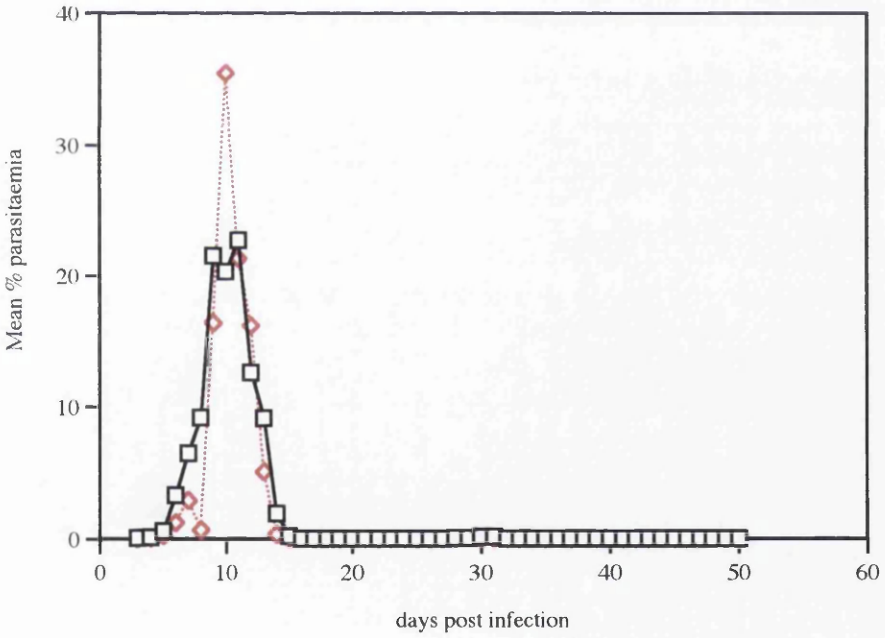

Figure 3.2.2. Adoptive transfer of liver LM cells, isolated at day 11 of a *P. chabaudi* infection, to irradiated recipients, significantly reduced the peak of the primary parasitaemia ($p<0.04$) to an homologous challenge compared with the transfer of PBMN cells isolated concomitantly. Each group of recipients consisted of six mice and were challenged with $1x10^5$ pRBCs of *P. chabaudi*. The mean log parasitaemia A) and percentage parasitaemia B) are presented.

this preliminary experiment appeared to be due to the fulminating parasitaemia, suggesting the liver LM cells were capable of suppressing parasite growth for a period but were able only to delay the time of death.

Previous studies have shown an increase in numbers of cells present in the liver (Kumararatne *et al.*, 1987) and associated this increase with protection (Dockrell, De Souza and Playfair, 1980) but the adoptive transfer studies reported here demonstrate the LM cells present in the liver during the acute phase of *P. chabaudi* infection are involved in a protective immune response against the blood-stage of infection. It has been recently suggested that the liver is a site where T cells migrate too in order to apoptose (Crispe and Mehal, 1996). The LM cells isolated from the liver are unlikely to be undergoing apoptosis because the adoptive transfer studies demonstrate that they are capable of mediating a protective immune response.

To understand what type of effector mechanism may be mediated by the LM cells present in the liver which possibly results in parasite destruction, it is important to know the types of cells which participate in this response. Lymphomyeloid cells isolated from the liver have been shown to contain unusual lymphocytes. The γδ T cell subset, which have been reported to expand during malaria infection (van der Heyde *et al.*, 1993b), appear to be a minor ingredient of the liver LM cells along with B cells, although this may be dependent upon the isolation technique (Crispe and Mehal, 1996). It is mainly T cells that have been characterised in cells isolated from the liver. A mixture of $CD8^+$, $CD4^+$ and $CD4^-$ $CD8^-$ T cells along with a subset termed NK1.1 T cells, which express TCRαβ at an intermediate level and IL-2 receptor along with the natural killer 1.1 cell marker, have been shown to be present in the intrahepatic T cell pool (Ohteki and MacDonald, 1994, Crispe and Mehal, 1996). The NK1.1 T cells have an unusual distribution with a high frequency of cells present in the liver, bone marrow and thymus suggesting that they may exert their functions in specialised locations (Vicari and Zlotnik, 1996). The morphology of these cells indicates that they are

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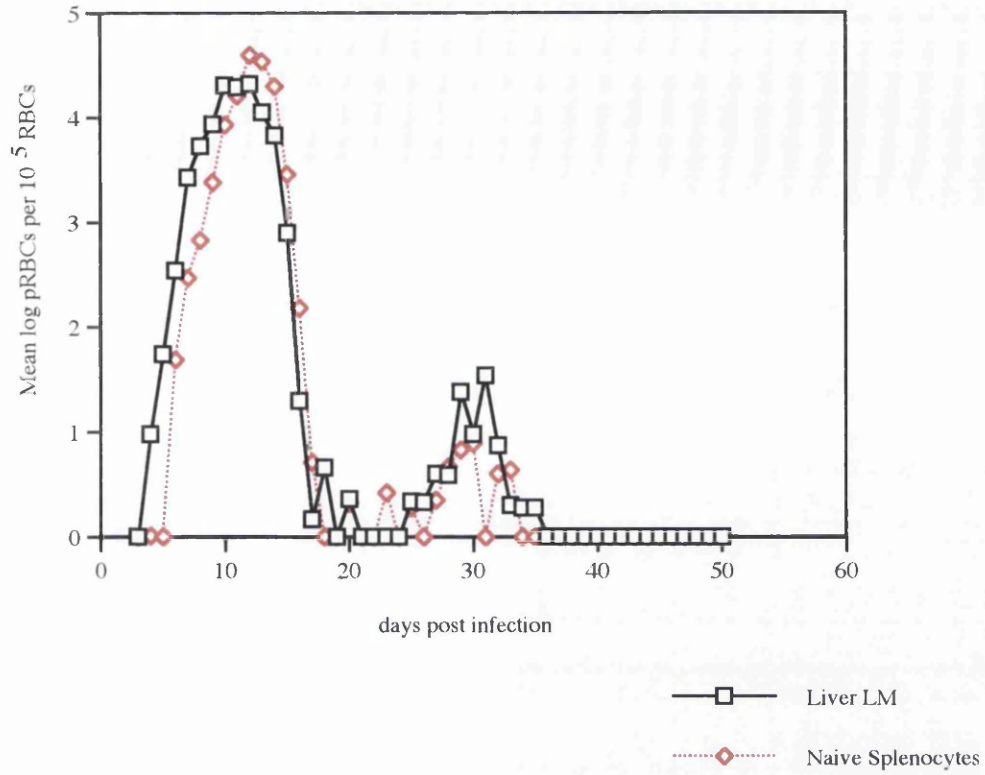

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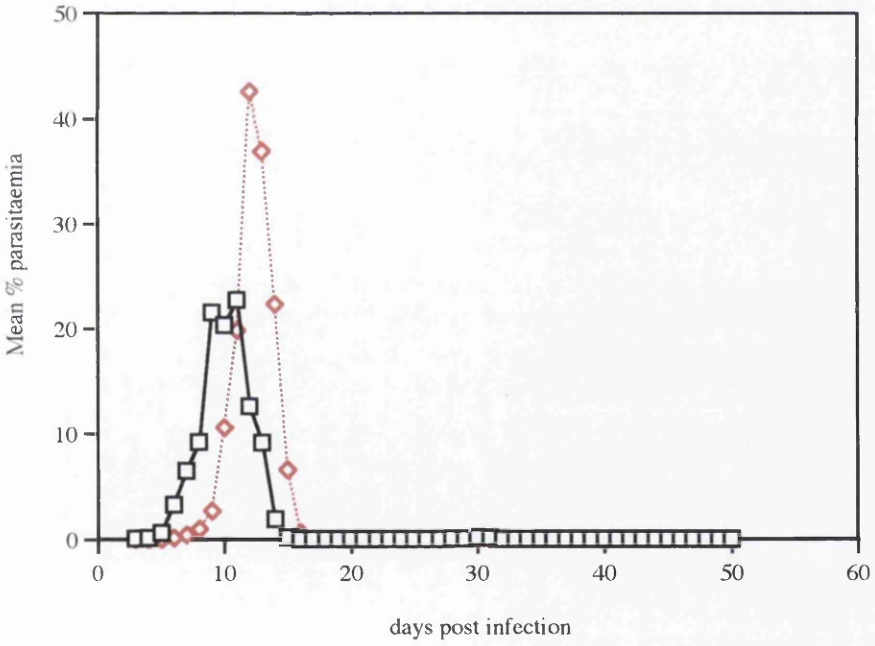

Figure 3.2.3. Adoptive transfer of liver LM cells, isolated at day 11 of a *P. chabaudi* infection, to irradiated recipients, significantly reduced the peak of the primary parasitaemia ($p<0.04$) to an homologous challenge compared with the transfer of splenocytes isolated from naive mice. Each group of recipients consisted of six mice and challenged with $1x10^5$ pRBCs of *P. chabaudi*. The mean log parasitaemia A) and mean percentage parasitaemia B) are presented.

intermediate between T cells and NK cells (Watanabe *et al.*, 1995). NK 1.1 T cells have been shown to produce both IL-4 and IFNγ (Arase, Arase and Saito, 1996) and can differentiate into cytotoxic effector cells upon activation with IL-2, with the ability to kill targets of NK cells (Koyasu, 1994). The function of this subset of T cells is unclear but because of their cytokine secretion profile and potential cytotoxic ability, they may be involved in not only directing the $CD4^+$ T helper cell mediated responses through IFNγ or IL-4 secretion but may also participate in parasite destruction within the liver.

Cytofluorometric analysis of the isolated LM cells during the acute phase of *P. chabaudi* infection, would enable identification of the cells present within this population and could be compared with changes in the lymphocyte subsets in the spleen. This would give a direct comparison between the lymphocyte populations within the liver and spleen, identifying the immune responses which are functional in both these organs during a primary *P. chabaudi* infection and perhaps reveal why LM cells from the liver confer significantly better protection than splenocytes upon adoptive transfer to recipient mice receiving an homologous challenge of *P. chabaudi.* However, it must be noted that at day 11 post infection, when the cells were isolated from both the spleen and liver, it has been reported that there is an exclusion of lymphocytes from the spleen and increased retention of lymphocytes in the liver (Kumararatne *et al.*, 1987), perhaps reflecting a change in the site of the protective immune response at this time point in the infection. Liver LM cells have been previously characterised by cytofluorometric analysis following infection of mice with *P. yoelii* sporozoites (Faure *et al.*, 1994). The majority of cells identified were lymphoid, with a rapid increase in $CD4^+$, $CD8^+$ and $CD4^-$ $CD8^-$ T cells plus B cells (Faure *et al.*, 1994). The increase in cell numbers in the livers was persistent throughout the subsequent blood stage infection following inoculation with sporozoites of *P. yoelii.* Cytological analysis of the same LM cells revealed that polymorphonuclear (PMN) cells and macrophage/monocytes were also present in high numbers (Faure *et al.*, 1994). Liver LM cells isolated from mice during a primary *P. chabaudi* infection and analysed cytologically, were found to have an

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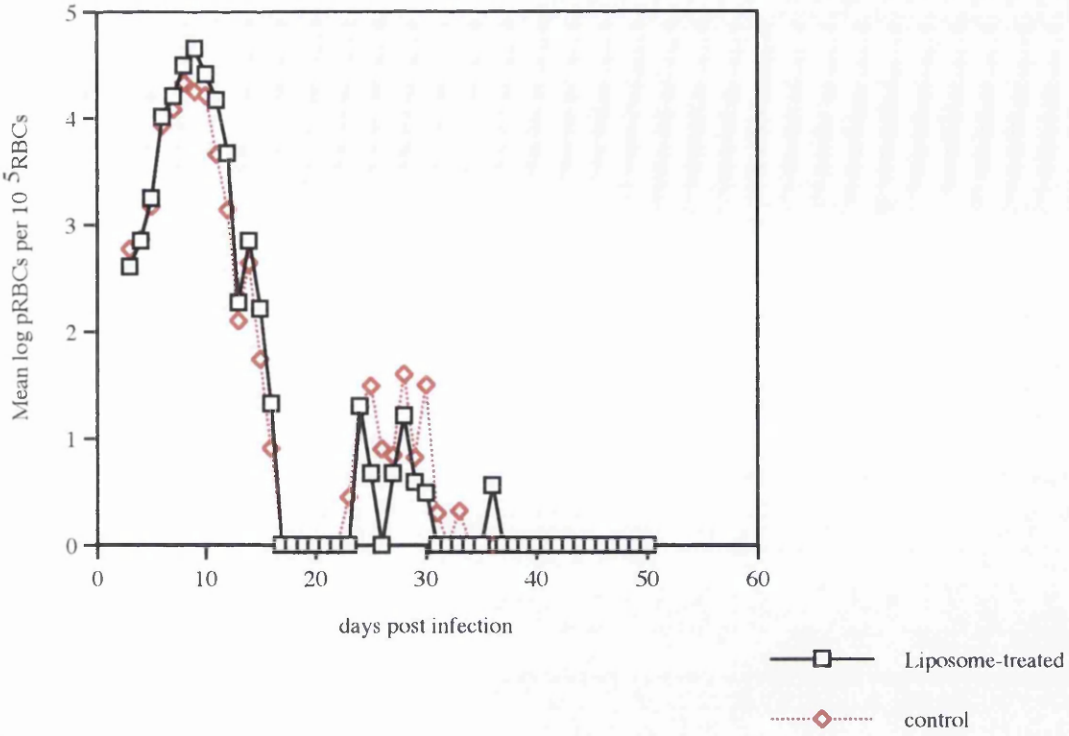

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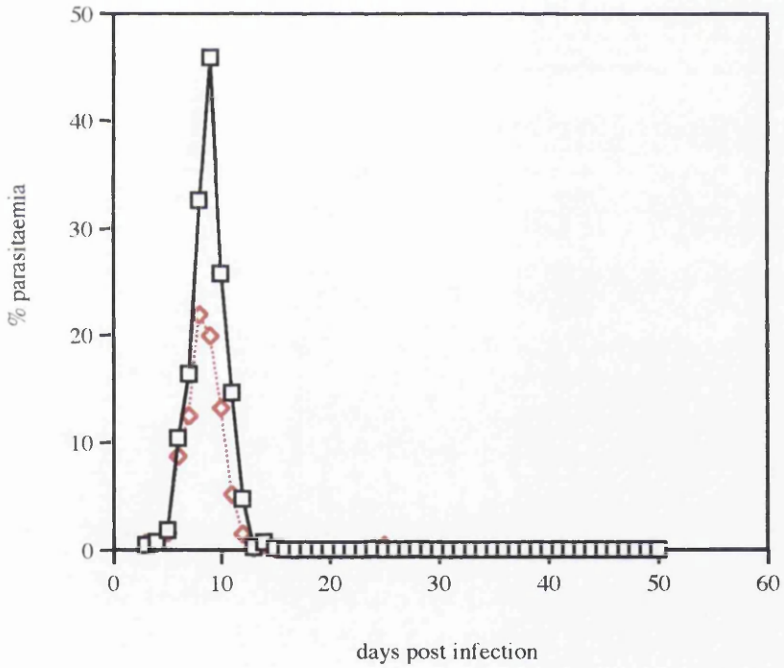


Figure 3.3. Kupffer cells were depleted before and during a *P. chabaudi* infection by administration of liposome-encapsulated clodronate. Control mice received no treatment. Each group consisted of six mice and infected with $1x10^5$ pRBCs of *P. chabaudi*. Depletion of Kupffer cells resulted in an exacerbation of the primary patent parasitaemia. The mean log parasitaemia A) and mean percentage parasitaemia B) are presented.

increase in lymphoid, monocyte and PMN cell populations (see Chapter 6 and 7). The absence of this increase, most notably in the monocyte and PMN cells, observed in IFNγ receptor and IL-4 gene deficient mice was associated with an exacerbation of the primary parasitaemia (see Chapter 6 and 7). Therefore, the recruitment of monocytes and PMN cells appears to be crucial to the protective effector mechanisms in the liver during the acute phase of *P. chabaudi* infection.

It has been estimated that more than half the circulating monocytes are destined to become the resident macrophages in the liver, known as Kupffer cells (Crofton, Diesselhoff-den Dulk and Van Furth, 1978). These cells can phagocytose parasitised erythrocytes (Shear, Nussenzweig and Bianco, 1979) and may act as antigen-presenting cells (Faure *et al.*, 1994). Hence, Kupffer cells appear to have a pivotal role in the immune response in the liver. Antigen presentation to T cells will result in an expansion of intrahepatic T cells or enhanced recruitment which will stimulate effector cells present in the liver, including Kupffer cells, to secrete various inflammatory cytokines and toxic molecules such as NO, associated with activated macrophages. When Kupffer cells were depleted *in vivo*, a significant exacerbation of the primary peak of parasitaemia was observed. This demonstrates the importance of Kupffer cells during the acute phase of infection but also illustrates that control of the primary parasitaemia includes effector mechanisms which are Kupffer cell independent. It must be noted however, that this observation is preliminary and requires further investigation.

Malaria is a very dynamic, systemic infection which induces the production of various inflammatory cytokines (Bate, Taverne and Playfair, 1988, Kwiatkowski *et al.*, 1990). Associated with the production of these cytokines, such as IL-1 and TNFα, is the activation of endothelial cells and the up-regulation of adhesion molecules (Schofield *et al.*, 1996). The presence of the parasite induces this inflammatory environment but both the parasite and the immune system utilise it, either in the process of parasite sequestration or the recruitment of immunocompetent cells to various sites. In the *P.*

chabaudi model, a major site of sequestration, as already mentioned, is the liver (Cox, Semoff and Hommel, 1987). This process is linked to antigenic variation and the establishment of chronic infection (Gilks, Walliker and Newbold, 1990) but it is unclear if sequestration is required to induce the recruitment of LM cells to the liver. The cytoadherence of the mature parasitised erythrocyte, in an environment which has the potential to mediate a protective immune response, provides a good opportunity for anti-parasite effector mechanisms. Analysis of LM cells present in the liver during infection with a non-sequestering clone of *P. chabaudi* would determine if sequestration is necessary to induce the increase in the numbers of LM cells present and the proposed protective effector mechanism occurring in the liver during a primary *P. chabaudi* infection.

The liver is potentially a site of parasite destruction during the acute phase of a *P. chabaudi* infection. An increase in the numbers of LM cells present in the liver coincides with the peak of the primary parasitaemia and these cells were able to confer protection to an homologous challenge. The importance of the liver was further defined by the observation that depletion of Kupffer cells resulted in an exacerbation of the primary patent parasitaemia. Identification of the cell types present in the isolated LM cells would elucidate the type of immune response which potentially is involved in parasite destruction during the asexual erythrocytic stage of *P. chabaudi* infection. Further investigations of the LM cells present in the liver during a *P. chabaudi* infection are planned. Cytofluorometric analysis is already underway with the aim of identifying the composition of the LM cells through the expression of surface molecules. Initially the work has focused on lymphoid cells, where both B and T cells have been identified, although this work will be expanded to include $CD3^{+}CD4^{-}CD8^{-}$ T cells and NK cells. As already mentioned, repeat studies of the adoptive transfer experiments are planned, including LM cells isolated from the liver of naive mice as a further control.

Chapter Four

The role of serum amyloid P during blood-stage malaria infection.

Introduction

A rapid inflammatory response is an essential part of the host's non-specific response to injury or microbial infection. This is characterised by fever and an increase in the synthesis in the liver of serum proteins termed acute phase reactants (APR). The major APR in humans is C-Reactive protein (CRP), originally named for its calcium dependent binding to the C-polysaccharide of pneumococcal bacteria (Volanakis and Kaplan, 1971). In mice, CRP is present at very low concentrations in the serum and has not yet been identified as an APR in mice (Pepys *et al.*, 1978, Le, Muller and Mortensen, 1982). Serum amyloid P (SAP) is the main APR in mice (Pepys *et al.*, 1979) and is a molecular homologue of CRP (Pepys *et al.*, 1978, Levo, Frangione and Franklin, 1977). Both CRP and SAP are pentraxins (Osmand *et al.*, 1977), with both coding genes found on chromosome 1 in humans and mice (Steel and Whitehead, 1994). The biological activity of these APRs is related to non-specific host defence mechanisms with both CRP and SAP having similar properties. However, the role of CRP has been better characterised. CRP can act as an opsonin for bacteria and immune complexes (Steel and Whitehead, 1994) mediated through the ability of CRP to bind to Fc receptors (Mortensen and Dusckiewicz, 1977), and activate the classical pathway of complement (Osmand *et al.*, 1975). SAP is the circulating precursor to amyloid P component which is a constituent of amyloid deposits (Benson *et al.*, 1976). Like CRP, SAP can enhance macrophage mediated phagocytosis via binding to glycoprotein receptors (Siripont, Tebo and Mortensen, 1988) and can activate the classical complement pathway via binding to C1q (Ying *et al.*, 1993). SAP has also been shown to regulate antibody responses *in vitro* (Sarlo and Mortensen, 1987). Both CRP and SAP have the capacity to bind chromatin, histones and DNA (Steel and Whitehead, 1994) and it has been suggested that the binding to and subsequent clearance of nuclear material which is released from necrotic tissue during inflammation would prevent the development of nuclear antigen specific autoimmunity (Steel and Whitehead, 1994).

An increased rate of APR synthesis can occur following various inflammatory stimuli. Rapid increases in hepatic SAP mRNA in mice following an intraperitoneal injection of thioglycollate have been reported (Zahedi and Whitehead, 1989). IL-1 and IL-6 have been shown to stimulate the production of SAP by mouse hepatocytes *in vitro* (Lin *et al.*, 1990). Administration of recombinant IL-1 and TNFα induces SAP production *in vivo* (Mortensen *et al.*, 1988). Hence, APRs are synthesised rapidly following inflammatory stimuli, which is important during an infection, when the non-specific immune response controls the initial stages of infection allowing the development of acquired, specific effector mechanisms.

Although mice do not synthesise CRP as part of their acute phase response, passively administered human CRP has been shown to protect mice from lethal infection with *Streptococcus pneumoniae* (Mold *et al.*, 1981). Transgenic mice, expressing human CRP, have increased protection to *S. pneumoniae* (Szalai, Briles and Volanakis, 1995). Elevated levels of SAP have been observed in the serum of mice infected with *Nippostrongyls braziliensis* (LaMontague *et al.*, 1984) and *Schistosoma mansoni* (Pepys *et al.*, 1979). *Trypanosomia cruzi* infection in mice induces an increase in SAP levels (Luz, van Leuven and Araujo-Jorge, 1994, Truyens *et al.*, 1994) and is associated with a delay in mortality following a lethal infection (Truyens *et al.*, 1994).

The exo-erythrocytic stage of malaria infection involves a developmental phase within the liver which induces a strong inflammatory response inducing the required stimuli for the production of APRs. CRP has been shown to bind to the surface membrane of *P. falciparum* and *P. yoelii* sporozoites probably via a phosphorylcholine-binding site (Mazier *et al.*, 1988). Addition of purified CRP to hepatocyte monolayers *in vitro*, at the time of inoculation with *P. yoelii* sporozoites inhibited parasite schizogony (Pied *et al.*, 1989). This effect was abrogated by the pre-treatment of cultures with anti-CRP antibodies (Pied *et al.*, 1989). Time-course experiments revealed that CRP-mediated

inhibition occurred at the early phase of infection (Mazier *et al.*, 1988). It is thought that CRP inhibits sporozoite penetration of the hepatocyte by masking recognition sites involved in the sporozoite-hepatocyte interaction (Nussler *et al.*, 1991c). CRP mediated inhibition was also observed after sporozoite penetration (Nussler *et al.*, 1991c). It is possible that CRP bound to sporozoites, penetrates the hepatocyte and subsequently prevents trophozoite division (Nussler *et al.*, 1991b). Induction of raised CRP levels in rats by injection with turpentine oil, protected against a challenge with *P. yoelii* sporozoites (Pied *et al.*, 1989). The susceptibility to *P. berghei* infection of two species of rat has been attributed to differences in the acute phase response induced by infection (Vreden *et al.*, 1995). Furthermore, sporozoites incubated in acute phase serum had reduced infectivity but this was abolished by the treatment of the cultures with anti-CRP antibodies (Pied *et al.*, 1989). The inhibition of the exo-erythrocytic stage of development may also involve indirect CRP mediated mechanisms such as the activation of complement or macrophages resulting in opsonisation of the parasite.

Raised serum levels of CRP have been observed in *P. falciparum* infected individuals (Gillespie *et al.*, 1991) and correlate with severity of disease. CRP has been shown to bind to merozoites (Pied *et al.*, 1989), probably in a similar manner to the calcium dependent binding to sporozoites. However, the role of APRs in the immune response induced by blood-stage malaria infection is unclear. This study investigated the production of SAP, the main APR of mice during the course of experimental erythrocytic malaria infection. *In vitro* assays were performed to analyse if SAP had any direct anti-parasite effect.

Results

SAP production during experimental blood-stage malaria infection

Levels of SAP in the serum of mice infected with *P. chabaudi*, *P. berghei* and *P. vinckei petteri.* were assayed by the technique described (Materials and Methods). Each group consisted of six mice and were infected with $1x10^5$ pRBCs of the respective parasite species. Serum samples were collected by tail bleeds from 3 mice of each experimental group and two mice from a non-infected control group. Each individual sample was assayed in triplicate and the mean value of SAP was calculated for each group. Elevated SAP levels were observed during the course of infection for all three malaria species compared to serum taken from non-infected controls (Figures 4.1, 4.2 and 4.3). Peak production of SAP during *P. chabaudi* and *P. vinckei* infection, occurred one or two days after the peak of the primary parasitaemia (Figures 4.1 and 4.2). SAP production during a lethal infection of *P. berghei* was elevated (Figure 4.3) and remained relatively high (compared to control levels) until the time of death (mortality data not shown).

The effect of SAP on the growth of *P. falciparum in vitro*

SAP was isolated from serum harvested from mice on day 11 of a *P. chabaudi* infection by the technique described (Materials and Methods). *P. falciparum* at 0.5% parasitaemia and 1.5% haematocrit was cultured in the presence of isolated SAP (100-5000 μg/ml) or chloroquine (0.1-10 μg/ml). The effect of either treatment on parasite growth was determined by the uptake of tritiated hypoxanthine. The addition of SAP, at 5 mg/ml, a concentration observed physiologically during a *P. chabaudi* infection, resulted in a significant decrease in tritiated hypoxanthine incorporation indicating an inhibitory effect of SAP on parasite growth (Figure 4.4).

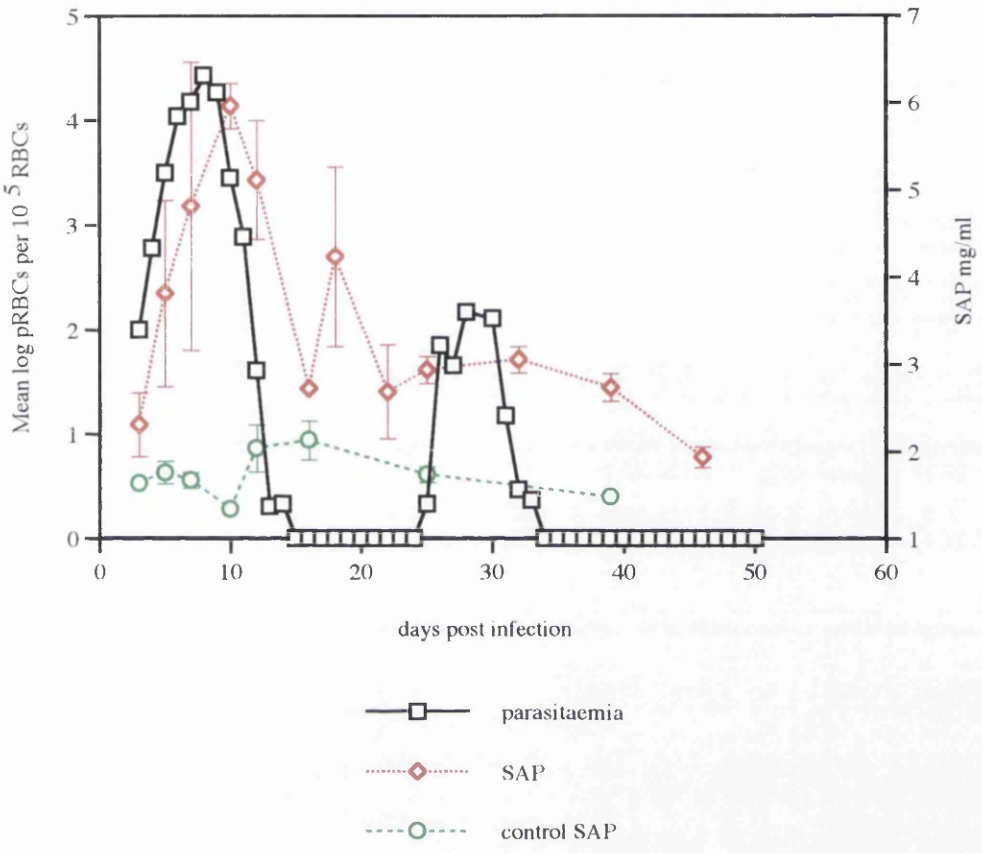


Figure 4.1. SAP was assayed in the serum of mice infected with 1x10 ^{5} pRBCs of *P. chabaudi* Serum was collected from 3 mice at each timepoint and each serum sample was assayed in triplicate. Control mice were non-infected and serum was collected from two mice at each timepoint and assayed in triplicate. Each data point is the mean of all the results obtained for each group. Peak production of SAP occurred one day after the peak of the primary parasitaemia.

The effect of SAP on the growth of *P. chabaudi in vitro*

P. chabaudi was harvested from an infected mouse and cultured at 0.5% parasitaemia and 1.5% haematocrit in the presence of isolated SAP (100-5000 μg/ml) or chloroquine (0.1-10 μg/ml). The effect of both treatments on the growth of *P. chabaudi in vitro* over a 24 hour period was determined by tritiated hypoxanthine incorporation. No effect on parasite growth was observed following the addition of SAP to *P. chabaudi* cultures (Figure 4.5).

The effect of SAP on proliferative responses of splenocytes

Splenocytes were harvested from naive mice and cultured at $5x10^6$ cells/ml. The cells were stimulated with either Concanavalin A or LPS (both 5 μg/ml) and concomitantly treated with SAP (1 mg/ml). The effect of SAP on the proliferative response by splenocytes to both stimulants was determined by tritiated thymidine incorporation. Treatment of splenocytes with SAP was found to inhibit the proliferative response to the T cell mitogen, Concanavalin A (Figure 4.6). However, it was observed that the proliferative response to LPS was enhanced in the presence of SAP (Figure 4.6).

Discussion

The data reported here demonstrates that experimental infection of mice with blood-stage malaria induces elevated production of SAP, the main APR in mice. During *P. chabaudi* and *P. vinckei* infection, both non-lethal infections in NIH mice, the peak production of SAP occurred one or two days after the peak of the primary patent parasitaemia. *P. berghei* infection in NIH mice has a lethal outcome, with death occurring approximately 19 to 20 days after infection. SAP levels were elevated in the serum of *P. berghei* infected mice and remained relatively high compared to the control

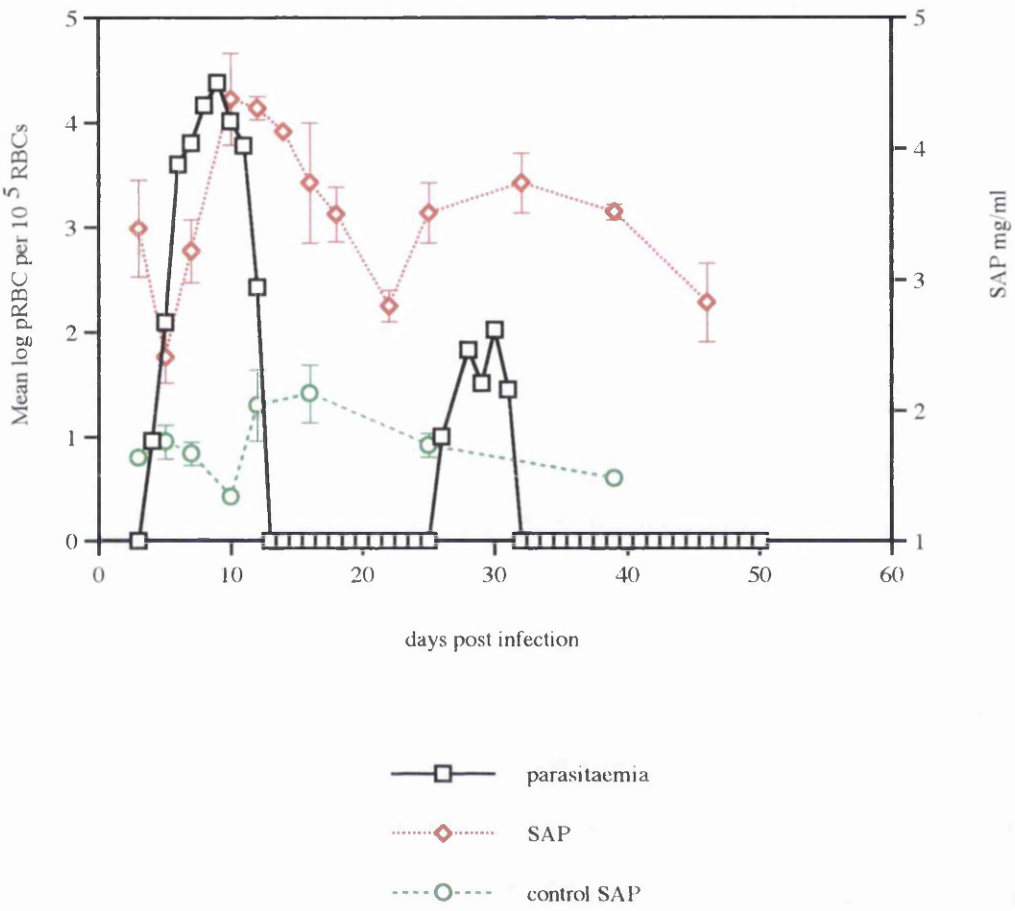


Figure 4.2. SAP was assayed in the serum of mice infected with $1x10^5$ pRBCs of *P. vinckei petteri*. Serum was collected from 3 mice at each timepoint and each serum sample was assayed in triplicate. Control mice were non-infected and serum was collected from two mice at each timepoint and assayed in triplicate. Each data point is the mean of all the results obtained for each group. Peak production of SAP occurred one day after the peak of the primary parasitaemia.

group. It is unclear if the stimulus for inducing the increased rate of SAP synthesis is derived from the presence of the parasite and/or the inflammatory environment created by the response of the host to infection. During *P. berghei* infection, the parasitaemia remains at a high level from day 6 onwards until the time of death, resulting in sustained production of SAP probably due to the stimulation of inflammatory mediators during the course of infection.

During the course of *P. chabaudi* and *P. vinckei* infections, SAP production is elevated throughout, with similar concentrations at peak production of SAP. As already discussed (see Chapter 3), mature asexual erythrocytic stages of *P. chabaudi* withdraw from the circulation and cytoadhere to endothelial linings of tissues such as the liver. Hepatocytes are the main source of SAP synthesis (Koj, 1974) and it was thought that perhaps the sequestration of the parasite to the liver, resulting in a localised immune response, may induce SAP production. However, it appears sequestration does not significantly enhance the production of SAP because *P. vinckei* does not undergo sequestration but the level of SAP production during a *P. vinckei* infection is similar to that induced by a *P. chabaudi* infection. *P. falciparum* infection in humans results in elevated CRP levels (Gillespie *et al.*, 1991) and taken with the observations made from the three rodent malarial infections reported here, demonstrate that blood-stage malaria infection induces a systemic acute phase response.

The actual biological role of APR during blood-stage malaria infection is unclear. Isolation of SAP from immune serum allowed the investigation of any direct anti-parasite effect. The addition of murine SAP, at a concentration which is found in the serum of mice at peak parasitaemia, to *P. falciparum* cultures, inhibited parasite development. However, no effect on the growth of *P. chabaudi in vitro* was observed in terms of the uptake of tritiated hypoxanthine. The lack of growth inhibition mediated by SAP against *P. chabaudi*, although disappointing, may actually give an insight into the mechanism of inhibition of *P. falciparum* growth. The normal culture conditions for

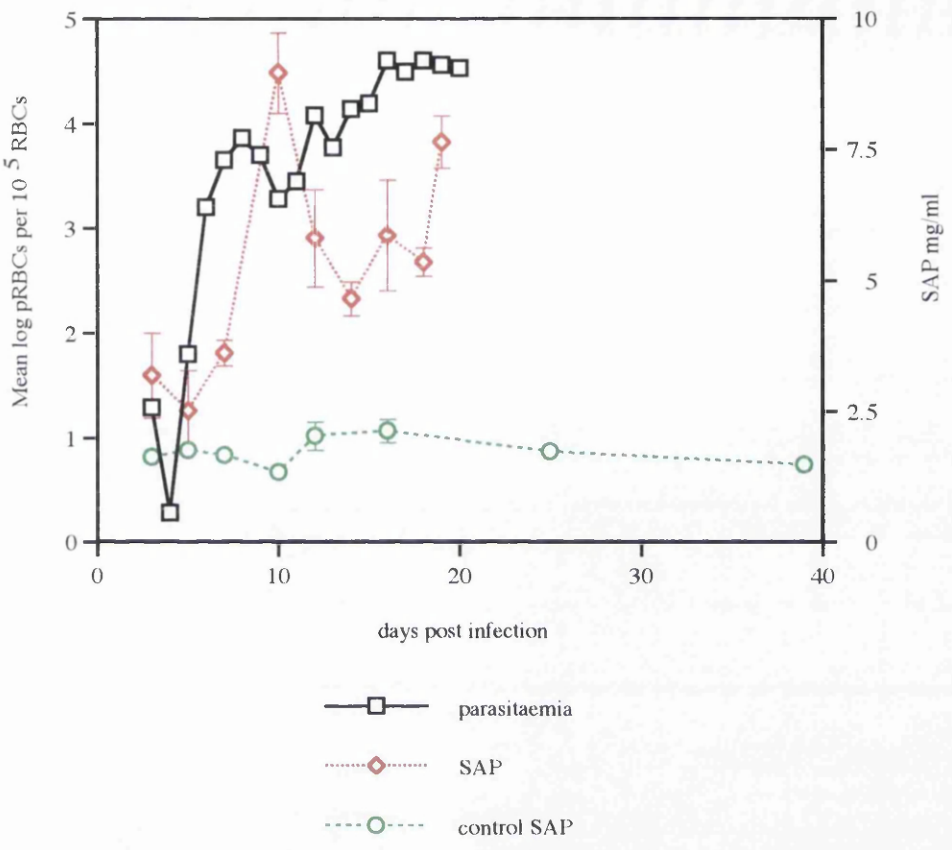


Figure 4.3. SAP was assayed in the serum of mice infected with $1x10^5$ pRBCs of *P. berghei*. Serum was collected from 3 mice at each timepoint and each serum sample was assayed in triplicate. Control mice were non-infected and serum was collected from two mice at each timepoint and assayed in triplicate. Each data point is the mean of all the results obtained for each group. SAP was elevated in the serum of mice infected with *P. berghei* until the time of death.

P. falciparum, are not ideal for the growth of *P. chabaudi in vitro*. *P. chabaudi* has been shown to have a preference for young erythrocytes and the best growth rate has been obtained using 10% rat serum instead of foetal calf serum which was used in this study (McNally, O'Donovan and Dalton, 1992). Furthermore, it has been reported that although schizogony does occur in *P. chabaudi in vitro* cultures, only a small proportion of merozoites re-invade and produce new rings (Sohal and Arnot, 1993). The growth rate of *P. chabaudi* was determined by tritiated hypoxanthine incorporation in this study but it has been shown that most of the uptake of this tracer occurs during the early phase of development and in fact the rate of incorporation decreases as schizogony commences (Newbold *et al.*, 1982, Sohal and Arnot, 1993). Therefore, if SAP mediated growth inhibition occurs during schizogony and release of merozoites, this would not be reflected by the determination of tritiated hypoxanthine uptake because the incorporation of the tracer would occur earlier in the parasites growth cycle. *P. falciparum* completes its life cycle under *in vitro* conditions and readily goes through schizogony allowing continuous culture (growth of *P. falciparum in vitro* is asynchronous). Hence, the inhibition of SAP may occur during schizogony when SAP has easier access to the cell. Even though SAP, at 230kDa, is a large glycoprotein it may enter the erythrocyte via endocytosis or a parasitophorous duct (J. Kusel, personal communication). The other possibility is that SAP may interfere with the invasion of the merozoite into the erythrocyte. CRP has been shown to bind to merozoites (Pied *et al.*, 1989) and can interfere with the sporozoite invasion of hepatocytes by masking important binding sites. SAP may prevent merozoite invasion of erythrocytes by either binding to the merozoite, probably in a calcium dependent manner or by masking important receptors on the erythrocyte because SAP has been shown to bind to complement coated erythrocytes (Hutchcraft *et al.*, 1981).

During *P. chabaudi* infection (and *P. falciparum* infection), the parasites undergo deep tissue schizogony in the liver. The merozoites released may be vulnerable to a high local concentration of SAP which may inhibit re-invasion of erythrocytes by merozoites. The

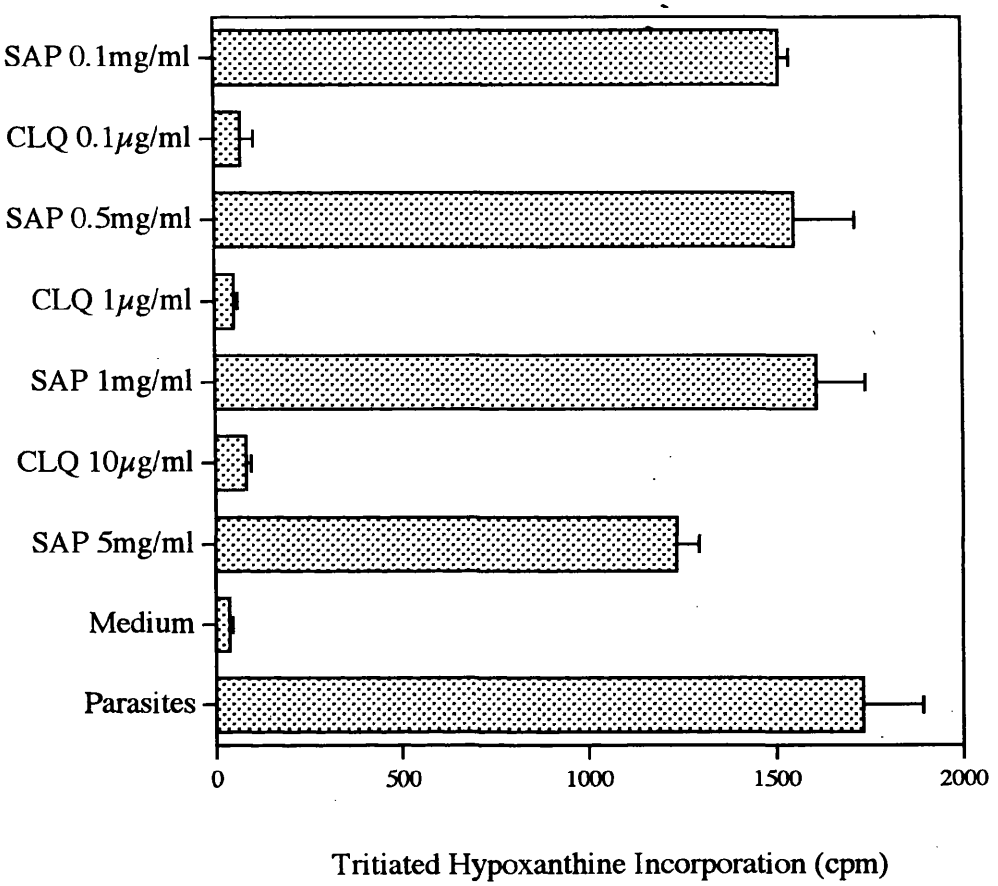


Figure 4.4. The effect of SAP on the growth of *P. falciparum in vitro*. SAP was isolated from the serum of *P. chabaudi* infected mice was added to *in vitro* cultures of *P. falciparum* at the concentrations shown. Chloroquine (CLQ) was used as a control indicating parasite killing. SAP at 5mg/ml,which is a concentration found in the serum of mice at peak parasitaemia, significantly inhibited*P. falciparum* growth ($p < 0.0003$).

possible direct inhibition of parasite growth mediated by SAP requires further investigation. Induction of the acute phase response and hence, elevated APR levels prior to infection with *P. chabaudi*, would determine if SAP is involved in controlling the primary parasitaemia but it would be difficult to elucidate if this was a direct or indirect mechanism mediated by SAP.

SAP may be involved in the response to *P. chabaudi* infection indirectly through the activation of several different protective mechanisms of the immune system. Macrophages have an important role in the immune response to malaria infection through their ability to present antigen, phagocytose parasitised erythrocytes and secrete various inflammatory mediators. SAP has been reported to activate macrophages, enhancing IL-1 production (Sarlo and Mortensen, 1985) and increasing the bactericidal activity of elicited, inflammatory macrophages (Singh *et al.*, 1986). Therefore, elevated production of SAP during malaria infection may inhibit the growth of the parasite throughout the activation of macrophage-mediated responses. The interaction between macrophages and the synthesis of APR is important because the macrophage is the major source of the inflammatory mediators such as IL-1 and IL-6 which induce the production of APR. Hence, activated macrophages will secrete products which induce APR synthesis, which in turn further activate macrophages creating a response which depends upon the constant stimulation of macrophages. Depletion of macrophages during a primary *P. chabaudi* infection (Chapter 3) demonstrated that the macrophage appears to be important in controlling the primary patent parasitaemia which is when the peak production of SAP occurs. It would be interesting to repeat the depletion of macrophages during the course of a *P. chabaudi* infection and monitor the effect this has on the production of SAP. The effect of Kupffer cell depletion on macrophage-derived molecules such as NO, would also have to be examined.

Acute phase reactants have been shown to modulate neutrophil function (Butcha *et al.*, 1987) with enhanced chemotaxis and phagocytosis by neutrophils following *in vitro*

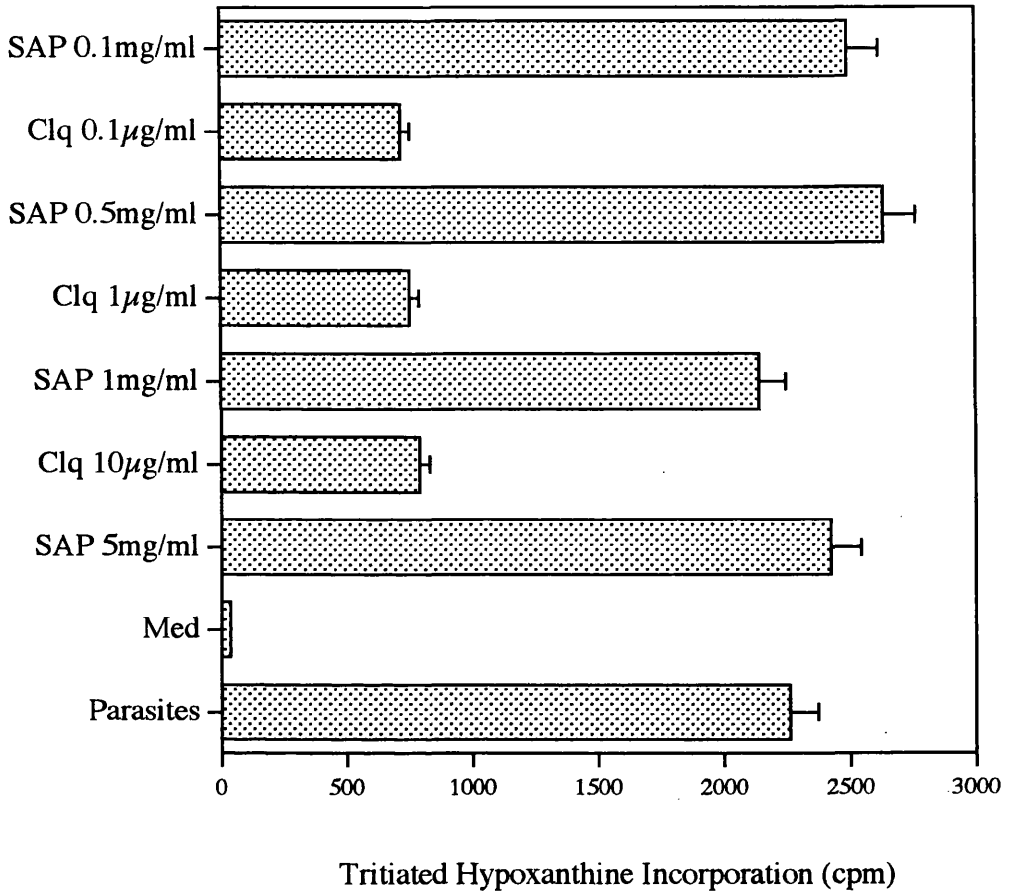


Figure 4.5. The effect of SAP on the growth of *P. chabaudi in vitro*. Isolated SAP was added to *in vitro* cultures of *P. chabaudi*, freshly obtained from an infected mouse, at the concentrations shown. Chloroquine (Clq) was used as a control of parasite killing. SAP had no effect on the growth of *P. chabaudi in vitro*

stimulation with CRP. Neutrophil activation has been reported during severe malaria infection (Mohamed *et al.*, 1996) and neutrophils can kill *P. falciparum in vitro* by a phagocytic mechanism (Celada, Cruchaud and Perrin, 1984). Hence, APR modulation of neutrophil function during a malaria infection may result in increased parasite killing through phagocytosis.

The evidence for an important role for complement in immunity to blood-stage malaria infection is conflicting. During acute phase infections complement levels have been reported to decrease (Greenwood and Brueton, 1974) and *in vitro* studies have demonstrated monocyte- and neutrophil-mediated phagocytosis of *P. falciparum* parasitised erythrocytes is independent of complement (Celada, Cruchaud and Perrin, 1984). However, another study reported that phagocytosis of *P. falciparum in vitro* was facilitated by complement (Schwarzer *et al.*, 1992) and required the presence of complement (Salmon *et al.*, 1986). SAP can activate complement by binding to C1q (Ying *et al.*, 1993) and it has also been shown to bind to C4-binding protein (De Beer *et al.*, 1981) and C5b6 (Barbashov, Wang and Nicholson-Weller, 1997). The production of APR may be involved in the protective immune response through the activation of the complement cascade which may be involved in the development of protective immunity to the erythrocytic stages of malaria infection.

One of the main mechanisms through which SAP could influence the response to malaria infection is by exerting an immunomodulatory effect on various cells of the immune system. The studies reported here indicate that SAP has the ability to modulate the responses of both T cells and B cells because the proliferative response of splenocytes to the T cell mitogen, Concanavalin A, was inhibited following treatment with SAP. Furthermore, the proliferation of splenocytes in response to LPS stimulation was enhanced following the addition of SAP. These observations confirm earlier reports investigating the effect of SAP on T cells (Levo and Wollner, 1985, Li *et al.*, 1984) where SAP was found to inhibit T cell responses to Concanavalin A and the

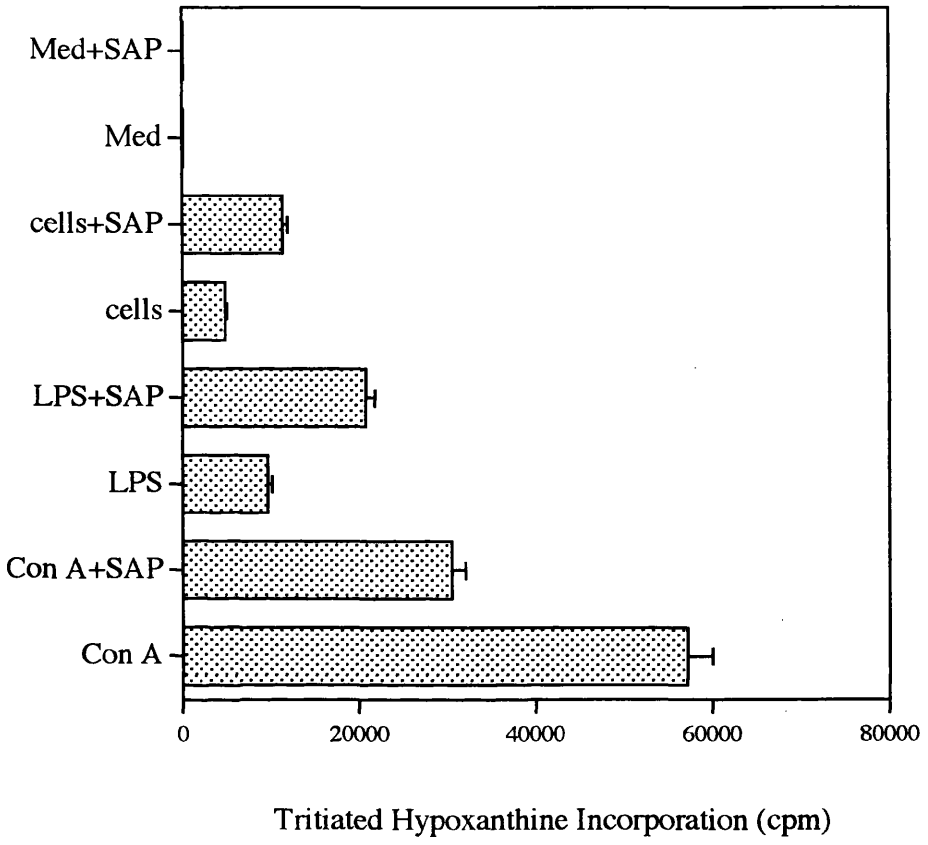


Figure 4.6. The effect of SAP on the proliferative responses of splenocytes. Isolated SAP (1mg/ml) was added to splenocytes harvested from naive mice. The cultures were stimulated with Concanavalin A (ConA) or LPS (both at 5μg/ml). SAP inhibited the proliferative response to Con A ($p < 0.001$) but actually enhanced the response of splenocytes to LPS ($p < 0.004$).

proliferative responses of human peripheral blood mononuclear cells to phytohaemagglutinin. These observations suggest that APR may exert an immunosuppressive effect on T cell responses. Splenocytes isolated from mice at peak parasitaemia of a *P. chabaudi* infection, display a suppressed response to T cell mitogens (Bernadette *et al.*, 1996). This coincides with peak production of SAP and suggests that the elevated levels of SAP may suppress T cell responses perhaps as a regulatory mechanism to dampen inflammatory responses which may be involved in the pathology associated with severe infection.

The immunosuppressive effect exerted on T cells by SAP, may not extend to B cells because addition of SAP to splenocytes, enhances the proliferative response to the B cell stimulant, LPS. Furthermore, SAP has been shown to suppress antibody responses *in vitro* to T cell dependent antigens (Sarlo and Mortensen, 1987). Another possible link between antibody production and SAP comes from the studies reported later (Chapter 5) on *P. chabaudi* infection of IL-6 gene deficient mice. As already mentioned, IL-6 is one of the main inducers of APR production (Heinrich, Castell and Andus, 1990) and subsequently, IL-6 deficient mice have an impaired acute phase response (Kopf *et al.*, 1994). During the course of a *P. chabaudi* infection, the normal production of SAP is absent and this correlates with an extension of the primary parasitaemia and a reduced humoral response to the infection in the IL-6 deficient mice. Therefore, it is possible that the absence of SAP production and subsequent loss of its immunosuppressive effect, may result in a delay in reaching the required level of humoral response which is necessary for parasite clearance after the peak of the primary parasitaemia. However, it must be noted that the IL-6 deficient mice still cleared the *P. chabaudi* infection indicating that the role of SAP, if any, during immunity to this experimental infection is a minor one.

The work presented here demonstrates that experimental infection of mice results in the systemic production of APR, namely SAP and that this acute phase protein may have a

direct inhibitory effect on the development of malaria parasites. SAP also has the potential to be an important immunoregulatory molecule which may influence the immune response to malaria infection.

Chapter Five

Course of *P. chabaudi* infection in IL-6 deficient mice.

Introduction

IL-6, a glycoprotein of 20-30 kDa, is a pleiotropic cytokine produced by many cell types including monocytes/macrophages, endothelial cells, T cells and B cells (Akira, Taga and Kashimoto, 1993). Although it is the major stimulus of acute phase production (Gauldie *et al.,* 1987, Andus *et al.,* 1987), IL-6 is also important for the growth of B cells (Akira, Taga and Kashimoto, 1993) and can act on mitogen-activated B cells to induce IgM, IgG and IgA production without stimulating B cell proliferation (Muraguchi *et al.,* 1988, Beagley *et al.,* 1989). T cell activation, growth and differentiation are all regulated partially by IL-6, including the proliferation of peripheral T cells and differentiation of CTLs (Takai *et al.,* 1988, Okada *et al.,* 1988, Renauld, Vink and van Snick, 1989). IL-6 may achieve these activities by converting T cells to an IL-2 responsive state through the upregulation of IL-2 receptor expression and inducing IL-2 production by T cells (Noma *et al.,* 1987, Le *et al.,* 1988, Garman *et al.,* 1987, Houssiau *et al.,* 1988). Hence, IL-6 can induce IL-2 secretion and proliferation of T cells directly, bypassing the requirement of expression of co-stimulatory molecules by antigen presenting cells (Kasahara *et al.,* 1990, Lorre *et al.,* 1990). It is a possibility that IL-6 may be a co-stimulatory factor when a macrophage is acting as an antigen presenting cell. Macrophage phagocytosis and expression of a number of macrophage differentiation antigens can be enhanced by IL-6 (Akira, Taga and Kashimoto, 1993, Shabo *et al.,* 1988, Lotem, Shabo and Sachs, 1989). In general, cells require stimulation to produce IL-6. LPS and IFN-γ can induce IL-6 production by monocytes (Helfgott *et al.,* 1987, Leeuwenberg *et al.,* 1990). IL-1 and TNF can also induce IL-6 production (Kohase *et al.,* 1986, Van Damme *et al.,* 1987) but IL-6 cannot induce IL-1 or TNF production. Indeed, IL-6 can suppress endotoxin induced IL-1 and TNF production by macrophages (Aderka, Le and Vilcek, 1989, Schindler *et al.,* 1990). Signal transduction pathways for the expression of IL-6 appear to involve protein kinase C and adenylate cyclase following induction by IL-1 or TNF (Zhang,

Lin and Vilcek, 1988, Sehgal, Walther and Tamm, 1987). Dexamethasone and other glucocorticoids can markedly suppress the production of IL-6 (Helfgott *et al.,* 1987): suppression occurs at both transcriptional and post-transcriptional levels.

IL-6 is involved in haematopoiesis, the inflammatory response, bone metabolism and can exert an influence on neuronal cells (Akira, Taga and Kashimoto, 1993). The activity of NK cells can be enhanced by IL-6 stimulation (Luger *et al.,* 1989). IL-6 induced production of acute phase proteins (APP) by hepatocytes is well established (Gauldie *et al.,* 1987, Andus *et al.,* 1987). The actual biological function of many of the acute phase proteins remains unclear. Several cytokines can synergise with IL-6 to induce acute phase protein production, including IL-1, IL-11, TNF-α and TGF-β (Akira, Taka and Kashimoto, 1993). IL-6 is also involved in the control of body temperature. It can act directly on the anterior hypothalamus altering the thermoregulatory set-point probably via the induction of PGE_2 synthesis (Kwiatkowski, 1995). This has obvious importance during a malarial infection where TNF, IL-1 and IL-6 are attributed with mediating/regulating the fever associated with schizont rupture. Although IL-6 appears to be a pro-inflammatory cytokine, anti-inflammatory activities have also been reported. IL-6 has been shown to inhibit TNF production and may have a role in a negative feedback mechanism which can inhibit endotoxin initiated, cytokine mediated acute inflammation (Ulich *et al.,* 1991).

Elevated levels of IL-6 have been detected in the serum of patients presenting with severe *P. falciparum* infection (Kern *et al.,* 1989). The levels correlate with parasite density and high serum TNF levels (Kern *et al.,* 1989). Soluble IL-6 receptor levels were raised in children with severe *P. falciparum* and appear to be a sensitive marker of cerebral malaria in conjunction with IL-6 serum levels (Jakobsen *et al.,* 1994). It has been suggested that elevated IL-6 levels may be related to hypergammaglobulinaemia observed in malaria (Grau *et al.,* 1990). The ability of IL-6

to inhibit hepatic gluconeogenesis (Hill, Stith and McCallum, 1990) may contribute to the hypoglycaemia observed during malarial infection (Clark *et al.,* 1981).

Whilst research concerning the cause of fever during a malaria infection has centred on the TNF inducing abilities of parasitised erythrocytes or supernatants from *in vitro* cultures (Taverne *et al.,* 1990a, Taverne *et al.*, 1990b), it has been shown that the same crude extracts can also induce IL-6 production by monocytes (Jakobsen *et al.,* 1993). *In vitro* experiments have illustrated that IL-6 can inhibit the development of exo-erythrocytic stages of parasites directly or indirectly (Pied *et al.,* 1992, Nussler *et al.,* 1991b). This has also been shown *in vivo* in rats infected with *P. berghei* (Vreden *et al.,* 1995). IL-6 levels were raised in rats resistant to *P. berghei* infection compared with a susceptible strain of rat.

A protective role for IL-6 during experimental human malaria has been suggested. Immunized volunteers, receiving irradiated *P. falciparum* sporozoites, were protected from a subsequent *P. falciparum* challenge (Harpaz *et al.,* 1992). IL-6 was the only cytokine measured where a significant rise from background levels was observed. IFN-γ, CRP, soluble CD8 and soluble IL-2 receptor were all raised in non-vaccinated individuals who all developed parasitaemia following a *P. falciparum* sporozoite challenge. It is interesting to note the absence of detectable CRP levels in the protected individuals suggesting that IL-6 may be involved at local level during first exposure to the infection but when individuals are vaccinated and then challenged the role of IL-6 may be to generate a specific humoral response. Serum analysis of individuals during the vaccination process may give an indication of the mechanisms which result in mediating protection against a live sporozoite challenge and elucidate the role of IL-6 during both responses.

Mice deficient in IL-6 production develop and breed normally (Kopf *et al.,* 1994). The numbers of thymocytes and peripheral T cells are reduced when compared with

controls but they were shown to have normal expression levels of characteristic T cell markers (Kopf *et al.*, 1994). B cell functions in the IL-6 deficient mice were deficient in response to infection with vesicular stomatitis virus (Kopf *et al.*, 1994). IgG production and mucosal IgA responses were reduced in IL-6 deficient mice but IgM responses were normal. Reduced generation and activity of cytotoxic T cells were demonstrated by inefficient control of vaccinia virus by IL-6 deficient mice (Kopf *et al.*, 1994). IL-6 deficient mice are more susceptible, than intact control mice, to *Listeria monocytogenes* (Kopf *et al.*, 1994, Dalrymple *et al.*, 1995), *Candida albicans* (Romani *et al.*, 1996), *Escherichia coli* infection (Dalrymple *et al.*, 1996), and have impaired resistance to the development of toxoplasmic encephalitis (Suzuki *et al.*, 1997). However, efficient immunity to *Leishmania major* infection develops in IL-6 deficient mice (Moskowitz, Brown and Reiner, 1997) demonstrating that IL-6 deficient mice mount an effective $CD4^+$ Th1 T cell response which mediates protective immunity to *L. major* infection (Locksley *et al.*, 1993) through the activation of macrophages to produce NO (Liew *et al.*, 1990). The IL-6 deficient mice produce comparable levels of IFNγ in lymph nodes to that of control mice following *L. major* infection (Moskowitz, Brown and Reiner, 1997) but reduced IFNγ production in the brains of *T. gondii* infected IL-6 deficient mice was observed (Suzuki *et al.*, 1997). This suggests that IL-6 may have different functions depending upon the site of the inflammatory response. Impaired recruitment of inflammatory cells to the brains of *T. gondii* infected IL-6 deficient mice may be a consequence of reduced IFNγ production due to the absence of IL-6 (Suzuki *et al.*, 1997).

One of the main detrimental effects of the absence of IL-6 during the response to an infection is a reduced peripheral blood neutrophilia. This has been observed following infection with *E. coli* (Dalrymple *et al.*, 1996), *C. albicans* (Romani *et al.*, 1996) , *L. monocytogenes* (Dalrymple *et al.*, 1995) and has been proposed as a possible deficiency in the inflammatory response to *T. gondii* (Suzuki *et al.*, 1997). However, the inefficient neutrophilia resulting from the absence of IL-6 appears to be

inconsequential to the control of *L. major* infection (Moskowitz, Brown and Reiner, 1997).

As already mentioned above, IL-6 is involved in the immune response to the intrahepatic stages of malaria (Pied *et al.*, 1992). Elevated levels of IL-6 have been observed in *P. falciparum* infected individuals but the actual role of IL-6, if any, during the erythrocytic stages of infection, is unclear. Hence, IL-6 deficient mice were used to investigate the role of IL-6 in immunity to *P. chabaudi* infection.

Results

The course of *P. chabaudi* infection in IL-6 deficient mice

Inbred IL-6 deficient mice on a 129SVJ background and inbred intact control mice (129SVJ) were infected with either $1x10^5$ pRBCs or $2x10^6$ pRBCs of *P. chabaudi* AS. Each group consisted of six mice and the parasitaemia was observed daily by microscopic examination of Giemsa's stained thin blood smears collected from the tail. IL-6 deficient mice receiving $1x10^5$ pRBCs of *P. chabaudi* consistently had a lower peak parasitaemia compared with control mice (Figure 5.1), although the difference was never statistically significant. A small but significant extension to the primary patent parasitaemia was observed in the IL-6 deficient mice (Figure 5.1). Infection of the IL-6 deficient mice with the higher challenge dose of $2x10^6$ pRBCs of *P. chabaudi* (Figure 5.2), abrogated the suppression of the peak parasitaemia observed following infection with the lower dose of $1x10^5$ pRBCs, but the significant extension of the primary patent parasitaemia remained in the IL-6 deficient mice (Figure 5.2). The appearance of recrudescent parasites in both the IL-6 deficient mice and control mice was inconsistent.

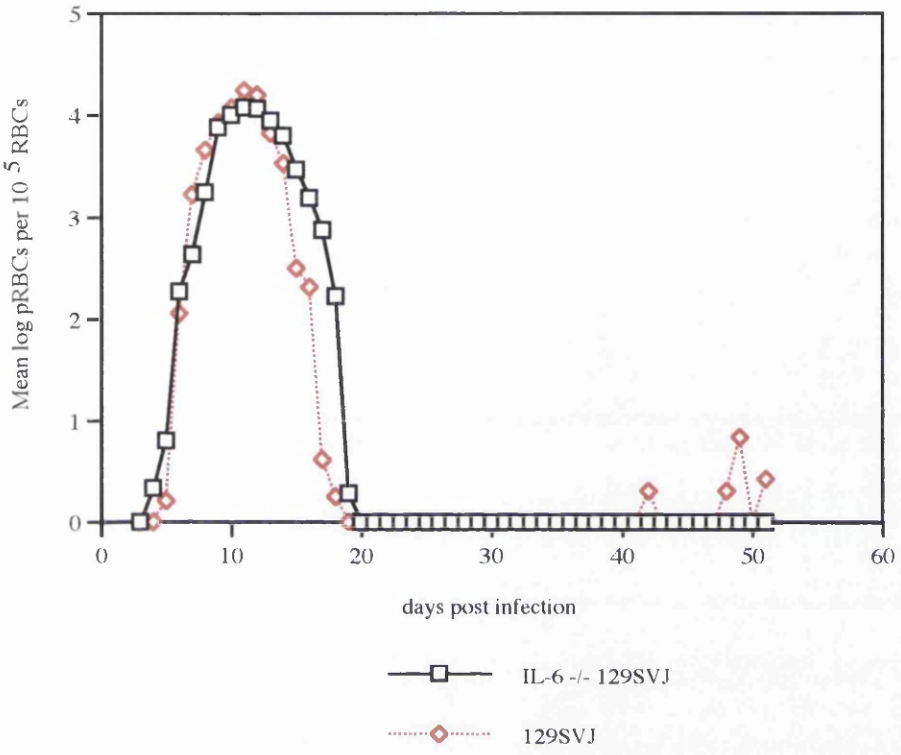


Figure 5.1. The course of infection in IL-6 deficient mice (IL-6 -/- 129SVJ) and control mice (129SVJ) following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. The parasitaemia is the mean of six mice.

Total IgG production in IL-6 deficient mice during *P. chabaudi* infection

Total IgG1 and IgG2a production was determined in the serum of IL-6 deficient mice during *P. chabaudi* infection. Both IL-6 deficient mice and intact control mice received either $1x10^5$ pRBCs or $2x10^6$ pRBCs of *P. chabaudi* AS. Three mice in each group were sacrificed at various time points and serum was collected, pooled and assayed for the production of total IgG1 and IgG2a by the protocol described (see Materials and Methods). Following infection with either $1x10^5$ pRBCs (Figure 5.3) or $2x10^6$ pRBCs (Figure 5.4) of *P. chabaudi*, production of both total IgG1 and IgG2a was delayed and reduced compared to control mice.

The production of parasite-specific IgG in IL-6 deficient mice during *P. chabaudi* infection

Parasite-specific IgG production in the serum of IL-6 deficient mice and control mice was determined following infection with $1x10^5$ pRBCs or $2x10^6$ pRBCs of *P. chabaudi* AS by the indirect fluorescent antibody test (IFAT) described in Materials and Methods. Serum was collected from 3 mice via the tail vein at the time points indicated and assayed for the production of parasite-specific IgG. No significant differences were observed between the two groups in terms of parasite-specific IgG produced in the serum following infection with either $1x10^5$ pRBCs or $2x10^6$ pRBCs of *P. chabaudi* (Tables 1 and 2).

***Ex vivo* analysis of the response of splenocytes taken from IL-6 deficient mice during *P. chabaudi* infection**

IL-6 deficient mice and intact control mice were infected with $1x10^5$ pRBCs of *P. chabaudi* AS. Three mice from each group were sacrificed at the various time points indicated. Splenocytes were harvested and pooled from the three individual mice of

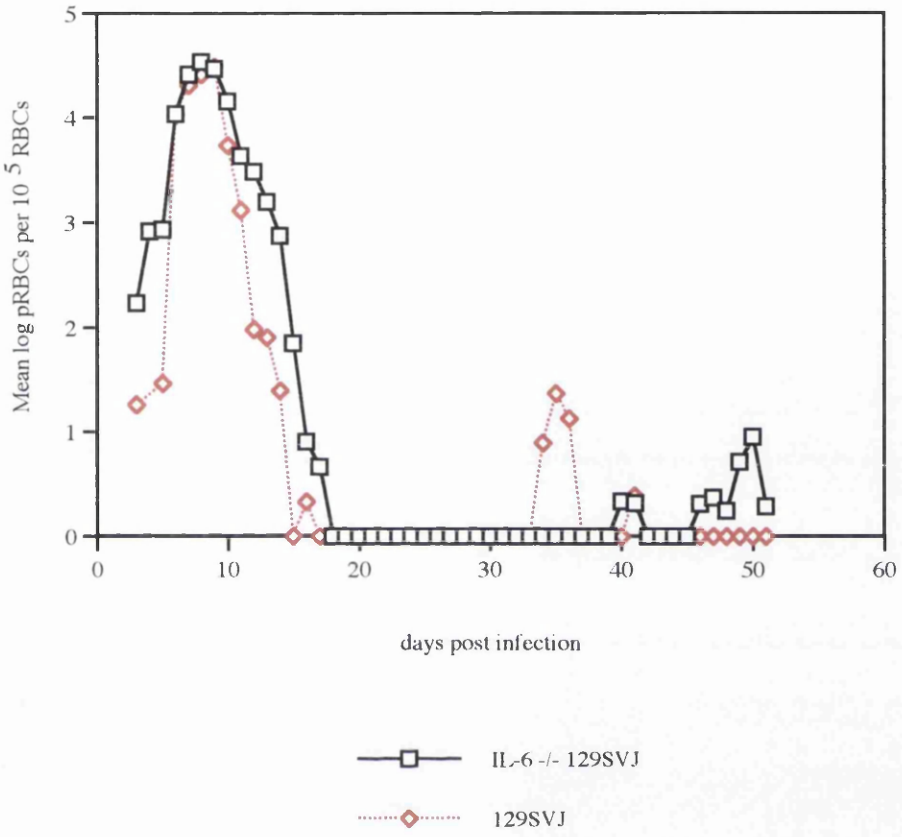


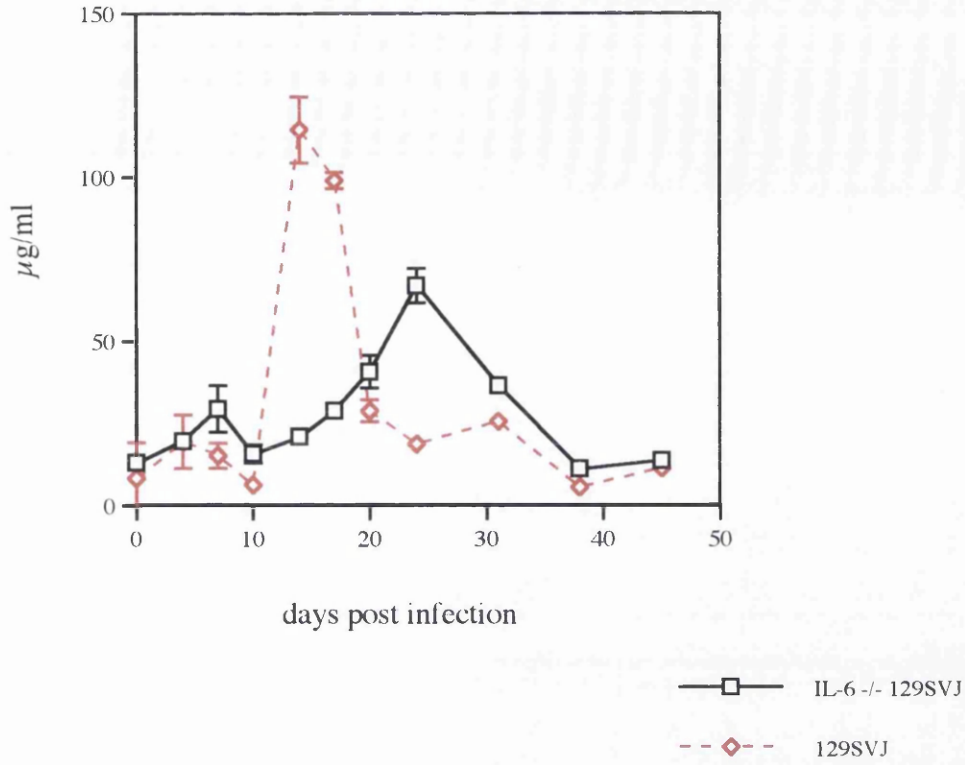
Figure 5.2. The course of infection in IL-6 deficient mice (IL-6 -/- 129SVJ) and control mice (129SVJ) following infection with $2x10^6$ pRBCs of *P. chabaudi*. The parasitaemia is the mean of six mice.

each group *in vitro* at $5x10^6$ cells/ml and stimulated with Concanavalin A (Con A) or LPS (both at 1μg/ml). The proliferative response of the splenocytes was determined by the incorporation of tritiated thymidine. Splenocytes taken from non-infected IL-6 deficient mice and control mice had similar proliferative responses to Con A and LPS stimulation (Figure 5.5). Splenocytes from IL-6 deficient mice at days 4 and 8 post infection had a greater proliferative response to both Con A and LPS compared with splenocytes from control mice (Figure 5.5). On day 15 post infection splenocytes from the control mice had an enhanced proliferative response to Con A compared to splenocytes from IL-6 deficient mice. Both groups displayed a suppressed response to Con A stimulation on day 11 post infection (Figure 5.5). No parasite-specific proliferation was observed for splenocytes from either the IL-6 deficient mice or control mice (data not shown).

Cytological analysis of cells present in the spleen and liver of IL-6 deficient mice during *P. chabaudi* infection

Cytological analysis was performed on leukocytes extracted from the spleen and liver of both IL-6 deficient mice and control mice during *P. chabaudi* infection. IL-6 deficient mice and control mice were infected with $1x10^5$ pRBCs of *P. chabaudi*. Three mice were sacrificed at the time points indicated, the leukocytes were extracted from the liver and spleen of individual mice and then pooled to give leukocytes extracted from either the liver or spleen for each group. Cytological analysis was performed as described in Materials and Methods and the results expressed as number of cells present per spleen or liver. It was observed that there was a delay in reaching peak numbers of lymphoid cells in the spleens of IL-6 deficient mice (Figure 5.6) but the monocyte and polymorphonuclear (PMN) cell numbers were similar in both groups. The data obtained from the liver is incomplete and therefore no definite conclusions can be made but there appears to be a trend of reduced monocyte and

A). Total IgG1



B). IgG2a

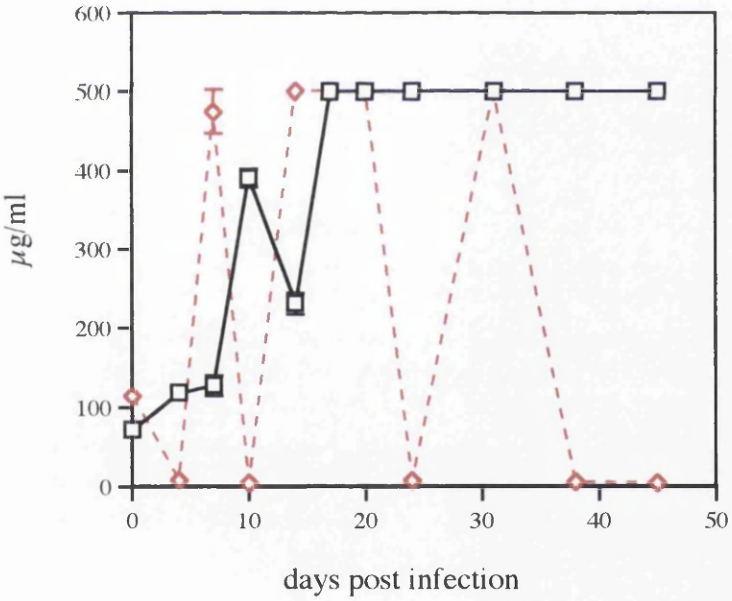


Figure 5.3. Total IgG1, A) and total IgG2a, B) production were determined in the serum of IL-6 deficient mice and control mice following inoculation with $1x10^5$ pRBCS of *P. chabaudi*. Serum from three mice was analysed individually for both groups, in triplicate and the mean ± SD antibody level was calculated.

PMN cells present in the liver during the early phase of infection (Figure 5.6) in the IL-6 deficient mice.

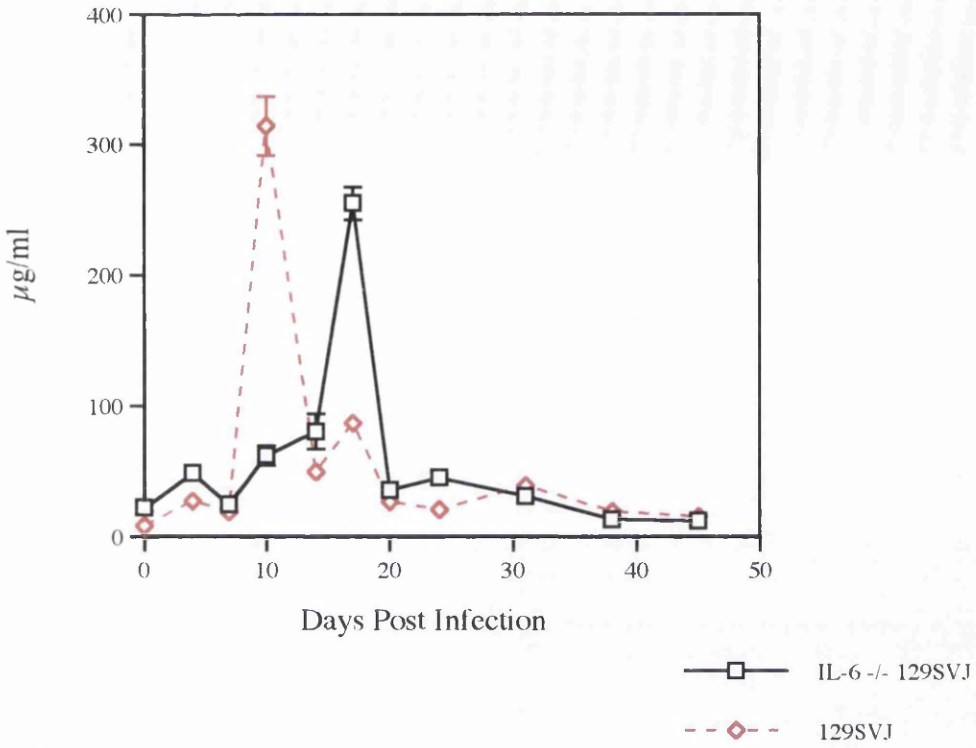
SAP production in IL-6 deficient mice during *P. chabaudi* infection

SAP production was determined in the serum of IL-6 deficient mice and control mice following infection with either $1x10^5$ pRBCs or $2x10^6$ pRBCs of *P. chabaudi*. Serum was collected from three mice via the tail vein at the time points indicated and assayed for SAP production individually. SAP production in the IL-6 deficient mice following infection with either dose of *P. chabaudi* did not rise significantly above background levels (Figure 5.7). In the control mice peak production of SAP coincided with the peak of the primary parasitaemia (Figure 5.7).

Discussion

The role of IL-6 during *P. chabaudi* infection appears to be a minor one. Infection of mice deficient in IL-6 production with $1x10^5$ pRBCs of *P. chabaudi*, consistently resulted in a lower peak parasitaemia when compared with wild type mice. Therefore, this result suggests that IL-6 is not involved in the control of the primary patent parasitaemia but may have a regulatory or negative role to play. The removal of IL-6 production from the repertoire of the immune response creates an imbalance in the cytokine network, resulting in B cell and T cell deficiencies and impaired acute phase protein production (Kopf *et al.*, 1994). The lower peak parasitaemia observed in the IL-6 deficient mice suggests that there is an increase in parasite killing. IL-6 is capable of down-regulating TNF production by monocytes and hence could result in a suppression of inflammatory responses mediated by macrophages in response to malarial antigens. The absence of IL-6 *in vivo* during *P. chabaudi* infection may result in an increase in synthesis of macrophage products which have been linked with parasite killing. The increased proliferative response of splenocytes from IL-6

A). Total IgG1



B). Total IgG2a

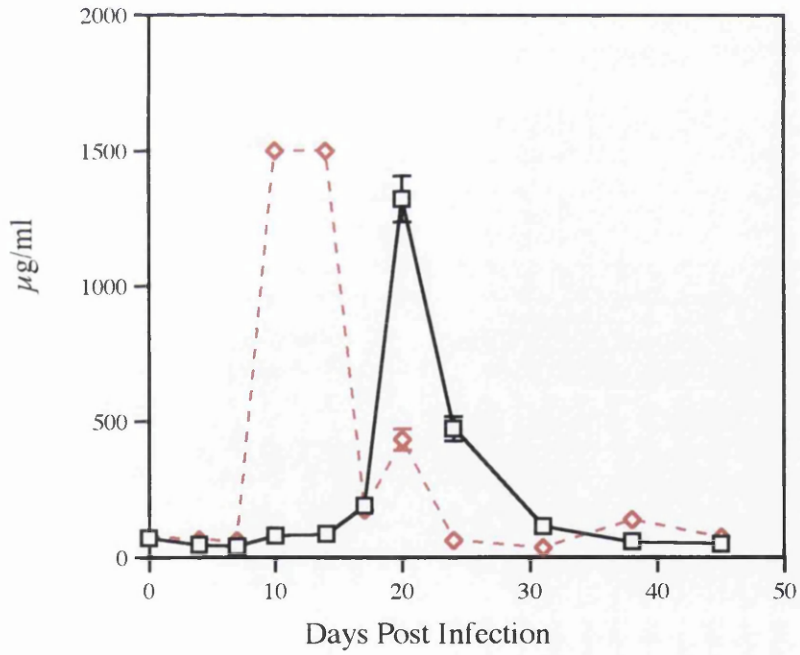


Figure 5.4. Total IgG1, A) and total IgG2a, B) production were determined in the serum of IL-6 deficient mice and control mice following inoculation with $2x10^6$ pRBCS of *P. chabaudi*. Serum from three mice was analysed individually for both groups, in triplicate and the mean ± SD antibody level was calculated.

deficient mice suggests greater T cell activation which may be responsible for the increased stimulation of macrophages to produce inflammatory products such as TNF, NO and IL-12 which have been implicated in the control of the primary patent parasitaemia (Stevenson *et al.,* 1995). The IL-6 deficient mice do not suffer from endotoxic shock, unlike IL-10 deficient mice which succumb to overproduction of inflammatory mediators following *P. chabaudi* infection (Linke *et al.,* 1995). Hence IL-6 is not the main down-regulator of the macrophage mediated inflammatory response, indeed evidence suggests that IL-10 may fulfil this role (Moore *et al.,* 1993). Depletion of macrophages during *P. chabaudi* infection results in an exacerbation of the primary peak parasitaemia (see Chapter 3). This illustrates that the macrophage has an important function in the control of the peak parasitaemia confirming that down-regulation of macrophage mediated inflammatory responses will result in an increase in the number of parasites at peak parasitaemia. Therefore it is feasible to suggest that the removal of the negative feedback mechanism mediated by IL-6 may result in an increase in the production of inflammatory products like TNF, IL-1 and NO by macrophages, leading to an increase in parasite killing. However the increase in parasite killing does not significantly reduce the peak of the primary parasitaemia which implies that the importance of the proposed negative feedback mechanism mediated by IL-6 is minor and probably secondary to IL-10 mediated suppression of inflammatory responses.

Another possible explanation of the lower peak parasitaemia observed in the IL-6 deficient mice could be related to the ability of IL-6 to alter body temperature (Kwiatkowski, 1995). Fever is a well documented symptom of a malarial infection. It has been suggested that although fever is distressing to the infected individual, the change in body temperature may interfere with parasite development because malarial parasites have optimal temperatures for growth (Kwiatkowski, 1989). During malaria infection in mice, the body temperature actually decreases and it is unclear how this process actually occurs. Fever in humans has been correlated with schizont rupture and

Days post infection	Group	
	IL-6 -/- 129SVJ	129SVJ
0	-	-
4	-	-
7	-	-
10	100	100
14	500	500
17	1000	1000
20	1000	1000
24	1000	1000
31	1000	1000
38	1000	1000
45	1000	1000

Table 1. Parasite-specific IgG production in IL-6 deficient mice and control mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Serum from three mice was pooled and the level of parasite-specific IgG was determined by indirect fluorescence. Results are the mean of three samples and are expressed as the reciprocal of antibody titre.

Days post infection	Group	
	IL-6 -/- 129SVJ	129SVJ
0	-	-
4	-	-
7	-	-
10	-	500
14	100	500
17	1000	500
20	500	500
24	1000	1000
31	500	500
38	1000	500
45	1000	1000

Table 2. Parasite-specific IgG production in IL-6 deficient mice and control mice following inoculation with $2x10^6$ pRBCs of *P. chabaudi*. Serum from three mice was pooled and the level of parasite-specific IgG was determined by indirect fluorescence. Results are the mean of three samples and are expressed as the reciprocal of antibody titre.

Note: Range of samples analysed was 1:50 - 1:1000.

the production of endogenous pyrogens, TNF, IL-1, and IL-6 (Kwiatkowski, 1995). It is possible that the removal of IL-6 production during *P. chabaudi* infection results in the temperature of IL-6 deficient mice being different to that of the normal intact control mice. This may mean that the body temperature of the IL-6 deficient mice is not optimal for the growth of the parasite and results in a slightly decreased peak parasitaemia.

Immunity to *P. chabaudi* in mice is regarded as a sequential Th1/Th2 mediated response. Following a challenge with $1x10^5$ pRBCs, IL-6 deficient mice had a slower rate of parasite clearance when compared with controls. To analyse if this slower clearance of the patent parasitaemia could be attributed to a delay in the switch from Th1 to Th2 T cell mediated responses, the mice were challenged with $1x10^5$ and $2x10^6$ pRBCs. It was possible that the IL-6 deficient mice receiving the $1x10^5$ pRBCs, had a reduced parasite load at peak parasitaemia compared with controls. Hence, there was less stimulation of effector mechanisms which would result in a slower clearance of parasites. This theory was shown to be incorrect because the IL-6 deficient mice, when challenged with $2x10^6$ pRBCs had approximately the same peak parasitaemia as control mice. Therefore the antigen load and stimulation of the effector mechanisms involved in the clearance of parasites was the same in both groups of mice but the IL-6 deficient mice still had a slower rate of clearance of the primary patent parasitaemia.

Increasing the challenge of inoculation appears to have abrogated the reduction in the peak parasitaemia observed in the IL-6 deficient mice. The beneficial effect during the peak parasitaemia attributed to the absence of IL-6 production may be lost due to the increased growth rate of the parasite and a greater antigen load. The resultant increase in the production of inflammatory products may stimulate compensatory, down-regulating mechanisms, hence reducing the rate of parasite killing. The increase in the parasite challenge and consequently parasite growth rate, overrides the mechanism which results in a reduction in the peak parasitaemia when IL-6 production is absent.

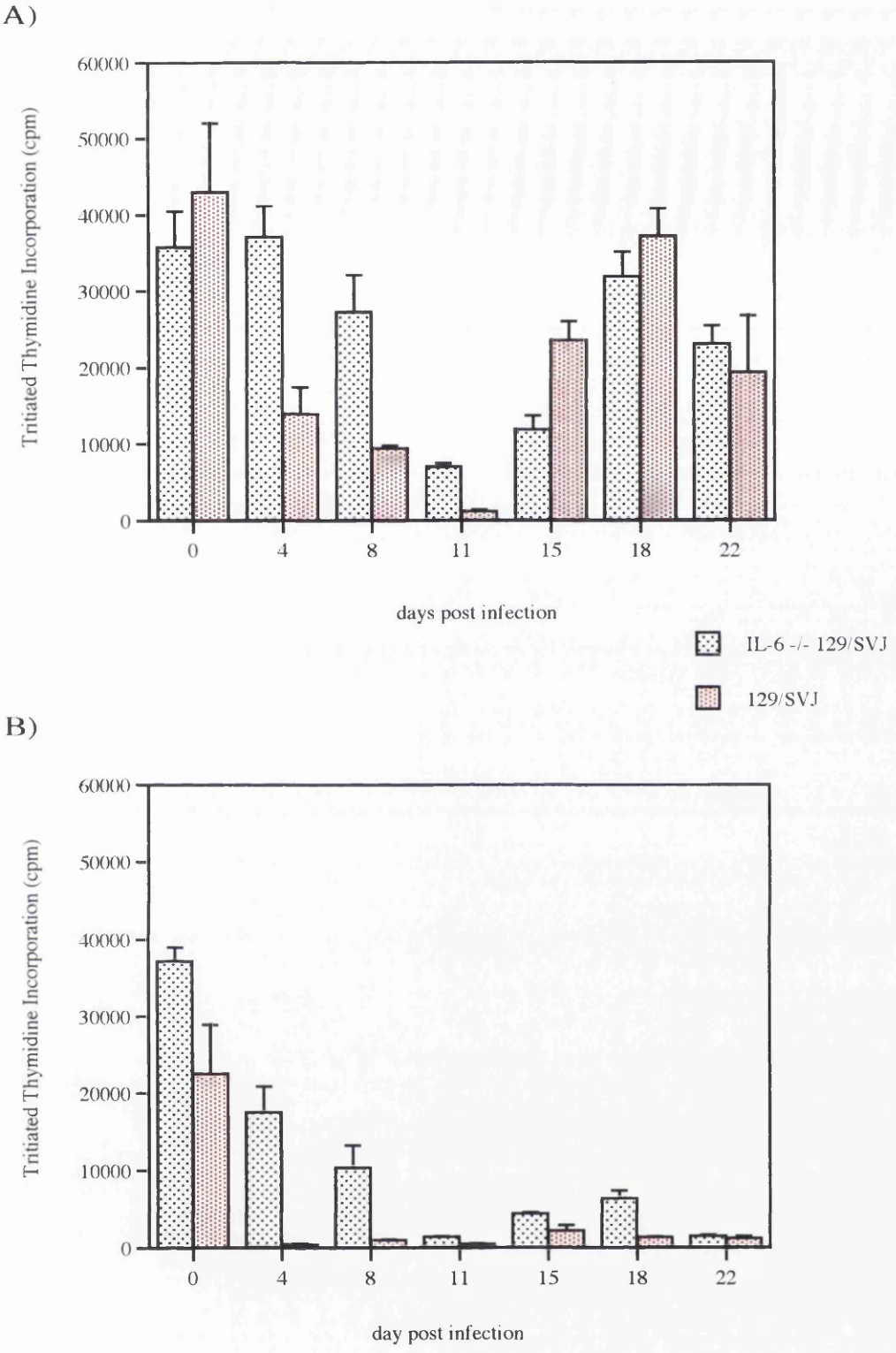


Figure 5.5. The proliferative response of splenocytes from IL-6 deficient mice and control mice harvested following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from both groups were sacrificed and the splenocytes were pooled within each group and stimulated with either A) Concanavalin A or B) LPS. Each data point is the mean ± SD for an experiment performed in triplicate.

The extension of the primary parasitaemia observed in the IL-6 deficient mice is associated with a delay and reduction of total IgG1 and IgG2a and an absence of acute phase protein production. The role of IL-6 in the maturation of B cells is well established and a deficiency in this process may explain the slower rate of parasite clearance as both IgG1 and IgG2a have been shown to be involved in immunity during a malarial infection (Troye-Blomberg, Berzins and Perlmann, 1994, Akanmori, Waki and Suzuki, 1994). However, there appears to be no significant reduction in the levels of parasite specific IgG produced in the IL-6 deficient mice during *P. chabaudi* infection although this data is subjective and requires further confirmation through the production of parasite-specific Ig isotypes. There is a reduced proliferative response of splenocytes taken from IL-6 deficient mice at a time which coincides with the extension of the primary parasitaemia. It is possible that the reduced activation of T cells at this time may result in a reduction of the $CD4^+$ Th2 T cell mediated assistance required to stimulate the production of the appropriate humoral response. Further investigation into the cytokine profiles of T cells at this time of infection would elucidate any deficiency in this process.

Administration of recombinant IL-6 to mice has been shown to boost anti-plasmodial IgG subtype production and suppress the secondary phase of *P. chabaudi* infection (Akanmori, Kawai and Suzuki, 1996). However, the absence of IL-6 does not conversely exacerbate the parasitaemia of *P. chabaudi* infection or reduce the production of parasite-specific IgG. Akanmori and colleagues (1996) proposed that IL-6 mediates the protective humoral response mounted during the secondary phase of *P. chabaudi* infection but in the studies performed here, the influence of IL-6, albeit a minor one, appears to be during the acute phase of infection. Inconsistent observations concerning the appearance of recrudescent parasites prevents any conclusions being made about the effect IL-6 absence has on the secondary phase of *P. chabaudi* infection.

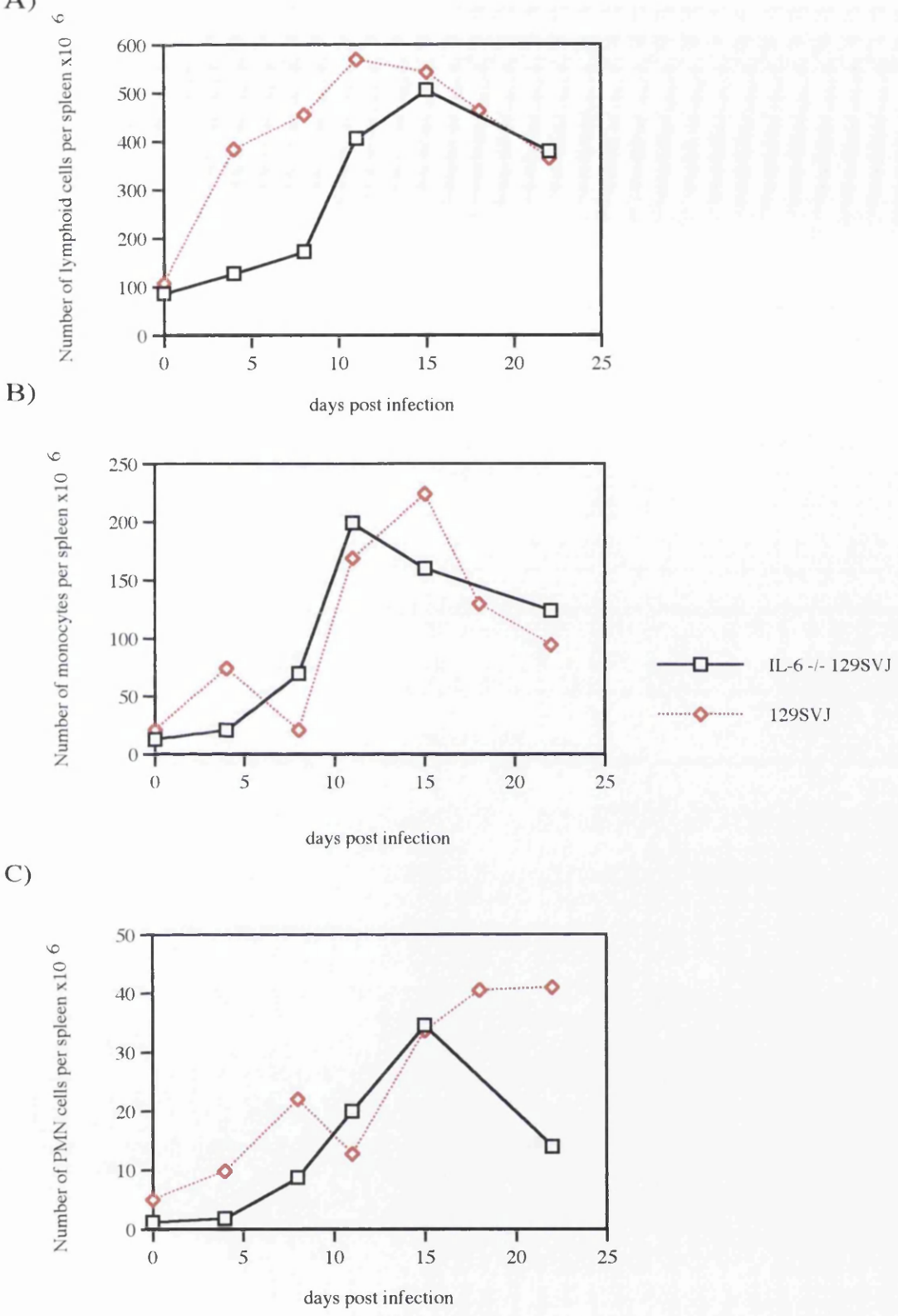


Figure 5.6. Cytological analysis of cells present in the spleen of IL-6 deficient mice and control mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at each timepoint and the cells harvested were pooled and centrifuged. 500 cells were counted on each Geimsa's stained cytospin smear. Each data point is calculated from one smear and the number of A) lymphoid, B) monocyte and C) polymorphonuclear (PMN) cells, are expressed per spleen.

Acute phase protein production, indicated by the measurement of SAP, was impaired during *P. chabaudi* infection in the IL-6 deficient mice. This demonstrates that the stimulation of the production of acute phase reactants is mainly via IL-6. Malaria infection cannot override this deficiency, nor can it stimulate the production of these inflammatory products either directly or through a compensatory mechanism. An interesting point is that the peak of SAP production in the control mice occurs just after the clearance of the primary parasitaemia. The possibility exists that the absence of the production of SAP at this time in the IL-6 deficient mice may contribute to the decreased rate of parasite clearance. This could be through a direct inhibition of the growth of the parasite, as SAP is known to bind to erythrocytes, the host cell for *Plasmodium* parasites during the asexual cycle in mammals. The other option is that SAP production may be an important stimulatory step in the process leading to effective parasite clearance (see Chapter 4).

Further work is required on this experimental model to determine the compensatory mechanisms which result from the absence of IL-6 production. An imbalance in the cytokine network is created but it is unclear if there is a cytokine which can replace the functions of IL-6 or if overproduction of several cytokines compensates for the IL-6 deficiency. Analysis of the response of splenocytes during the peak parasitaemia and the remission of the primary patent parasitaemia to specific and non-specific antigens may reveal why the deficiency in the cytokine network results in a lower peak parasitaemia and reduced rate of parasite clearance observed in the IL-6 deficient mice.

IL-6 is a pleiotropic cytokine involved in many different areas of the immune response, bridging specific and non-specific protective mechanisms. It is possible that the diverse functions of IL-6 may contribute to the minor detrimental effect observed in IL-6 deficient mice during *P. chabaudi* infection. Efficient compensatory mechanisms are probably already functional in an immunocompetent host but in fact

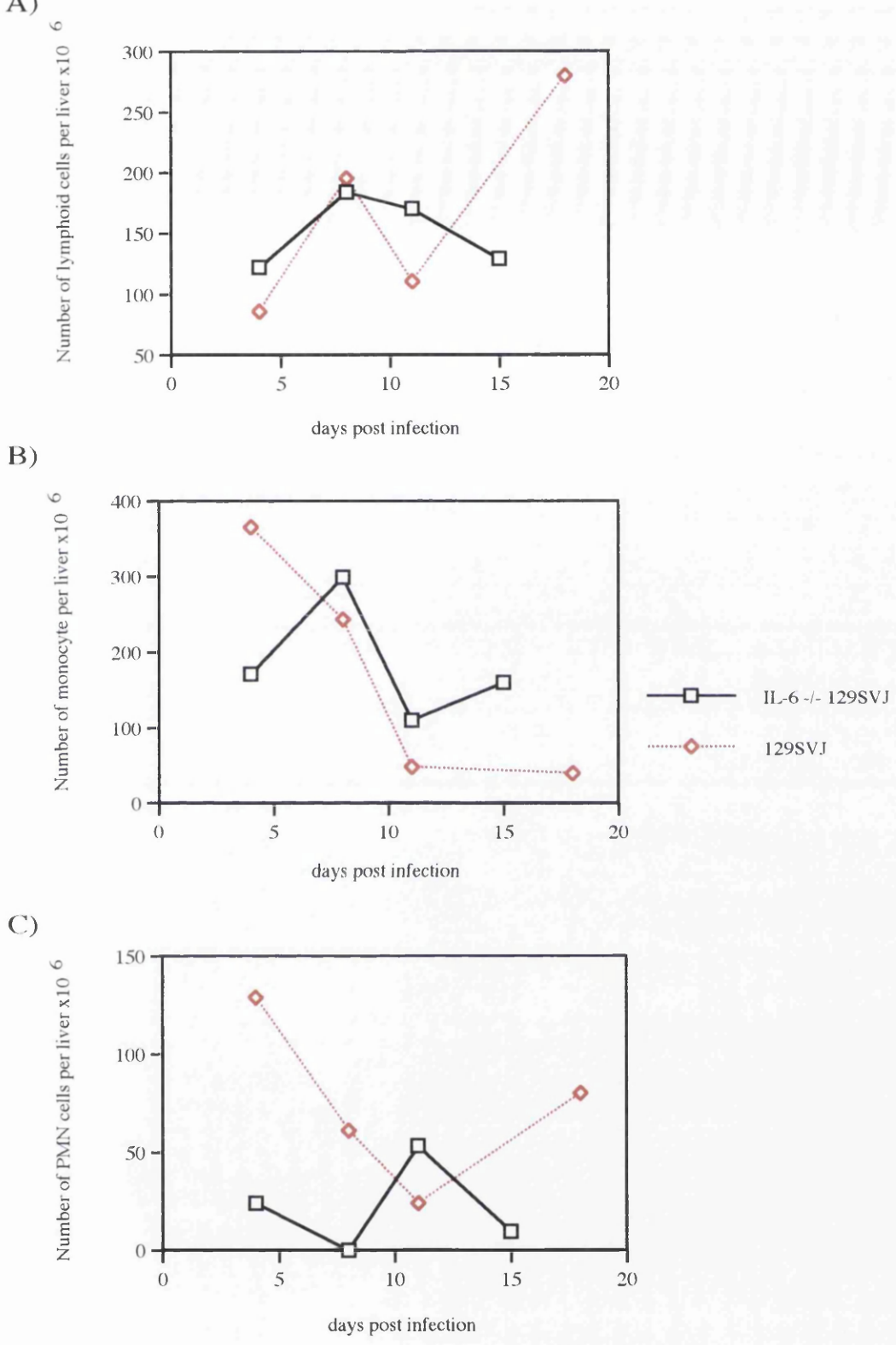
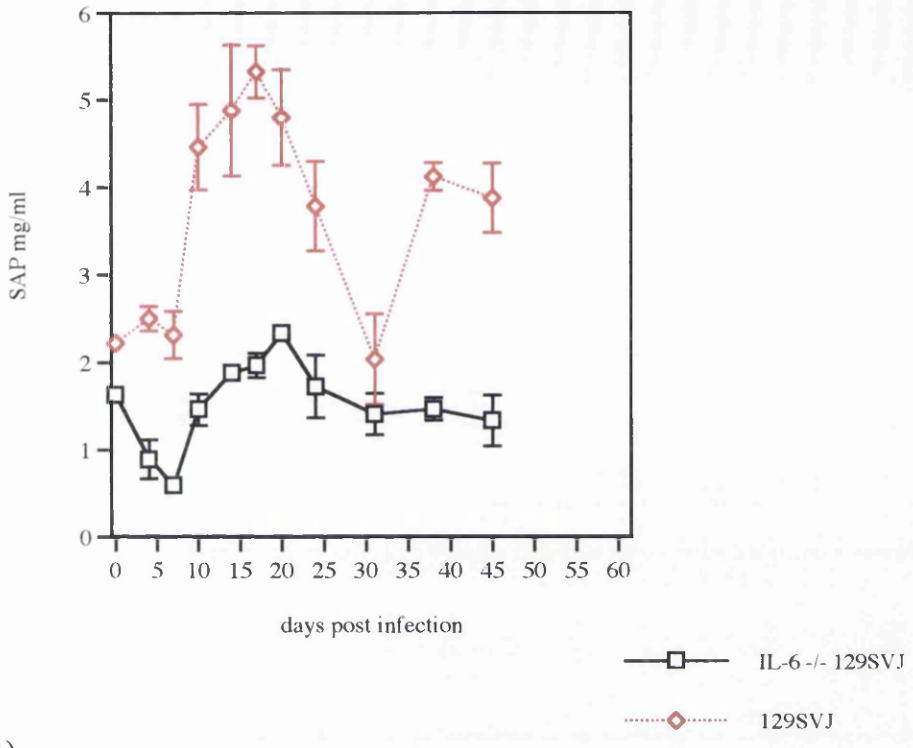


Figure 5.7. Cytological analysis of cells present in the liver of IL-6 deficient mice and control mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at each timepoint and the cells harvested were pooled and centrifuged. 500 cells were counted on each Geimsa's stained cytospin smear. Each data point is calculated from one smear and the number of A) lymphoid, B) monocyte and C) polymorphonuclear (PMN) cells, are expressed per liver.

synergise with IL-6 to stimulate the required response under normal conditions. Therefore, the absence of IL-6 would result in an over-expression of the synergistic mechanisms which may explain the minor role for IL-6 observed in the experiments performed here. IL-6 deficient mice can mount efficient immunity to *L. major* infection (Moskowitz, Brown and Reiner, 1997) even though the macrophage, a major source of IL-6, has a pivotal role during leishmaniasis. However, the absence of IL-6 results in increased susceptibility to *L. monocytogenes* (Kopf *et al.,* 1994) and *E. coli* infection (Dalrymple *et al.,* 1996). The determining factor in the outcome of different infections in IL-6 deficient mice will be the influence IL-6 exerts on the definitive microbicidal mechanism. NO production is known to be the effector molecule of anti-parasitic activity during leishmaniasis (Liew *et al.*, 1990), whereas, the identity of the microbicidal mechanism during malaria infection remains elusive. However, it is clear that the process is complex and involves innate and acquired immune responses. Hence, the outcome of a malaria infection may not be significantly altered by the loss of IL-6 production.

A)



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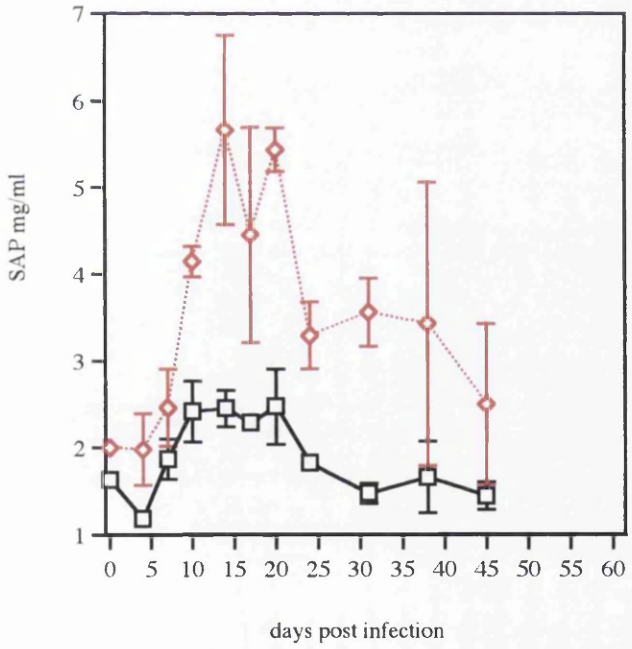


Figure 5.8. The production of SAP in IL-6 deficient mice and control mice following infection with either A) $1x10^5$ pRBCS or B) $2x10^6$ pRBCs of *P. chabaudi*. Serum from three mice in each group was analysed for SAP levels in triplicate and the mean ± SD was calculated.

Chapter Six

The course of *P. chabaudi* infection in IL-4 deficient mice

Introduction

IL-4 is a 19 kDa glycoprotein, which has pleiotropic effects on various components of the immune response. The gene for IL-4 is found on chromosome 5 (human) or chromosome 11 (mouse) and is part of a cytokine complex which includes genes for IL-3, IL-5 and GM-CSF (Paul, 1991). IL-4 can influence several types of cells including B cells, T cells, monocyte/macrophages and mast cells and mediates its function by binding to a receptor expressed on the target cell. The receptor is a 140 kDa transmembrane protein (Mosley *et al.*, 1989, Idzerda *et al.*, 1990) which is known to activate at least two distinctive pathways following interaction with IL-4 (Kaplan *et al.*, 1996). One involves the activation of Stat 6 (signal transducer and activator of transcription 6), which appears to be the critical pathway for mediating IL-4 induced responses in lymphocytes (Kaplan *et al.*, 1996). The second pathway involves the phosphorylation of an insulin receptor substrate termed 4 PS which associates with phosphatidylinositol 3-kinase (Keegan *et al.*, 1994).

IL-4, originally identified by its ability to induce B cell differentiation (Howard *et al.*, 1982) is essential for Ig class switching to IgG1 production (Snapper and Paul, 1987a and 1987b) and is essential for the induction of IgE production (Snapper, Finkleman and Paul, 1988). Transgenic mice, which overexpress IL-4, have elevated levels of both IgE and IgG1 (Tepper *et al.*, 1990). IL-4 stimulation of B cells results in increased expression of MHC class II (Noelle *et al.*, 1984), IL-4 receptor (Ohara and Paul, 1988) and CD23, which is a low affinity IgE receptor (Conrad *et al.*, 1987). The influence of IL-4 extends beyond B cell functions. Increased expression of Ia antigen and MHC class I and II induced by IL-4 stimulation, can result in the enhancement of the ability of macrophages to present antigen (Zlotnik *et al.*, 1987, Stuart *et al.*, 1988). IL-4 has been shown to enhance the proliferation of precursors of cytotoxic T cells and differentiation into CTL (Widner and Grabstein, 1987, Trenn *et al.*, 1988) and it has been proposed

that IL-4 may have a role in the development of the T cell repertoire within the thymus (Tepper *et al.*, 1990).

One of the principal functions of IL-4 is the polarisation of the $CD4^+$ T helper subsets. Th1 $CD4^+$ T cells secrete IFNγ and TNFβ whereas Th2 $CD4^+$ T cells produce IL-4, IL-5 and IL-10 (Mosmann *et al.*, 1986). The different subsets have been the focus of extensive research into their involvement in various mechanisms of the immune system and host defence responses. However, evidence is emerging that there is not such a clear definition of Th1 and Th2 subsets, with some T cells secreting both sets of cytokines (Romagnani, 1996). The microenvironment can influence the polarisation of the $CD4^+$ T cell response, with the presence of IL-4 early in the development being a potent stimulus for Th2 differentiation. Potential sources of rapid IL-4 production include a subset of $CD4^+$ NK 1.1 cells or naive Th $CD4^+$ T cells (Romagnani, 1997). As the activation of T cells increases, the production of IL-4 by naive Th cells would increase and reach a threshold level, polarising the differentiation of $CD4^+$ T cells to the Th2 phenotype because IL-4 is capable of dominating the effects of the other cytokines produced by naive Th cells (Romagnani, 1997).

The polarisation of the $CD4^+$ Th subsets has been extensively investigated during infectious diseases, with IFNγ and IL-4 reflecting the involvement of Th1 and Th2 mediated responses respectively. Hence, the role of IL-4 (and Th2 mediated responses) during infectious diseases has been analysed by anti-IL-4 treatment, addition of recombinant IL-4, detection of mRNA expression or protein secretion and more recently by the generation of IL-4 gene deficient mice.

Susceptibility to *L. major* infection in mice is associated with IL-4 production rather than IFNγ (Reiner and Locksley, 1995). Depletion of IL-4 in susceptible mice by administration of anti-IL-4 antibody results in resistance to *L. major* (Sadick *et al.*, 1990). In contrast, IL-4 mediates a protective response to the gastrointestinal nematodes

Heligmosomoides polygyrus (Urban *et al.*, 1991) and *Trichuris muris* (Else *et al.*, 1994). Mouse strains which produce Th2 associated cytokines including IL-4 are resistant to *T. muris* infection whereas mice which produce Th1 cytokines are susceptible (Else and Grencis, 1991, Else *et al.*, 1994). Treatment of susceptible mice with IL-4, induces Th2 mediated responses, preventing the establishment of a chronic *T. muris* infection and can also cure an established *T. muris* infection (Else *et al.*, 1994). Furthermore, IL-4 treatment of immunodeficient mice can cure established *Nippostrongylus brazliensis* infection (Urban *et al.*, 1995).

The generation of IL-4 deficient mice has confirmed the results of studies where the role of IL-4 was examined previously by depletion of IL-4 by antibodies or addition of exogenous IL-4. The early production of IL-4 in Balb/c mice predisposes a susceptible phenotype to *L. major* infection and hence IL-4 deficient mice upon infection with *L. major* were found to be resistant with reduced Th2 responses (Kopf *et al.*, 1996). Infection of IL-4 deficient mice with *L. mexicana* demonstrated that IL-4 is involved in down-regulating a protective Th1 response as observed during *L. major* infection (Satoskar, Bluethmann and Alexander, 1995). IL-4 deficient mice are more susceptible than control mice during the initial phase of a *T. gondii* infection, where there is a rapid proliferation of tachyzoites, but have decreased pathology associated with chronic infection (Roberts *et al.*, 1996). Hence, IL-4 appears to have a protective role during the initial phase of a *T. gondii* infection, probably through down-regulation of inflammatory responses but the long-term effects of IL-4 are detrimental on the outcome of the development of protective mechanisms to bradyzoites, which encyst in tissues and initiate the chronic phase of infection. Immunocompetent mice can mount a protective immune response to a secondary *H. polygyrus* infection following drug cure of a primary infection (Urban *et al.*, 1992), unlike IL-4 deficient mice which are unable to reduce fecundity and expel adult worms during a secondary infection (Kopf *et al.*, 1995).

IL-4 and IL-5 are produced during *Schistosoma mansoni* infection in mice but only after an initial IFNγ mediated Th1 response (Paul, 1991). The sequential involvement of Th1/ Th2 mediated responses is also apparent in experimental malaria infection of mice with *P. chabaudi*. During a primary infection with *P. chabaudi*, Th1 and Th2 cells appear sequentially during the course of infection (Langhorne *et al.*, 1989) and $CD4^+$ T cell clones isolated from mice at day 16 post infection and after clearance of a secondary *P. chabaudi* infection, which were of a Th1 and Th2 subset respectively, were able to confer protection to an homologous challenge (Taylor-Robinson and Phillips, 1994b). These results imply that control of the acute phase of *P. chabaudi* infection is mediated by Th1 responses, essentially antibody-independent mechanisms and that this is superceded by antibody-dependent Th2 mediated response which is important for parasite elimination. Early production of IL-4 correlates with susceptibility to a *P. chabaudi* infection (Jacobs, Radzioch and Stevenson, 1995), plus IL-4 appears to have no role in the protective response of mice to *P. vinckei vinckei* infection (Perlmann *et al.*, 1995). Furthermore, IL-4 does not appear to be involved in anti-sporozoite immunity (White, Jarboe and Krzych, 1994) even though antibody-dependent mechanisms are thought to participate in a protective response (Nussenzweig *et al.*, 1967). Hence, IL-4 deficient mice have been used to examine the role of IL-4 and consequently Th2 mediated responses during experimental blood-stage malaria infection (von der Weid *et al.*, 1994b, van der Heyde *et al.*, 1997). Th2 associated cytokines (IL-5, IL-9 and IL-10) are reduced in the IL-4 deficient mice following *N. braziliensis* infection (Kopf *et al.*, 1993) and IgE responses to the same nematode infection are also absent (Kuhn, Rajewsky and Muller, 1991).

IL-4 deficient mice have been used to investigate the role of IL-4 and IL-4 driven Th2 responses to experimental malaria infection. The outcome of the previous investigations using IL-4 deficient mice infected with either *P. chabaudi chabaudi AS*, *P. chabaudi adami* or *P. yoelii* was that the absence of IL-4 has no significant exacerbating effect on the course of infection (von der Weid *et al.*, 1994b, van der Heyde *et al.*, 1997). In the

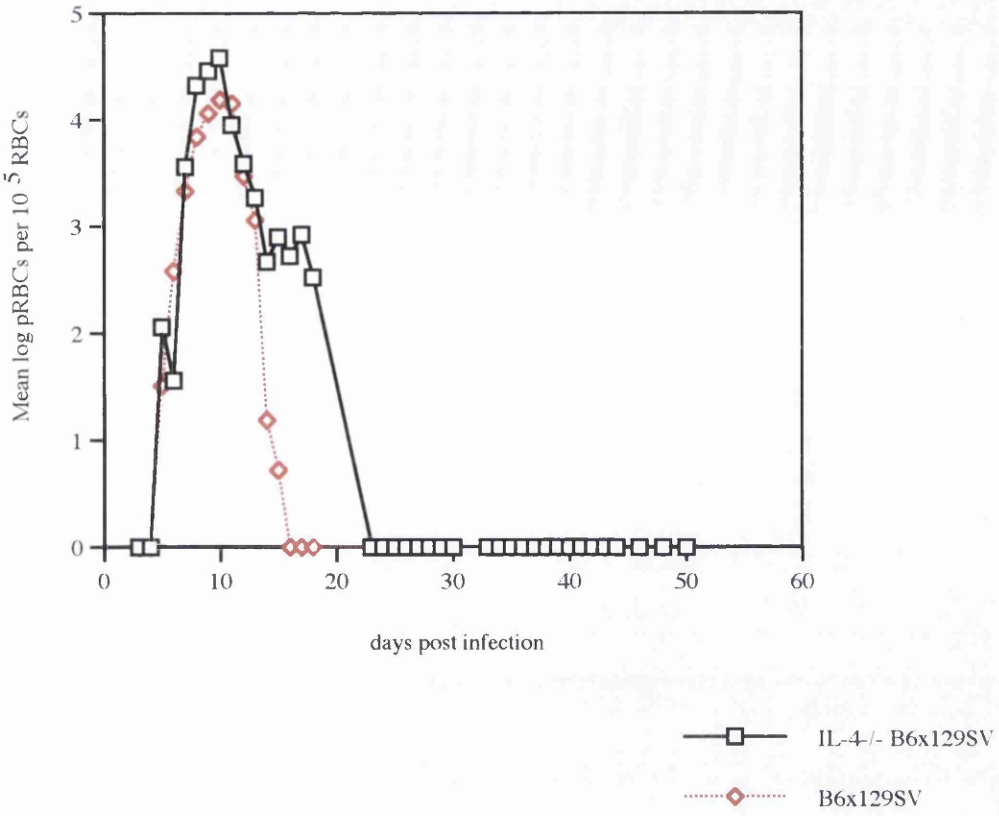
studies described here infection of IL-4 deficient mice on three different backgrounds, one outbred (B6x129) and two inbred (129SV and Balb/c) strains, with *P. chabaudi chabaudi* AS was performed in order to dissect the roles of Th1 and Th2 mediated responses and complement the investigation of the outcome of malaria infection in mice lacking the receptor for the Th1 associated cytokine, IFNγ (see Chapter 7).

Results

The course of *P. chabaudi* infection in IL-4 deficient mice

IL-4 deficient mice and corresponding intact control mice were infected with $1x10^5$ pRBCs of *P. chabaudi* AS. Mice of three different backgrounds with the disruption of the IL-4 gene were used, one outbred (B6x129SV) and two inbred strains (129SV and Balb/c). Each group consisted of six mice and the parasitaemia was observed daily by microscopic examination of Giemsa's stained thin blood smears collected from the tail. *P. chabaudi* infection of IL-4 deficient B6x129 mice resulted in a significant exacerbation at peak parasitaemia [$p<0.04$ at day 10 post infection] (Figure 6.1). The same result was observed following *P. chabaudi* infection of IL-4 deficient 129SV [$p<0.002$ at day 12 post infection] and Balb/c mice [$p<0.0004$ at day 9 post infection] (Figures 6.2 and 6.3 respectively). Clearance of the primary patent parasitaemia occurred at a similar rate in the IL-4 deficient mice (all three backgrounds) and the control mice. Recrudescent parasites appeared only in the IL-4 deficient 129SV mice (Figure 6.2). No data was obtained on the recrudescence parasitaemia for IL-4 deficient Balb/c mice because the course of infection was followed only for 20 days.

A)



B)

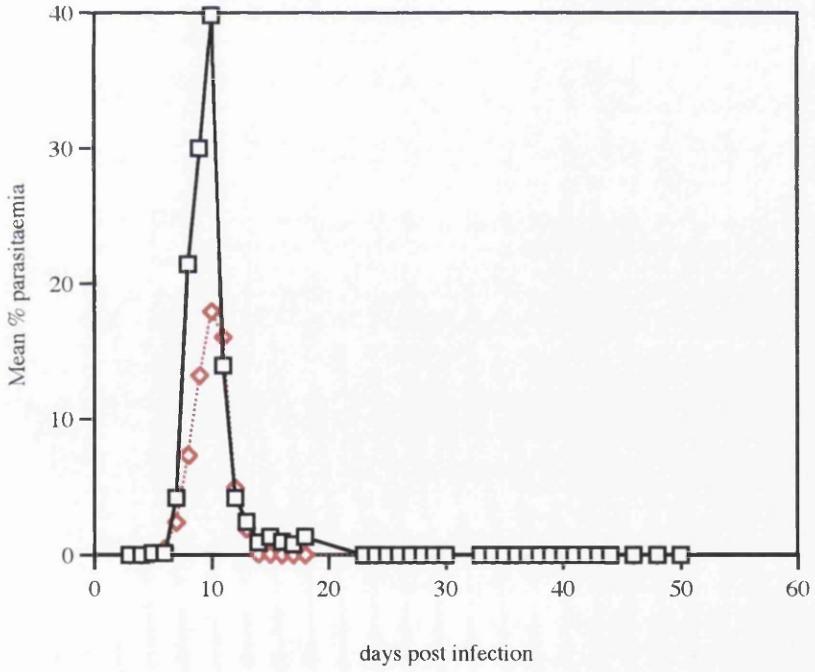


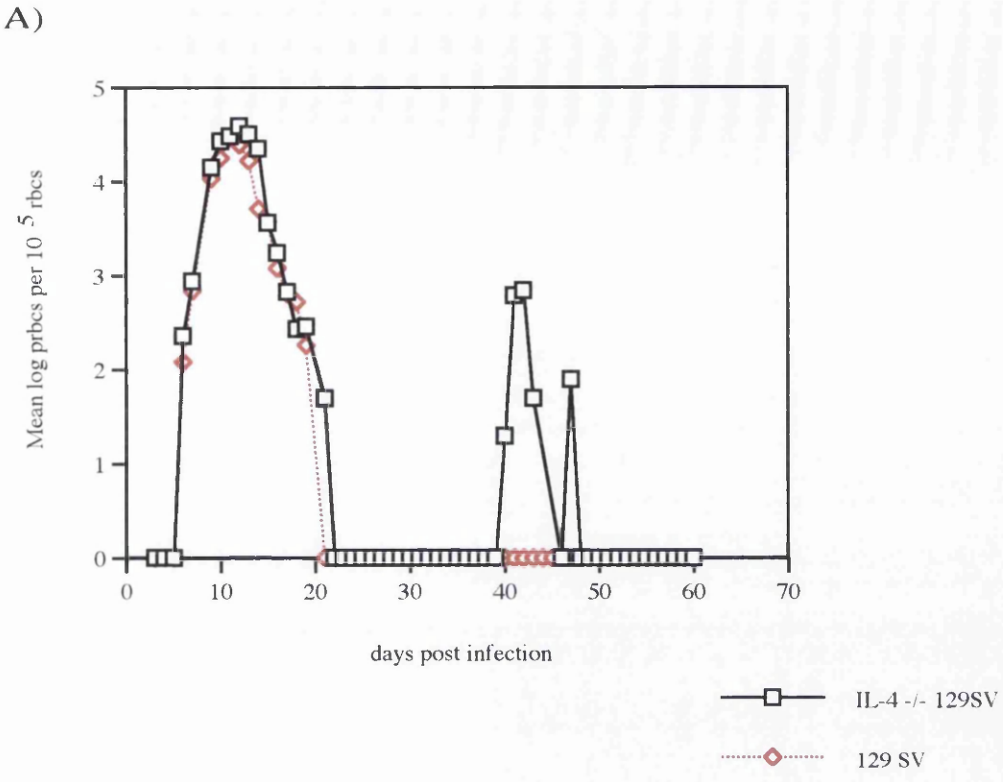
Figure 6.1. The course of infection in outbred IL-4 deficient mice (IL-4 -/- B6x129SV) and control mice (B6x129SV) following infection with $1x10^5$ pRBCs of *P. chabaudi*. The mean log parasitaemia, A) and percentage parasitaemia B), of six mice are presented.

Total IgG and IgE production in IL-4 deficient mice during *P. chabaudi* infection

Total IgG1, IgG2a and IgE production was determined in the serum of IL-4 deficient mice during *P. chabaudi* infection. IL-4 deficient 129SV mice and control 129SV mice received $1x10^5$ pRBCs of *P. chabaudi.* Three mice in each group were sacrificed at various time points and serum was collected, pooled and assayed for the production of total IgG1, IgG2a or IgE by the protocol described (see Materials and Methods). Total IgG1 was reduced in IL-4 deficient 129SV mice compared with control 129SV mice during the early phase of the primary parasitaemia (Figure 6.4). The level of total IgG2a production in the IL-4 129SV deficient mice was similar to that in the serum of control 129SV mice (Figure 6.4). Neither IL-4 deficient 129SV mice nor control 129SV mice produced significant levels of total IgE (Figure 6.4).

The production of parasite-specific IgG in IL-4 deficient mice during *P. chabaudi* infection

Parasite-specific IgG production in the serum of IL-4 deficient 129SV mice and control 129SV mice was determined following infection with $1x10^5$ pRBCs of *P. chabaudi* AS by the indirect fluorescent antibody test (IFAT) described in Materials and Methods. Three mice from each group were sacrificed at the time points indicated and serum was collected, pooled and assayed for the production of parasite-specific IgG. No significant differences were observed between the two groups in terms of parasite-specific IgG produced in the serum during *P. chabaudi* infection (Table 3).



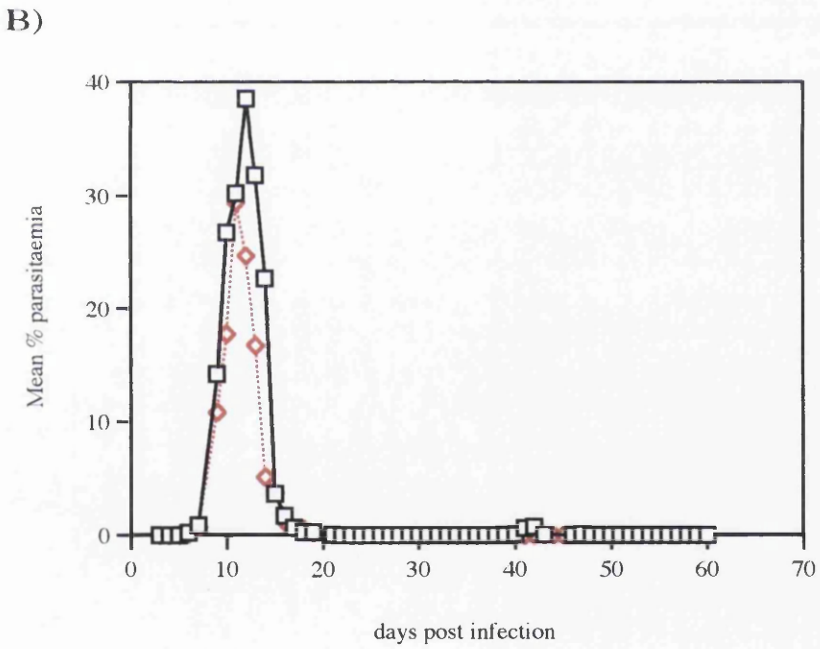


Figure 6.2. The course of infection in inbred IL-4 deficient mice (IL-4 -/- 129SV) and control mice (129SV) following infection with $1x10^5$ pRBCs of *P. chabaudi*. The mean log parasitaemia A), and percentage parasitaemia B), of six mice are presented.

Ex vivo analysis of the response of splenocytes taken from IL-4 deficient mice during *P. chabaudi* infection

IL-4 deficient 129SV mice and intact control 129SV mice were infected with $1x10^5$ pRBCs of *P. chabaudi* AS. Three mice from each group were sacrificed at the various time points indicated. Splenocytes were harvested and pooled from the three individual mice of each group, cultured *in vitro* at $5x10^6$ cells/ml and stimulated with Con A (1μg/ml). The proliferative response of the splenocytes was determined by the incorporation of tritiated thymidine. Splenocytes from non-infected IL-4 deficient 129SV mice and control 129SV mice had similar proliferative responses to Con A stimulation (Figure 6.5). Splenocytes from both groups had similar responses to Con A stimulation during the course of *P. chabaudi* infection except for day 6 post infection where splenocytes from IL-4 deficient 129SV mice had a reduced proliferative response [$p<0.002$] (Figure 6.5).

Cytological analysis of cells present in the spleen and liver of IL-4 deficient mice during *P. chabaudi* infection

Cytological analysis was performed on leukocytes extracted from the spleen and liver of both IL-4 deficient 129SV mice and control 129SV mice following infection with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at the time points indicated, the leukocytes were extracted from the liver and spleen of individual mice and then pooled to give leukocytes extracted from either the liver or spleen for each group. Cytological analysis was performed as described in Materials and Methods and the results expressed as the number of cells present per spleen or liver. A small reduction in the numbers of lymphoid cells present in the spleen of IL-4 deficient 129SV mice was observed but both groups had similar numbers of monocytes and PMN cells present in the spleen (Figure 6.7). There was a reduction in the numbers of lymphoid, monocyte

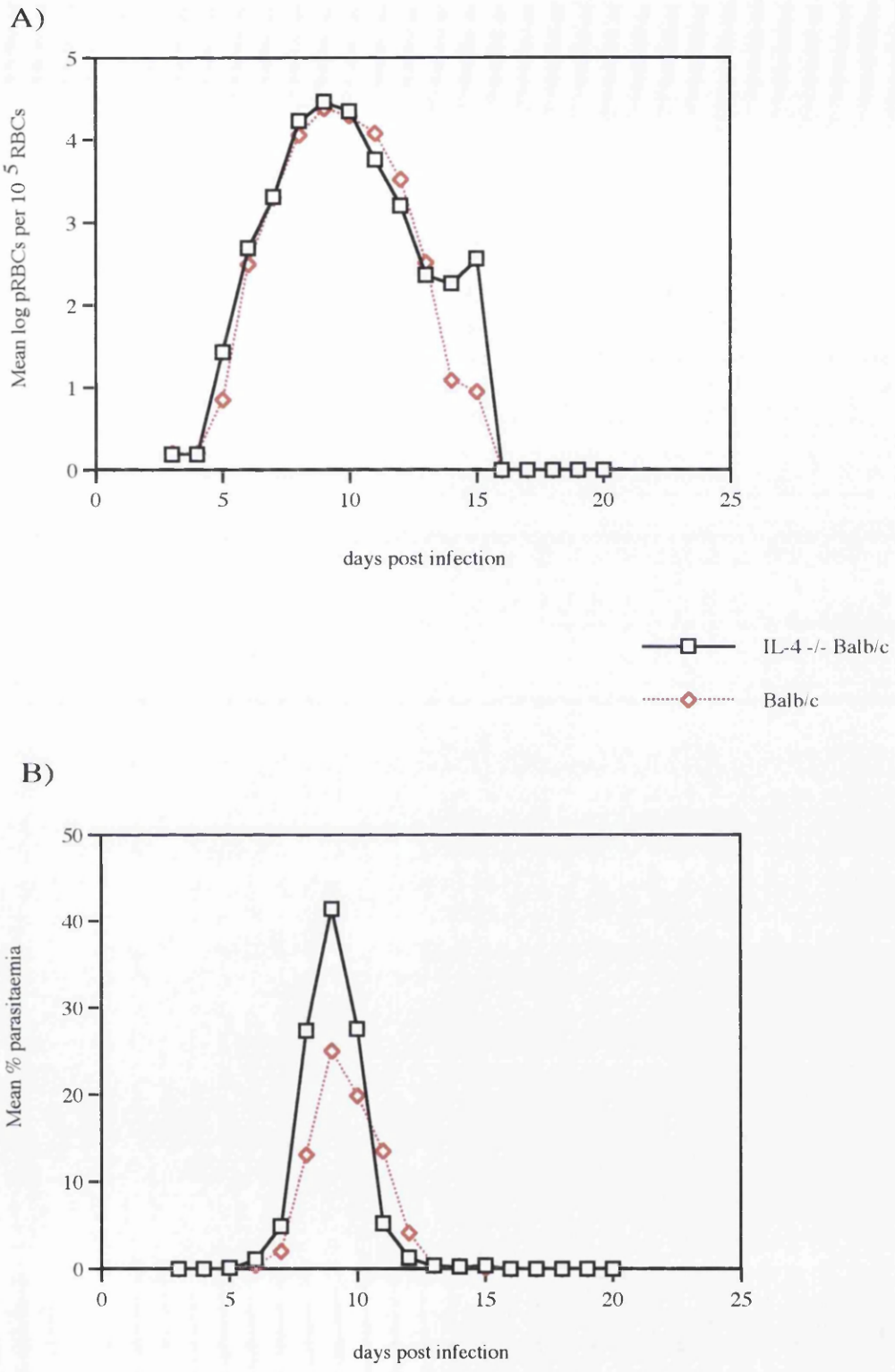


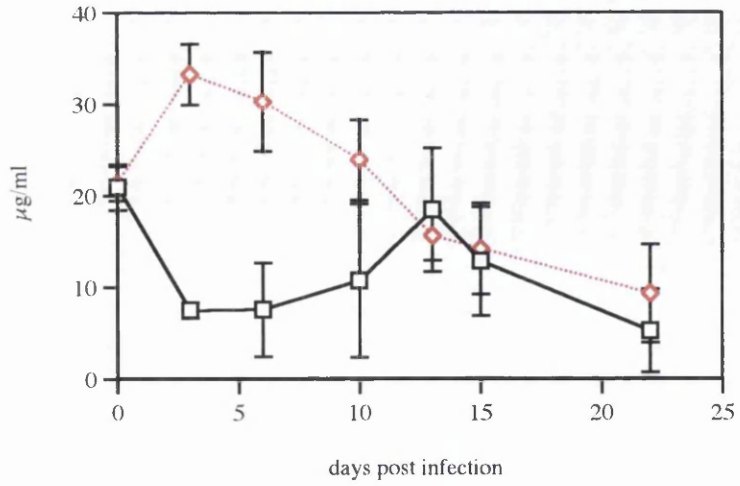
Figure 6.3. The course of infection in inbred IL-4 deficient mice (IL-4 -/- Balb/c) and control mice (Balb/c) following infection with 1x10 ^{5} pRBCs of *P. chabaudi*. The mean log parasitaemia A), and percentage parasitaemia B), of six mice are presented.

and PMN cells present in the liver of IL-4 deficient 129SV mice during *P. chabaudi* infection (Figure 6.8).

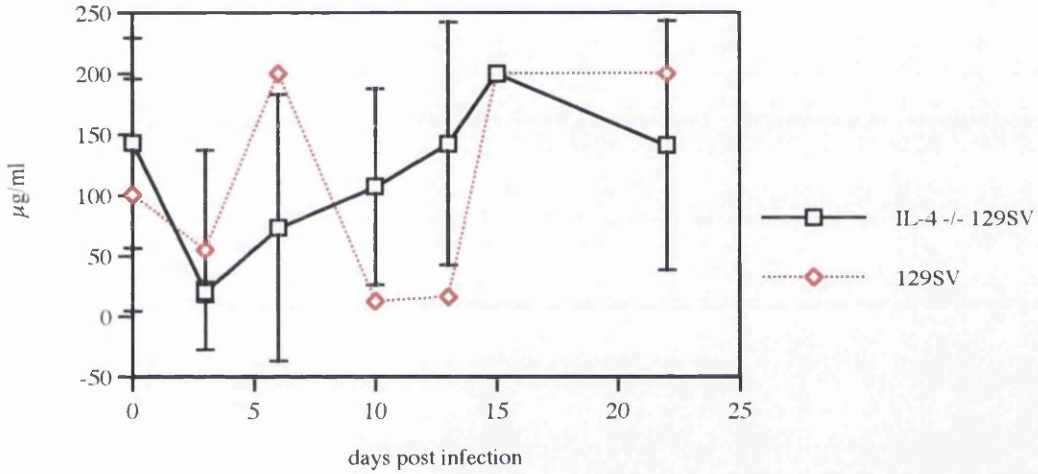
Discussion

IL-4 deficient mice can control and clear a primary infection of *P. chabaudi* with similar efficiency to that of controls. However, a small but significant exacerbation of the peak of the primary patent parasitaemia was observed in the IL-4 deficient mice. This result was consistently observed in IL-4 deficient mice on three different backgrounds. The work reported here confirms previous studies (von der Weid *et al.*, 1994, van der Heyde *et al.*, 1997) in that the presence of IL-4 is not essential for the efficient elimination of a patent erythrocytic stage malarial infection in mice.

The sequential involvement of Th1 and Th2 associated cytokines and the related cell mediated immunity (antibody-independent) and antibody-dependent immunity during *P. chabaudi* infection is well documented (Langhorne *et al.*, 1989, Taylor-Robinson and Phillips, 1992). The importance of IL-4 in the development of Th2 cells dictates that the absence of this Th2 associated cytokine during a *P. chabaudi* infection might result in a deficiency in the clearance of the parasites, a mechanism attributed to antibody mediated immunity (Meding and Langhorne, 1991, Stevenson and Tam, 1993). However, the studies presented here and elsewhere (von der Weid *et al.*, 1994b, van der Heyde *et al.*, 1997), using IL-4 deficient mice, contradict the proposed role for IL-4 in mediating Th2 associated effector mechanisms thought to be involved in the elimination of blood-stage infection. Infection of IL-4 deficient mice with *P. yoelii*, resulted in no difference compared to control mice (van der Heyde *et al.*, 1997) even though immunity to *P. yoelii* is traditionally thought to be mainly antibody-dependent (Weinbaum, Evans and Tigelaar, 1976). Furthermore, in the studies reported here, an exacerbation of the primary patent parasitaemia was observed in the IL-4 deficient mice despite this phase of the *P. chabaudi* infection being thought to be predominately a Th1 and IFNγ mediated



B). Total IgG2a



C). Total IgE

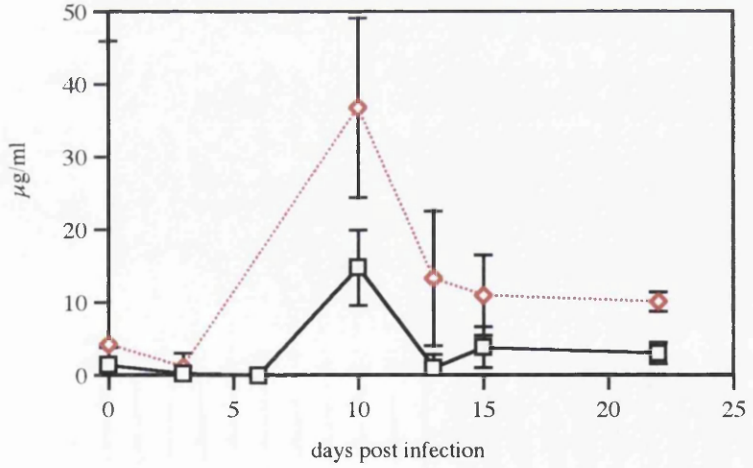


Figure 6.4. Total levels of IgG1, A), IgG2a, B) and IgE, C), were determined in the serum of IL-4 deficient 129SV mice and control 129SV mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Serum from three mice was analysed individually for both groups, in triplicate and the mean ± SD antibody level was calculated.

response (Langhorne *et al.*, 1989, Taylor-Robinson and Phillips, 1992). Previously, infection of IL-4 deficient mice resulted in a sustained Th1 response indicated by prolonged IFNγ production and elevated IL-12 levels (von der Weid *et al.*, 1994b). Analysis of cytokine production in the present studies, by splenocytes *in vitro*, in the serum or mRNA expression, would indicate if there was a similar sustained Th1 response. The exacerbation of the peak parasitaemia observed in the absence of IL-4 suggests that the acute phase of *P. chabaudi* is not solely under the control of Th1 mediated responses. However, the assumption that IL-4, although the main cytokine involved in the development of Th2 $CD4^+$ T cells, is essential for Th2 mediated responses is now regarded unlikely. von der Weid and colleagues (1994b) demonstrated that despite the absence of IL-4 during *P. chabaudi* infection, other Th2 associated cytokines (IL-5, IL-6, IL-10) were still produced and that $CD4^+$ T cells from the IL-4 deficient mice were only delayed in their switch from the Th1 to Th2 phenotype.

Infection of IL-4 deficient mice with *P. chabaudi* in the studies reported here and elsewhere (von der Weid *et al.*, 1994b) demonstrate the importance of the humoral response to malaria infection because even in the absence of IL-4, compensatory mechanisms ensure that a protective humoral response is still induced albeit either reduced or delayed. Total IgG1 levels were reduced in IL-4 deficient mice confirming previous reports (von der Weid *et al.*, 1994b) and a reduction in parasite-specific IgG1 has also been documented (von der Weid *et al.*, 1994b). These observations are not surprising because of the importance of IL-4 in class switching to IgG1 production (Snapper and Paul, 1987a). It is interesting that IL-4 deficient mice did not have a low grade persistent parasitaemia which is observed in B cell deficient mice following a *P. chabaudi* infection (Taylor-Robinson and Phillips, 1994a). A sustained Th1 mediated response, indicated by IFNγ and IL-12 production (Taylor-Robinson and Phillips, 1994a) and by the frequency of Th1 $CD4^+$ T cells following limited dilution analysis (von der Weid and Langhorne, 1993) was observed in *P. chabaudi* infected B cell deficient mice. Concomitantly, a reduction in IL-4 was observed (von der Weid and

Days post infection	Group	
	IL-4 -/- 129SV	129SV
0	-	-
6	100	-
10	100	100
13	1000	1000
15	500	1000
22	1000	1000

Table 3. Parasite-specific IgG production in IL-4 deficient 129SV mice and control 129SV mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Serum from three mice was pooled and the level of parasite-specific IgG was determined by indirect fluorescence. Results are the mean of three samples and are expressed as the reciprocal of antibody titre.

Note: Range of samples analysed was 1: 50 - 1: 1000.

Langhorne, 1993, Taylor-Robinson and Phillips, 1994a) and is probably a result of the loss of B cells as antigen presenting cells which may influence the development of $CD4^+$ T cells towards Th2 differentiation and subsequently IL-4 production (Gajewski *et al.*, 1991). A loss of B cell production of IL-10, a cytokine which can suppress Th1 development (Moore *et al.*, 1993), would also result in a reduction of IL-4 production because the response would now have a Th1 bias. However, the persistent patent parasitaemia observed in B cell deficient mice does not appear to be a consequence of the reduced Th2 associated cytokine production but is more likely due to the loss of the protective humoral response. IL-4 deficient mice efficiently clear a *P. yoelii* infection which is thought to induce antibody-dependent immunity (van der Heyde *et al.*, 1997). Furthermore, *P. chabaudi* infection of IL-4 deficient mice results in a similar sustained Th1 response as seen in the B cell deficient mice (von der Weid *et al.*, 1994b) but there is a humoral response still induced in the IL-4 deficient mice and subsequently the IL-4 deficient mice can efficiently control the infection unlike B cell deficient mice. These observations suggest that parasite elimination following the acute phase of a primary *P. chabaudi* infection, requires B cell production of parasite-specific antibodies whereas the involvement of IL-4 and Th2 mediated responses is not synomonous with antibody-dependent immunity. Evidence for this hypothesis is provided by the observation of an exacerbated *P. yoelii* infection in IFNγ deficient mice (van der Heyde *et al.*, 1997), which suggests that IFNγ can contribute to antibody-dependent immunity. Hence, the chronic parasitaemia observed in B cell deficient mice can be attributed to the absence of the humoral response and not the failure to switch from Th1 to Th2 mediated responses.

The reason why IL-4 deficient mice in this study have an exacerbated peak of the primary parasitaemia is unclear. It is most likely that the reduction in the humoral response, indicated by reduced levels of total IgG may contribute to this observation. Another possibility is that the alteration in the balance of cytokine interactions which regulate Th1/Th2 development caused by the absence of IL-4 may result in reduced anti-parasite immunity at this time of infection. IL-4 mediated induction of IgG1 has been

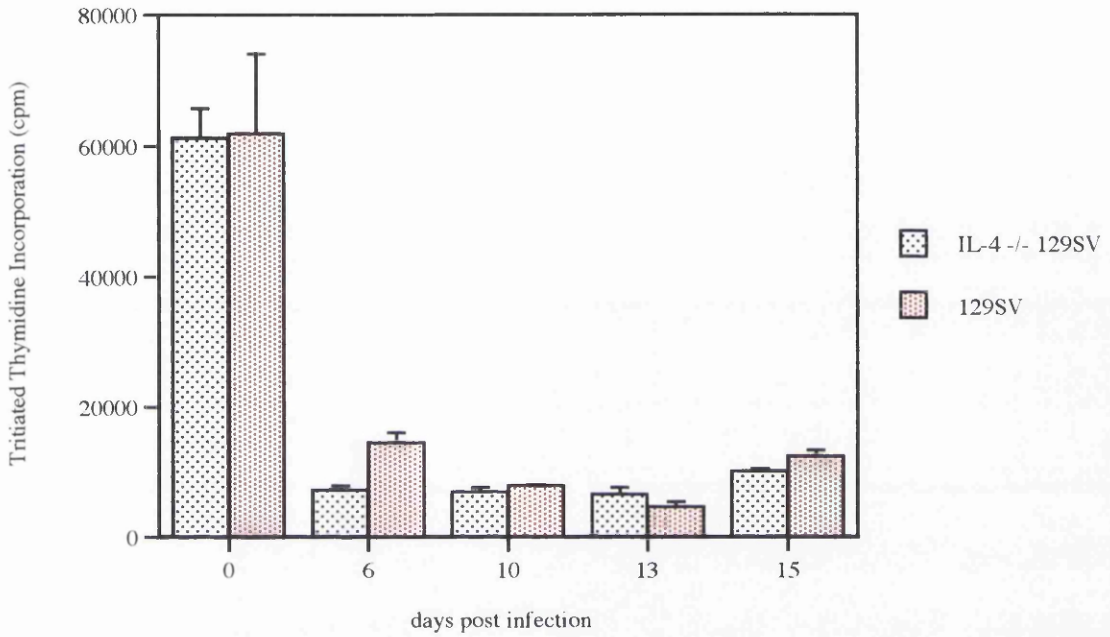


Figure 6.5. The proliferative response of splenocytes from IL-4 deficient 129SV mice and control 129SV mice harvested following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from both groups were sacrificed and the splenocytes were pooled within each group and stimulated with Con A. Each data point is the mean ± SD for an experiment performed in triplicate.

shown to be involved in the protection mediated by a Th2 $CD4^+$ T cell clone to $CD4^+$ T cell depleted mice upon challenge with *P. chabaudi* (Taylor-Robinson *et al.*, 1993). The reduction of IgG1 in IL-4 deficient mice during the course of *P. chabaudi* infection may lead to a decrease in antibody-dependent cellular cytotoxicity (ADCC) which has been proposed to inhibit *P. falciparum* growth *in vitro* (Brown and Smalley, 1980, Brown, Greenwood and Terry, 1986). An increase in ADCC has been reported in *P. chabaudi* infected mice (McDonald and Phillips, 1978a) and hence, the exacerbated peak of parasitaemia may be due to decreased IgG1 mediated ADCC.

IL-4 does exert effects on the immune system outwith Th2 T cell development and stimulation of IgG1 production. The stimulation of endothelial cells by IL-4, resulting in the enhanced adhesion of lymphocytes could explain, in part, the reduced numbers of lymphoid cells present in the liver of IL-4 deficient mice at peak parasitaemia. As previously discussed (see Chapter 3), lymphomyeloid cells present in the liver may be involved in a protective immune response at this point of a *P. chabaudi* infection. Hence, in the absence of IL-4, the reduced numbers of lymphomyeloid cells present in the liver may contribute to the exacerbated peak of parasitaemia observed in *P. chabaudi* infected IL-4 deficient mice.

The infection of IL-4 deficient mice with *P. chabaudi* demonstrates that either the requirement for IL-4 induction of Th2 mediated antibody-dependent immunity during a primary infection is of less importance than previously thought or that significant mechanisms compensate for the loss of IL-4 function. IL-10 or IL-13, alone or in combination, may replace IL-4 function and induce the required protective immunity during a malaria infection. However, no significant detrimental effect on the outcome of a *P. chabaudi* infection has been observed in mice deficient of Th2 associated cytokines (van der Heyde *et al.*, 1997, see Chapter 5), promoting the hypothesis that Th1 associated cytokine mediated antibody-dependent immunity may be sufficient to control a primary *P. chabaudi* infection and that the contribution of IL-4 and Th2 associated

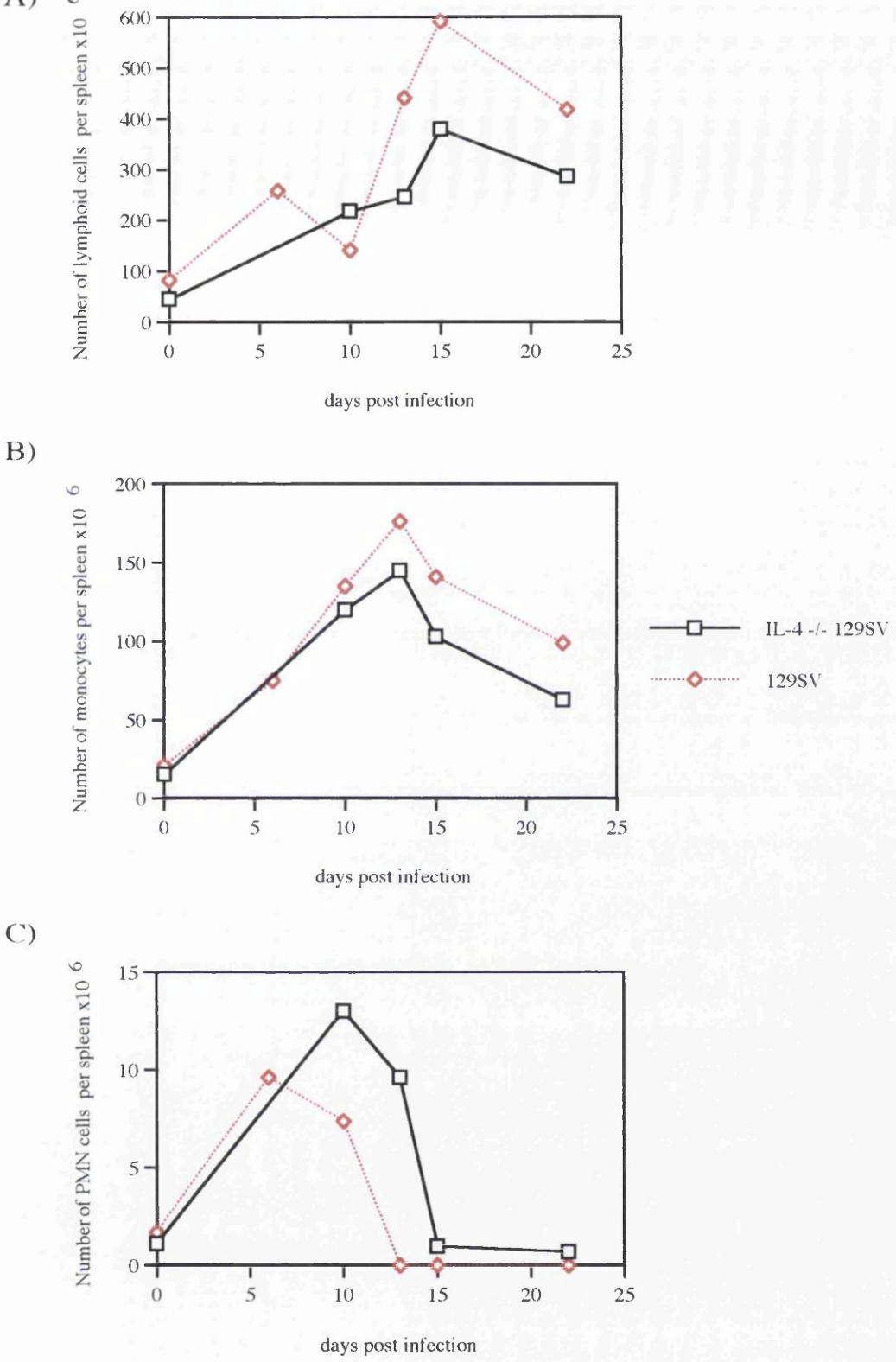


Figure 6.6. Cytological analysis of cells present in the spleen of IL-4 deficient 129SV mice and control 129SV mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at each timepoint and the cells harvested were pooled and centrifuged. 500 cells were counted on each Giemsa's stained cytospin smear and the number of A) lymphoid, B) monocyte and C) PMN cells, are expressed per spleen.

cytokines is of secondary importance. Further analysis of both the profile of cytokine production and the humoral response, in particular the production of parasite-specific antibody isotypes is necessary to elucidate the contribution of IL-4 to immunity to experimental malaria infection and the compensatory mechanisms replacing the function of IL-4.

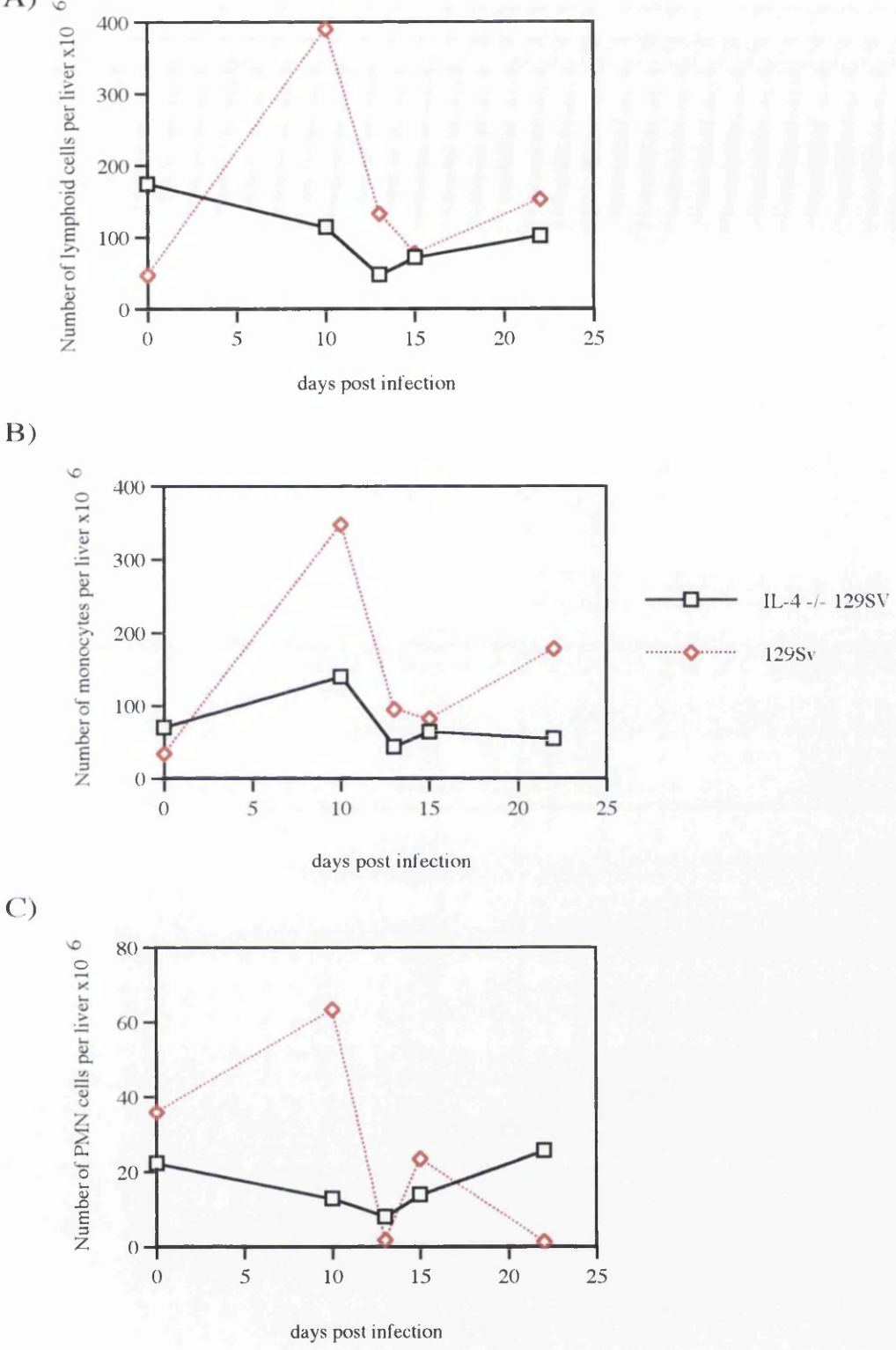


Figure 6.7. Cytological analysis of cells present in the liver of IL-4 deficient 129SV mice and control 129SV mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at each timepoint and the cells harvested were pooled and centrifuged. 500 cells were counted on each Giemsa's stained cytospin smear and the number of A) lymphoid, B) monocyte and C) PMN cells, are expressed per liver.

Chapter Seven

The course of *P. chabaudi* infection in IFNγR deficient mice

Introduction

IFNγ was first identified as an antiviral agent in the supernatants of leukocytes cultures stimulated by PHA (Wheelock, 1965). Subsequently, IFNγ has been shown to orchestrate a wide range of cellular responses which demonstrates the importance of IFNγ in the regulation of the immune system and the control of infectious disease. The main sources of IFNγ are activated Th1 $CD4^+$ T cells (Mosmann and Coffman, 1989), activated NK cells (Perussia, 1991) and $CD8^+$ cytotoxic T cells of the type 1 cytokine phenotype (Sad, Marcotte and Mosmann, 1995). Cross-linking of the T cell receptor is the major stimulus of IFNγ production by T cells (Ullman *et al.*, 1990). IFNγ production by NK cells is stimulated by TNFα and IL-12 (Trinchieri, 1995) and can also be autostimulated by IFNγ itself (Hardy and Sawada, 1989). Biologically active IFNγ is a 34 kDa homodimer (Greenlund, Wenner and Schreiber, 1992, Fountoulakis *et al.*, 1992) which interacts with a specific cell receptor ubiquitously expressed on all nucleated cells (Valente *et al.*, 1992). The IFNγ receptor (IFNγR) consists of two subunits, a 90 kDa α chain (Farrar and Schreiber, 1993) and a 314 amino acid (60-65 kDa) β chain (Bach, Aguet and Schreiber, 1997). Each chain of the IFNγR is associated with a specific Janus kinase [JAK] (Igarashi *et al.*, 1994). The IFNγ homodimer binds to 2 α chains of the IFNγR, resulting in α chain dimerisation (Fountoulakis *et al.*, 1992) and consequent association of the β chains which leads to activation of the JAKs (Boehm *et al.*, 1997). This induces binding of STAT 1α (signal transducer and activator of transcription 1α) to the receptor complex (Greenlund *et al.*, 1994, Heim *et al.*, 1995). STAT1α homodimers are formed following phosphorylation of the bound STAT1α (Greenlund *et al.*, 1995). The STAT1α homodimers (also known as Gamma Activated Factor, GAF) translocate to the nucleus and bind to gamma activated sites and initiate transcription (Boehm *et al.*, 1997). Through this signaling pathway, IFNγ can initiate transcription of a number of genes bearing suitable gamma activated sites in their promoter regions. Currently there are over 200 known IFNγ-regulated genes (Boehm *et al.*, 1997).

The rapid response to IFNγ induced transcription is due to the activation of the latent cytosolic transcription factor STAT1α and hence it does not require the synthesis of new transcription factors. However, some cellular responses to IFNγ require the synthesis of IFNγ induced transcription factors to initiate the transcription of the desired product (Boehm *et al.*, 1997). These secondary transcription factors are termed interferon regulatory factors (IRF) (Boehm *et al.*, 1997). IRF-1 is induced by IFNγ via a gamma activated site, participates in several IFNγ specific cellular responses (Sims *et al.*, 1993, Coccia *et al.*, 1995) and is also induced by TNFα (Fujita *et al.*, 1989). IRF-2 is antagonistic to IRF-1 and acts as a transcription inhibitor (Harada *et al.*, 1989, Harada *et al.*, 1994).

The major function of IFNγ is immunoregulatory, where it is involved in the development of the $CD4^+$ T cell response which determines the response to an antigen or pathogen. The dichotomy of the $CD4^+$ T cell response is well established. IL-12 and IFNγ are implicated in the decision to adopt a Th1 phenotype whereas IL-4 induces Th2 differentiation (Seder and Paul, 1994). The actual role of IFNγ in this process appears to be secondary to IL-12 which has emerged as one of the main immunoregulatory molecules directing cell-mediated immunity (Trinchieri, 1995). Th1 differentiation is maintained by a positive feedback loop in which IL-12 production from stimulated macrophages, induces IFNγ production by naive $CD4^+$ T cells resulting in the adoption of a Th1 phenotype. IFNγ from the Th1 cells or IL-12 activated NK cells, induces IL-12 production by macrophages providing a constant stimulus for the differentiation of the $CD4^+$ T cell response to the Th1 phenotype and links innate recognition of pathogens to acquired immunity. IFNγ has been shown *in vitro* to enhance the Th1 differentiating effects of IL-12 (Wenner *et al.*, 1996, Bradley, Dalton and Croft, 1996). It is possible that this phenomenon may be attributed to IFNγ induced expression of the IL-12 receptor on naive T cells (Boehm *et al.*, 1997). The costimulatory ligands B7-1 and B7-2 are thought to have a role in the induction of T cell responses (Lenchow, Walunas and

Bluestone, 1996). Both are upregulated by IFNγ (Freedman *et al.*, 1991, Hathcock *et al.*, 1994) but they may have differential effects on Th cell development with B7-1 favouring Th1 and B7-2 favouring Th2 cell development (Freeman *et al.*, 1995, Kuchroo *et al.*, 1995).

One feature of the Th1/Th2 paradigm is the antagonistic effect mediated by IFNγ on Th2 development and associated functions. Conversely, IL-4 is antagonistic to IFNγ mediated responses. IFNγ can inhibit the growth of Th2 $CD4^+$ T cell clones *in vitro* (Gajewski and Fitch, 1988, Maggi *et al.*, 1992) but it is unclear if IFNγ can suppress IL-4 gene transcription during the establishment of Th1 development. The antagonistic effects of IFNγ and IL-4 are present throughout Th1/Th2 mediated responses. IFNγ is the main switch factor regulating IgG2a switching in the mouse (Snapper and Paul, 1987a) and IL-4 induces IgG1 and IgE class switching (Vitetta *et al.*, 1985, Coffman *et al.*, 1986) with both cytokines antagonising the class switching mediated by the reciprocal cytokine. The expression of FcγRI on macrophages is upregulated by IFNγ stimulation and results in an increase in IgG2a mediated opsonisation (Boehm *et al.*, 1997). IL-4 induction of FcεRII (CD23) on basophils and mast cells is down regulated by IFNγ (Boehm *et al.*, 1997).

IFNγ is a critical mediator of early immune responses during the acute phases of various infections. The generation of mice with a disruption of the gene for IFNγ or the IFNγR has enabled the confirmation of the importance of IFNγ induced cellular responses to viral, bacterial and parasitic infections. IFNγR deficient mice are susceptible to vaccinia virus (Huang *et al.*, 1993) and mycobacterial infection (Kamijo *et al.*, 1993) demonstrating the requirement for IFNγ mediated functions in controlling infectious pathogens. The primary responses to *L. monocytogenes* infection are IFNγ dependent (Huang *et al.*, 1993) and early IFNγ production is associated with resistance to *L. major* infection (Heinzel *et al.*, 1991). Hence, IFNγ is a critical mediator of enhanced

microbicidal activity to acute infection mainly through the activation of macrophages to increase antigen presentation or the secretion of antimicrobial products such as NO.

The role of IFNγ during experimental malaria infection has been extensively studied. The intrahepatocytic development of *P. berghei in vitro* was inhibited upon the addition of recombinant human IFNγ (Schofield *et al.*, 1987a). IFNγ is produced prior to the peak parasitaemia of *P. chabaudi* infection (Slade and Langhorne, 1989). Treatment of mice infected with *P. yoelii* with recombinant IFNγ increases resistance to infection (Shear *et al.*, 1989). The addition of recombinant IFNγ to co-cultures of *P. falciparum* and human monocyte-derived macrophages increases the appearance of crisis forms of *P. falciparum in vitro* (Ockenhouse, Shulman and Shear, 1984). Control of the acute phase of *P. chabaudi* infection is Th1 mediated and partly IFNγ dependent (Meding *et al.*, 1990). Mice resistant to *P. chabaudi* infection have been shown to produce an early IFNγ response whereas susceptible mice have an early IL-4 response (Stevenson and Tam, 1993). However, anti-IFNγ treatment during *P. chabaudi* infection does not abrogate protective immunity suggesting that IFNγ independent mechanisms are also involved in the control of the acute phase of infection (Stevenson *et al.*, 1990).

IFNγR deficient mice displayed no significant increased susceptibility to *P. yoelii* and *P. chabaudi adami* infection (Tsuji *et al.*, 1995). However, IFNγ deficient mice have been shown to be more susceptible to *P. yoelii* and *P. chabaudi adami* (van der Heyde *et al.*, 1997), illustrating that the response of the two types of deficient mice perhaps cannot be directly compared. Recently, IFNγR deficient mice were shown to be more susceptible to *P. chabaudi chabaudi* infection displaying a high mortality rate and an increased leukocytosis compared to control mice (Favre *et al.*, 1997, published while these studies were in progress). No significant differences were observed between the two groups during the primary parasitaemia, but, IFNγR deficient mice developed a pronounced secondary parasitaemia. Hence, IFNγ dependent cellular responses are crucial to the development of protective immunity during a *P. chabaudi* infection. The studies reported

here aim to elucidate why the absence of the IFNγR results in an increased susceptibility to *P. chabaudi* infection.

Results

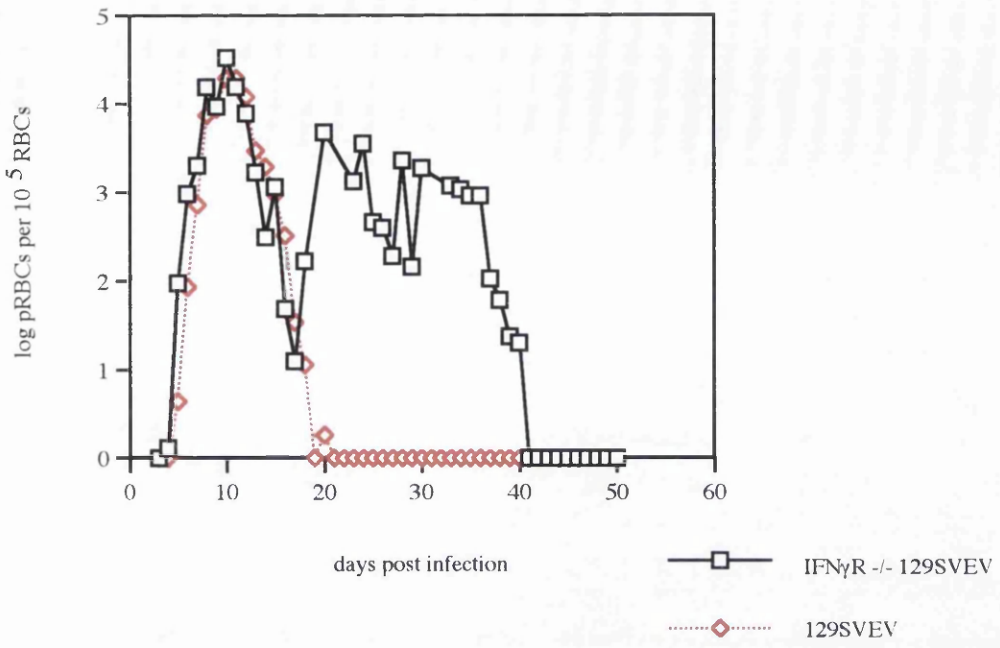
The course of *P. chabaudi* infection in IFNγR deficient mice

IFNγR deficient 129SVEV mice and intact control 129SVEV mice were infected with either $1x10^5$ pRBCs or $1x10^1$ pRBCs of *P. chabaudi* AS. Each group consisted of at least six mice and the parasitaemia was observed daily by microscopic examination of Giemsa's stained thin blood smears collected from the tail. Infection of IFNγR deficient mice with both $1x10^5$ pRBCs and $1x10^1$ pRBCs of *P. chabaudi* resulted in a significant secondary peak of parasitaemia (no recrudescence parasitaemia was observed in the control mice) observed after 20 days post infection (Figures 7.1 and 7.2). There was no significant difference between the IFNγR deficient mice and control mice during the primary parasitaemia although there was a consistent trend in that the peak of the primary parasitaemia was greater in the IFNγR deficient mice. In control mice the infection became sub-patent (determined by microscopic examination) around 20 days post infection whereas in IFNγR deficient mice, clearance of the parasites to sub-patency did not occur until approximately day 40 post infection (Figures 7.1 and 7.2).

Survival in IFNγR deficient mice during *P. chabaudi* infection

P. chabaudi infection of IFNγR deficient mice resulted in an increase in the mortality rate compared with control mice. 75% of IFNγR deficient mice succumbed to infection with $1x10^5$ pRBCs of *P. chabaudi* (Figure 7.3) and 43% died following infection with $1x10^1$ pRBCs of *P. chabaudi* (Figure 7.4). No mortality was observed in the control mice with either dose of *P. chabaudi* infection (Figures 7.3 and 7.4).

A)



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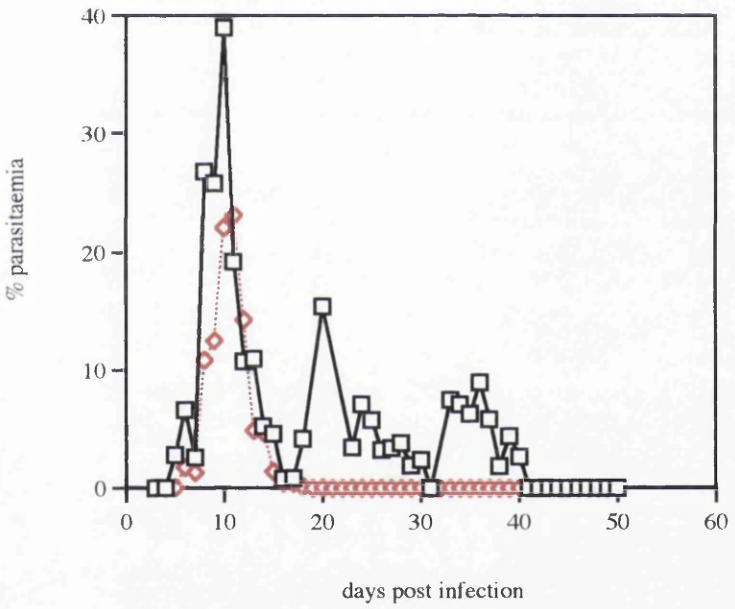


Figure 7.1. The course of infection in IFN γR deficient mice (IFNγ -/- 129SVEV) and control 129SVEV mice following infection with $1x10^5$ pRBCs of *P. chabaudi*. The mean log parasitaemia A), and percentage parasitaemia B), of six mice are presented. The results are combined from three replicate experiments.

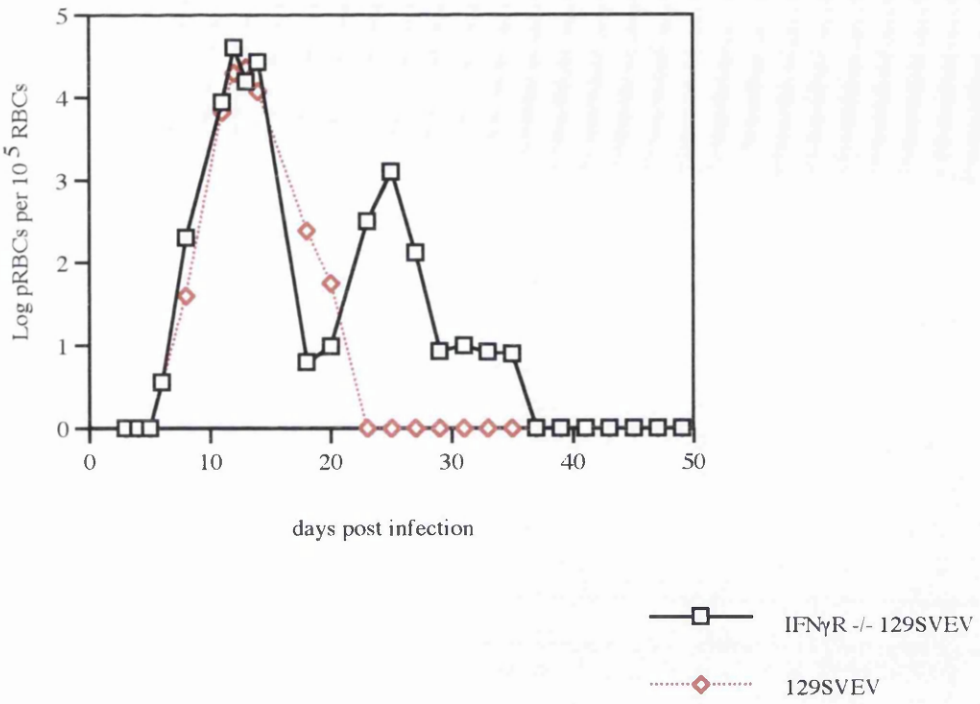
Total IgG and IgE production in IFNγR deficient mice during *P. chabaudi* infection

Total IgG1, IgG2a and IgE production was determined in the serum of IFNγR deficient mice during *P. chabaudi* infection. IFNγR deficient mice and control mice received $1x10^5$ pRBCs of *P. chabaudi*. Three mice in each group were sacrificed at various time points and serum was collected, pooled and assayed for the production of total IgG1, IgG2a or IgE (see Materials and Methods). Total IgG1 levels in the serum of IFNγR deficient mice were similar to that of control mice (Figure 7.5A). There was a delay and a reduction in the total IgG2a response of IFNγR deficient mice to *P. chabaudi* infection compared to control mice (Figure 7.5B). IFNγR deficient mice produced a significant amount of total IgE in response to *P. chabaudi* infection whereas no significant levels of total IgE were observed in the serum of control mice (Figure 7.5C).

The production of parasite-specific IgG in IFNγR deficient mice during *P. chabaudi* infection

Parasite-specific IgG production in the serum of IFNγR deficient mice and control mice was determined following infection with $1x10^5$ pRBCs of *P. chabaudi* AS by IFAT described in Materials and Methods). Three mice from each group were sacrificed at the time points indicated and serum was collected, pooled and assayed for the production of parasite-specific IgG. The level of parasite-specific IgG in the serum of IFNγR deficient mice during *P. chabaudi* infection was reduced compared to control mice throughout the period of infection analysed (Table 4).

A)



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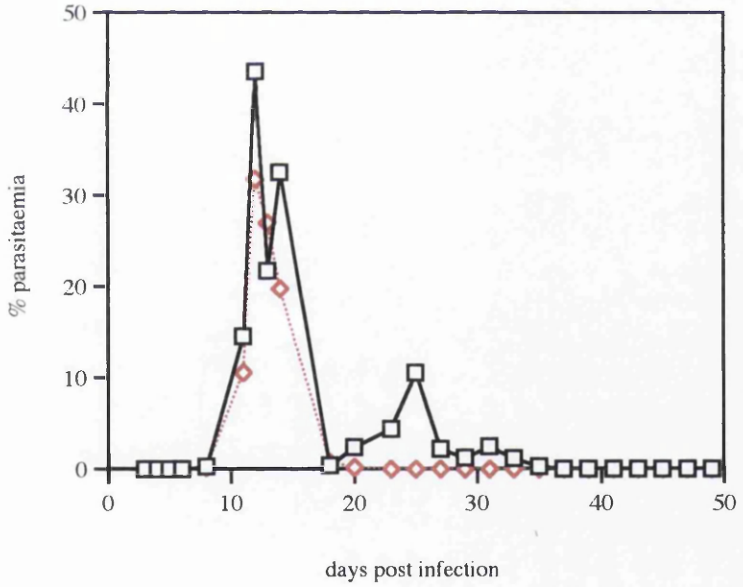


Figure 7.2. The course of infection in IFN γR deficient mice (IFNγ -/- 129SVEV) and control 129SVEV mice following infection with 1x10 ^{1} pRBCs of *P. chabaudi*. The mean log parasitaemia A), and percentage parasitaemia B), of six mice are presented.

Ex vivo analysis of the response of splenocytes taken from IFNγR deficient mice during *P. chabaudi* infection

IFNγR deficient mice and control mice were infected with $1x10^5$ pRBCs of *P. chabaudi* AS. Three mice from each group were sacrificed at the various time points indicated. Splenocytes were harvested and pooled from the three individual mice of each group, cultured *in vitro* at $5x10^6$ cells/ml and stimulated with Con A (1μg/ml). The proliferative response of the splenocytes was determined by the incorporation of tritiated thymidine. Splenocytes from IFNγR deficient mice had a greater proliferative response to Con A than splenocytes from control mice on day 0 post infection [$p<0.002$] (Figure 7.6). On days 6, 10 and 15 post infection, splenocytes from IFNγR deficient mice had a significantly increased proliferative response compared with splenocytes from control mice to Con A stimulation [$p<0.002$] (Figure 7.6). Splenocytes taken from control mice had a significantly greater proliferative response to Con A than splenocytes from IFNγR deficient mice on day 13 post infection [$p<0.001$] (Figure 7.6). No parasite-specific proliferative response was observed following stimulation of splenocytes with a pRBC lysate of *P. chabaudi* (data not shown).

Cytological analysis of cells present in the spleen or liver of IFNγR deficient mice during *P. chabaudi* infection

Cytological analysis was performed on leukocytes extracted from the spleen and liver of both IFNγR deficient mice and control mice following infection with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at the time points indicated, the leukocytes extracted from the liver and spleen of individual mice and then pooled to give leukocytes extracted from either the liver or spleen for each group. Cytological analysis was performed as described in Materials and Methods and the results expressed as the number of cells present per spleen or liver. There was a reduction in the number of lymphoid cells present in the spleen of IFNγR deficient mice but both

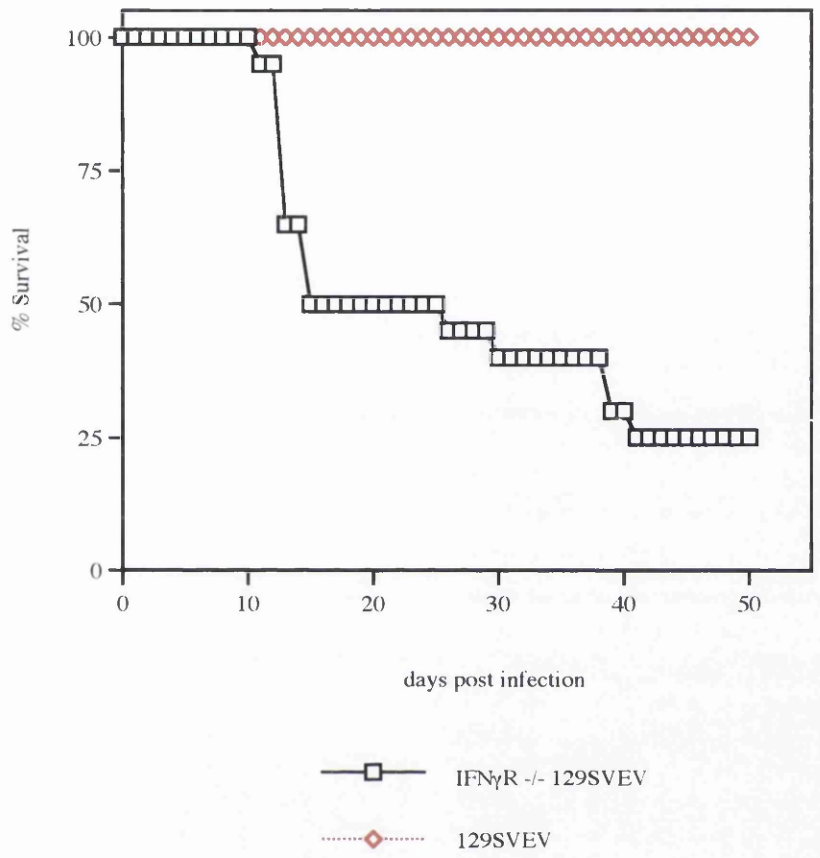


Figure 7.3. Survival of IFNγR deficient mice and control mice following infection with $1x10^5$ pRBCs of *P. chabaudi*. Each group consisted of at least six mice and the results are combined from three replicate experiments.

groups had similar numbers of monocytes and PMN cells present in the spleen (Figure 7.7). In the liver, a reduction in lymphoid, monocyte and PMN cells present was observed in IFNγR deficient mice compared to control mice during *P. chabaudi* infection (Figure 7.8).

Discussion

The data presented in this study demonstrate that IFNγR deficient mice are more susceptible to *P. chabaudi* infection. A high mortality rate in the IFNγR deficient mice was observed whereas there were no deaths in the control groups. The acute phase of the *P. chabaudi* infection was controlled by both IFNγR deficient mice and control mice with a similar efficiency. However, the infection never became sub-patent in the IFNγR deficient mice and this was followed by a pronounced secondary parasitaemia unlike the control mice where no recrudescence occurred during the period of infection observed. These observations confirm previous results (Favre *et al.*, 1997, van der Heyde *et al.*, 1997) illustrating the importance of IFNγ dependent cellular responses to malaria infection.

The use of IFNγR deficient and IFNγ deficient mice has extended the studies of IFNγ mediated response to malaria infection whereas in previous studies this took the form of either deletion of IFNγ by antibody treatment or addition of exogenous IFNγ (Meding *et al.*, 1990, Stevenson *et al.*, 1990). Anti-IFNγ treatment of mice resulted in a significantly higher primary peak parasitaemia of *P. chabaudi* infection Meding *et al.*, (1990), and Stevenson *et al.*, (1990). However, Jacobs and colleagues (1996) did not observe significant differences in the course of the parasitaemia of a *P. chabaudi* infection. The results obtained in this study and elsewhere (Favre *et al.*, 1997) demonstrate that the absence of the IFNγR results in a pronounced secondary peak of parasitaemia which is different to other published observations (Tsuji *et al.*, 1995) in which IFNγR deficient mice had only a moderately prolonged parasitaemia and no

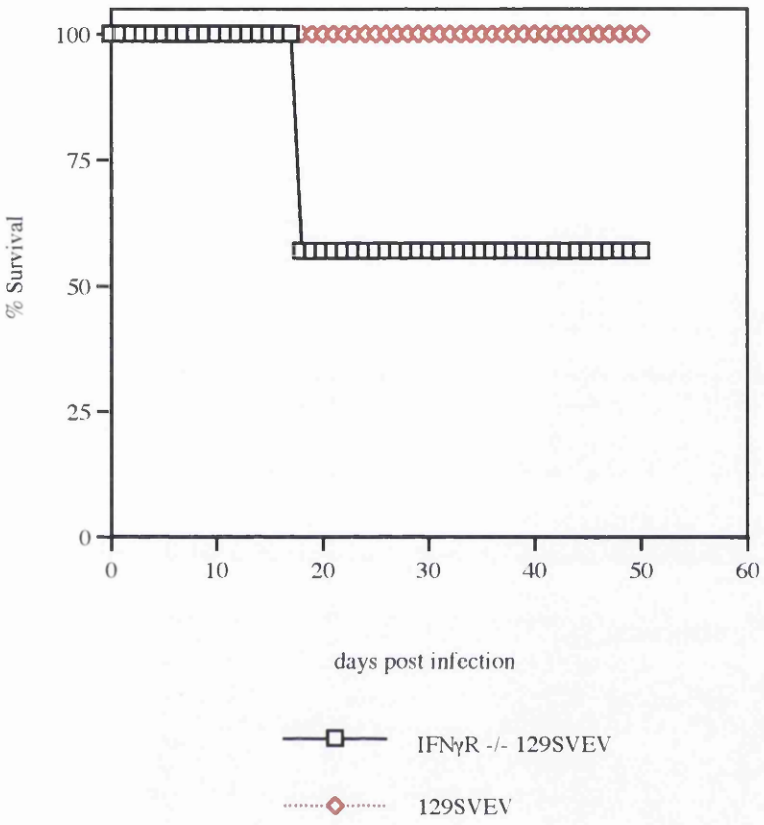
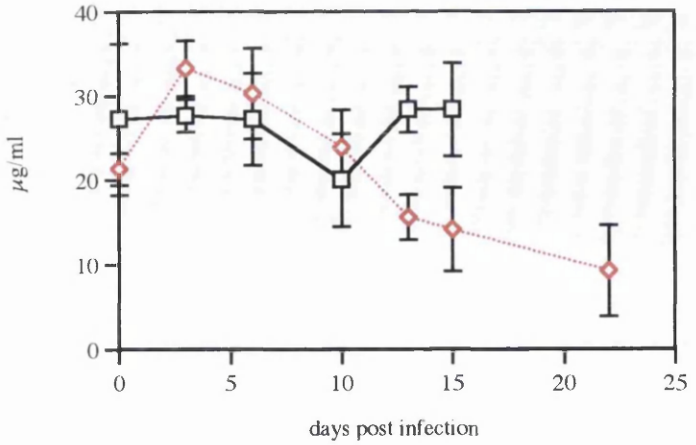


Figure 7.4. Survival of IFNγR deficient mice and control mice following infection with $1x10^1$ pRBCs of *P. chabaudi*. Each group consisted of six mice.

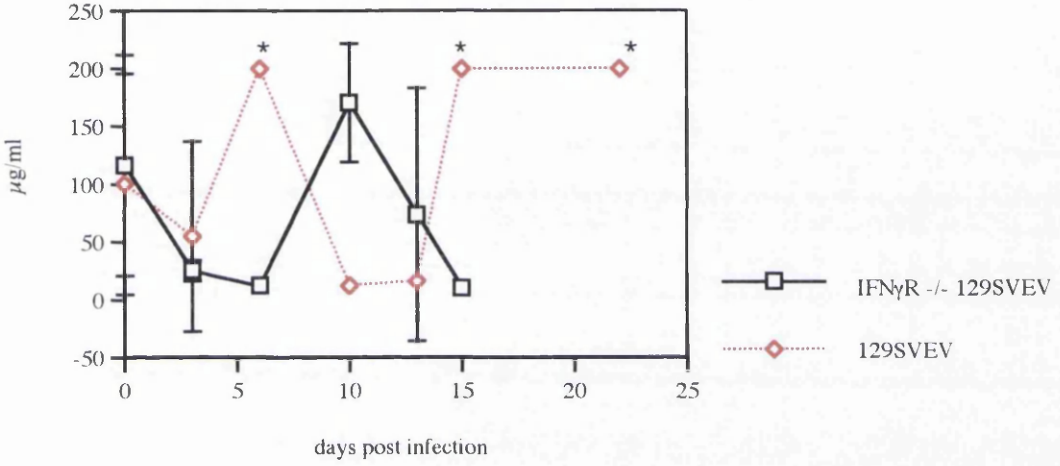
mortality associated with infection. These discrepancies could have several explanations. Different genetic backgrounds of the IFNγR deficient mice and infection with different murine malaria parasite species may contribute to the contradictory results obtained using IFNγR deficient mice. The degree of depletion of IFNγ mediated function by treatment with antibodies may vary from study to study and is probably not as absolute as the gene disruption strategy. Conversely, IFNγR deficient mice may have developed mechanisms to compensate for the loss of IFNγ function, thus making comparisons between studies more difficult.

The pronounced secondary peak of parasitaemia observed in the IFNγR deficient mice infected with *P. chabaudi* is in accord with a previous report (Favre *et al.*, 1997) and is similar to the outcome of infection of IFNγ deficient mice with *P. chabaudi adami* in that clearance of infection is significantly delayed when compared to controls (van der Heyde *et al.*, 1997). Mice treated with anti-IL-12 monoclonal antibodies have also been shown to have a pronounced secondary parasitaemia following *P. chabaudi* infection (Yap, Jacobs and Stevenson, 1994) which when taken together with the results reported here and previous studies (Favre *et al.*, 1997, van der Heyde *et al.*, 1997) supports the hypothesis that IL-12 mediated protection is partly dependent upon IFNγ stimulated cellular responses (Stevenson *et al.*, 1995).

The course of *P. chabaudi* infection in IFNγR deficient mice is similar to that observed in immunocompromised mice such as SCID or nude mice and mice depleted of $CD4^+$ T cells or B cells. All of these mice demonstrate an element of control during the acute phase of the infection but the development of the acquired protective immunity is absent or delayed and a chronic parasitaemia develops (Stevenson, Tam and Rae, 1990 Meding and Langhorne, 1991, Podoba and Stevenson, 1991, Taylor-Robinson and Phillips, 1994a). Hence, the outcome of experimental malaria infection in immunocompromised mice is similar to that of IFNγR deficient mice in that a high mortality rate and a pronounced secondary parasitaemia are observed, although the



B). Total IgG2a



C). Total IgE

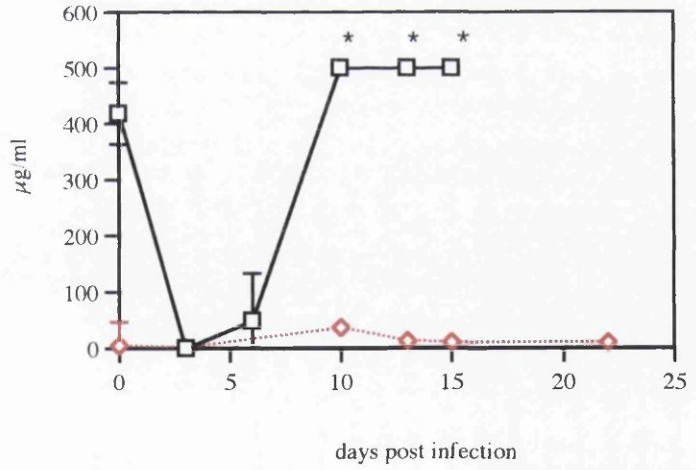


Figure 7.5. Total levels of A) IgG1, B) IgG2a and C), IgE were determined in the serum of IFN γR deficient mice following inoculation with 1x10 ^{5} pRBCs of *P. chabaudi*. Serum from three mice was analysed individually for both groups, in triplicate and the mean ± SD was calculated. Note: * signifies that the sample was over the range of the assay.

IFNγR deficient mice are able to clear the infection by day 40 post infection in this study and by day 23 post infection in the studies performed by Favre *et al.* (1997). The importance of the sequential involvement of Th1 and Th2 mediated responses in the development of protective immunity to *P. chabaudi* infection was confirmed by the studies using immunocompromised hosts. However, they also demonstrated the requirement for not only $CD4^+$ T cells but B cells to confer efficient resolution of infection in reconstituted immunocompromised hosts (Meding and Langhorne, 1991, Taylor-Robinson and Phillips, 1993). Hence, Th1 cells are important in the control of the acute phase of infection through the stimulation and amplification of non-specific inflammatory responses and Th2 cells and B cells are responsible for the total clearance of parasites. The mechanism of the switch between Th1 and Th2 mediated responses during a primary *P. chabaudi* infection is unclear. It has been proposed that B cells are required for this switch in the $CD4^+$ T cell response because of the development of a chronic parasitaemia in B cell deficient mice following the control of the acute phase of infection (Taylor-Robinson and Phillips, 1994a). Analysis of the $CD4^+$ helper T cell profiles during a *P. chabaudi* infection of B cell deficient mice revealed that a persistent Th1 response is present in these mice with a failure to switch to Th2 mediated responses. The role of B cells in antigen presentation is an important one which will influence the Th1/Th2 dichotomy during a *P. chabaudi* infection but it may be the production of the appropriate humoral response which is the main contributory factor to the development of the chronic parasitaemia observed in B cell deficient mice. It is feasible that the absence of IFNγR mediated function in B cells may prevent the desired humoral response being produced in the IFNγR deficient mice during a *P. chabaudi* infection. Analysis of the $CD4^+$ T cell helper response during infection of IFNγR deficient mice with *P. chabaudi* would determine if there is a failure to switch from Th1 to Th2 mediated responses or if there is an earlier appearance of Th2 and IL-4 mediated responses which has been associated with susceptibility to *P. chabaudi* infection (Stevenson and Tam, 1993). An indication may be given by the significant total IgE response observed in the serum of IFNγR deficient mice which may reflect an aberrant

Days post infection	Group	
	IFNγR -/- 129SVEV	129SVEV
0	-	-
6	-	-
10	50	100
13	100	1000
15	100	1000
22	N/A	1000

Table 4. Parasite-specific IgG production in IFNγR deficient 129SVEV mice and control 129SVEV mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Serum from three mice was pooled and the level of parasite-specific IgG was determined by indirect fluorescence. Results are the mean of three samples and are expressed as the reciprocal of antibody titre.

Note: Range of samples analysed was 1:50 - 1:1000.

Th2 response in these mice. Indeed, it may be that there is a requirement for the inhibitory interaction between IFNγ and IL-4 to induce the sequential Th1/Th2 mediated responses. It must be noted, however, that IL-12 is now regarded as the main cytokine involved in Th1 development and that IFNγ is a product of Th1 response which induces the cellular responses associated with Th1 $CD4^+$ T cell immunity (Manetti *et al.*, 1993). IFNγ dependent functions may have a pivotal role in the control of the switch between Th1 and Th2 regulated responses and hence the IFNγR deficient mice may have a dysfunctional $CD4^+$ T cell response because of the lack of IFNγ dependent cellular responses.

There are several explanations why a *P. chabaudi* infection of IFNγR deficient mice does not become sub-patent at the same time as in control mice. Increased parasite survival may occur due to the absence of IFNγ induced expression of adhesion molecules which could reduce not only the level of sequestration of mature pRBCs but reduce the recruitment of cells to the liver, which has been proposed as a site of a possible protective immune response (see Chapter 3). This is reflected in the reduced numbers of lymphoid, monocyte and PMN cells observed in the liver of *P. chabaudi* infected IFNγR deficient mice and may contribute to an increase in parasite survival. The IFNγR deficient mice do have an increased leukocytosis compared to control mice following a *P. chabaudi* infection (Favre *et al.*, 1997 and personal observations) which may be a consequence of a disruption in the redistribution of immune cells to organs such as the liver or spleen.

The failure of IFNγR deficient mice to clear the infection as efficiently as control mice could, as already mentioned, be attributed to the deficiency in IFNγ dependent humoral responses. In this study, there is a reduction in parasite-specific IgG production and total IgG2a levels in the IFNγR deficient mice. IgG2a has been proposed to have a protective role in experimental malaria infection (Waki *et al.*, 1995). It is possible that during the acute phase of infection, the parasite-specific isotype produced is IgG2a, a

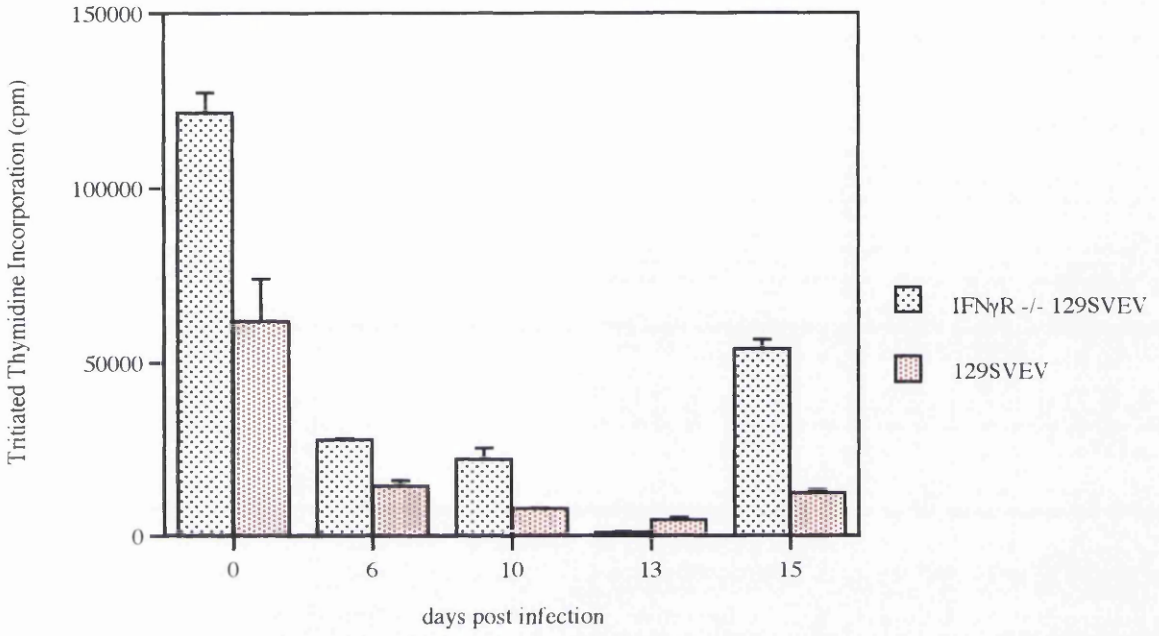


Figure 7.6. The proliferative response of splenocytes harvested from IFNγR deficient mice and control 129SVEV mice following inoculation with $1x10^{5}$ pRBCs of *P. chabaudi*. Three mice from both groups were sacrificed and the splenocytes pooled within each group and stimulated with Con A. The proliferative response was determined by the uptake of tritiated thymidine and each data point is the mean ± SD for an experiment performed in triplicate.

IFNγ dependent isotype (Snapper and Paul, 1987), which could explain the lower level of parasite-specific antibody production and the failure to resolve a *P. chabaudi* infection efficiently. However, it must be noted that the IFNγR deficient mice do produce detectable levels of total IgG2a demonstrating an IFNγ independent pathway of stimulation for IgG2a production.

The pleiotropic effects of IFNγ mean that there are deficiencies in several functions of the immune system in IFNγR deficient mice which could contribute to the inefficient development of protective immunity to *P. chabaudi* infection. IFNγ activation of macrophages and its influence on antigen presentation are processes which will be dysfunctional in the IFNγR deficient mice and will have a bearing on the outcome of a *P. chabaudi* infection. However, the possibility of compensatory mechanisms cannot be ignored and IFNγ independent pathways of activation of macrophages and NK cells, together could contribute to the degree of protective immunity observed during *P. chabaudi* infection in IFNγR deficient mice.

It is unclear why there is a high mortality rate in the IFNγR deficient mice following infection with the normally self-resolving *P. chabaudi.* Mortality is not consistently linked with fulminating parasitaemia and occurs throughout the course of a primary infection. The extent of the anaemia in the IFNγR deficient mice following *P. chabaudi* infection was the same as control mice (Favre *et al.*, 1997) and does not appear to contribute to the high mortality rate observed. It is unknown if there is an over-production of other inflammatory mediators such as IL-1 or TNF to compensate for the lack of IFNγ stimulated inflammatory responses. This would be similar to *P. chabaudi* infection of IL-10 mice which appear to succumb to an increase in pathology attributed to a combination of malaria toxins and an exacerbated inflammatory response (Linke *et al.*, 1996). However, recently IL-10 deficient mice were shown to survive and control both *P. yoelii* and *P. chabaudi adami* infection with a similar efficiency as control mice with no mortality observed (van der Heyde *et al.*, 1997). The increase in the

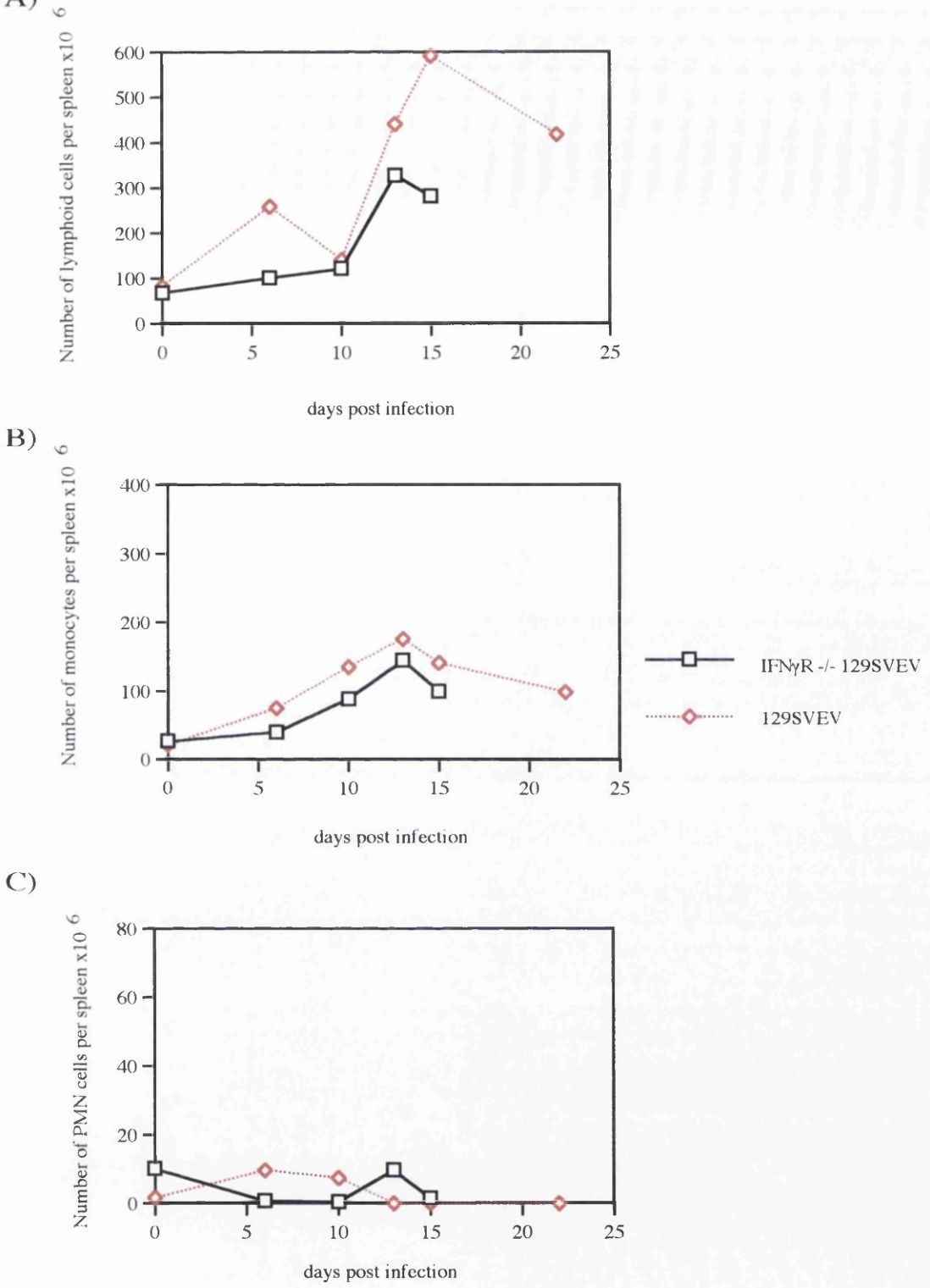


Figure 7.7. Cytological analysis of cells present in the spleen of IFN γR deficient 129SVEV mice and control 129SVEV mice following inoculation with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at each time point and the cells harvested were pooled and centrifuged. 500 cells were counted on each Giemsa's stained cytospin smear and the number of A) lymphoid, B) monocyte and C) PMN cells, is expressed per spleen.

proliferative response of splenocytes taken from *P. chabaudi* infected IFNγR deficient mice to Con A stimulation suggests that they may be primed to produce a greater response to infection but that this response is inappropriate because efficient control of the infection is not observed. Indeed it may be indicative of over-production of inflammatory mediators.

A significant quantity of total IgE was observed in the serum of IFNγR deficient mice during a *P. chabaudi* infection. IgE has recently been proposed as a pathogenic factor in *P. falciparum* infection (Perlmann *et al.*, 1997). Patients with cerebral malaria were shown to have significantly higher IgE levels compared to those with uncomplicated malaria (Perlmann *et al.*, 1994). The elevation of IgE may contribute to the pathology of severe disease without cerebral involvement and may be a pathogenic factor in *P. falciparum* infection in general (Perlmann *et al.*, 1997). The proposed mechanism involved in IgE mediated pathology is the interaction of IgE with the low-affinity FcεRII (CD23) receptor which is expressed on a variety of cells including monocytes/macrophages, eosinophils and B cells (Perlmann *et al.*, 1997). This process can induce the production of cytokines such as TNF which has been shown to be involved in the pathogenesis of malaria infection (Grau *et al.*, 1987, Clark, Rockett and Cowden, 1991). There does not appear to be a correlation between the level of parasite-specific IgE and pathogenicity but rather, it is the quantity of IgE that correlates with disease severity (Perlmann *et al.*, 1994, Perlmann *et al.*, 1997). Hence although the quantity of parasite-specific IgE in the IFNγR deficient mice during a *P. chabaudi* infection is unknown, the significant total IgE response may contribute to the pathology observed (high mortality rate) through the cross-linking of CD23 receptors on effector cells such as monocytes, resulting in the over-production of inflammatory mediators. Measurement of the level of TNF and NO production would confirm if this is a mechanism which is a contributory factor in the high mortality rate observed in the IFNγR deficient mice following a *P. chabaudi* infection.

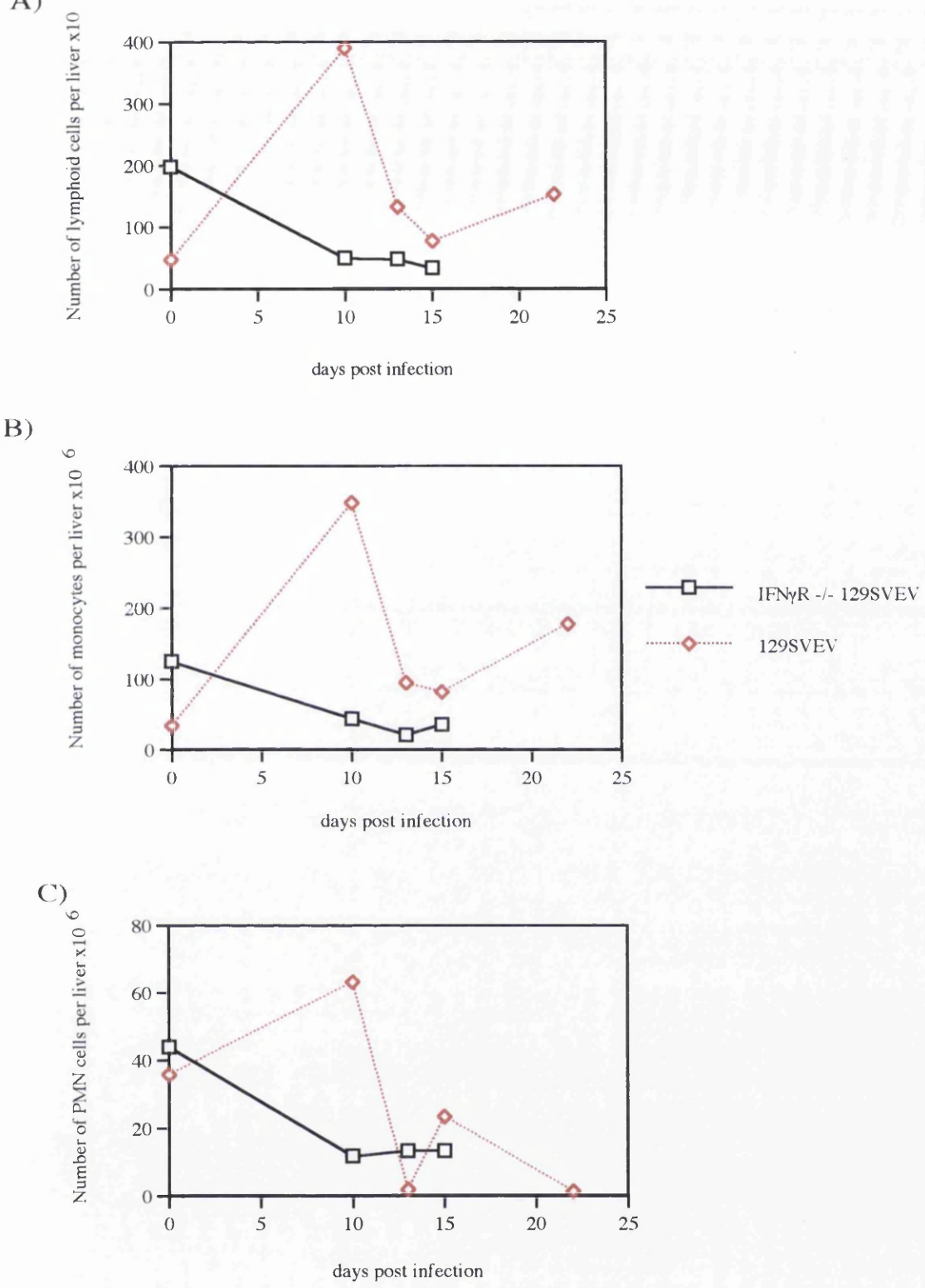


Figure 7.8. Cytological analysis of the cells present in the livers of IFNγR deficient 129SVEV mice and control 129SVEV mice following infection with $1x10^5$ pRBCs of *P. chabaudi*. Three mice from each group were sacrificed at each time point and the cells harvested were pooled and centrifuged. 500 cells were counted on each Giemsa's stained cytospin smear and the number of A) lymphoid, B) monocyte and C) PMN cells, is expressed per liver.

The infection of IFNγR deficient mice with *P. chabaudi* provides a model which may contribute to the understanding of the development of protective immunity to blood-stage malaria. Analysis of cytokine production by re-stimulation of splenocytes *in vitro*, tissue-specific mRNA expression and levels in the serum is planned and may identify if there is a bias to a Th2 response indicated by the significant IgE production or if it is an over-production of inflammatory cytokines such as IL-1, TNF and IL-6 that is detrimental to the host's response to infection.

Chapter Eight

The effect of the nitric oxide donor, SNAP, on the growth of malaria parasites *in vitro*.

Introduction

NO is produced when L-arginine is oxidised to produce one molecule each of L-citrulline and NO. This process is catalysed by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS are expressed in mammals, two of which, NOS1 and NOS3, are dependent on elevated intracellular Ca^{2+} (MacMicking, Xie and Nathan, 1997) and are expressed constitutively. The third isoform, NOS2, is independent of intracellular Ca^{2+} concentration and is termed inducible NOS (iNOS) (Xie *et al.*, 1992, Green and Nacy, 1993). NOS1 and NOS3 are often termed cNOS and produce a low output of NO which mediates the physiological functions of NO. NOS2 (iNOS) produces a high output of NO which is stimulated during inflammation and infection. Production of NO by NOS requires the attachment of several molecules including haeme, tetrahydrobiopterin, calmodulin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (MacMicking, Xie and Nathan, 1997). The difference in NO output between cNOS and iNOS can be attributed first to the ability of iNOS to bind calmodulin tightly independent of intracellular Ca^{2+} concentration and secondly to the sustained response of iNOS to inflammatory stimuli (Green and Nacy, 1993, MacMicking, Xie and Nathan, 1997).

Activation of iNOS requires a priming agent and a secondary signal. IFNγ, LPS, TNF and other cytokines have all been shown to induce iNOS expression and subsequent NO production (Weinberg, Chapman and Hibbs, 1978, Amber *et al.*, 1988, Ding, Nathan and Stuehr, 1988, Drapier, Weitzerbin and Hibbs, 1988). Induction of iNOS by IFNγ and LPS occurs at the level of transcription (Xie, Whisnant and Nathan, 1993, Martin, Nathan and Xie, 1994). The promoter region of the iNOS gene contains consensus sequences for the binding of transcription factors which are involved in the induction of several IFNγ inducible genes (Harada *et al.*, 1989, Xie, Whisnant and Nathan, 1993, Kamijo *et al.*, 1994, Martin, Nathan and Xie, 1994). Inhibition of iNOS expression by cytokines such as IL-4, IL-10 and TGFβ (Ding *et al.*, 1990, Gazzinelli *et al.*, 1991, Cunha, Moncada and Liew, 1992, Oswald *et al.*, 1992) is an important control

mechanism which prevents inappropriate production of NO. Inducible NOS activity is also inhibited by NO (Assruey *et al.*, 1993), demonstrating an important self-regulatory feedback mechanism.

NO can interact with other free radicals to yield various intermediates which are usually short-lived molecules but can influence the biological function of NO (MacMicking, Xie and Nathan, 1997). The physiological function(s) of NO includes vasodilation, enzyme regulation and neurotransmission (Moncada, Palmer and Higgs, 1991). The role of NO in response to inflammation or infection has been the focus of extensive research. NO has been shown to have microbicidal activity against bacteria, protozoan and helminth parasites, fungi and tumour cells. The cytotoxic potential of NO, produced by activated macrophages was first observed in tumour immunology studies (Hibbs *et al.*, 1988). The target of NO mediated inhibition of tumour cell growth was iron-sulphur complexes present in enzymes essential for mitochondrial respiration (Hibbs *et al.*, 1988). Subsequently, NO was shown to inhibit DNA synthesis in tumour cells through inhibition of ribonucleotide reductase which is the rate-limiting step in DNA synthesis (Kwon, Stuehr and Nathan, 1991).

NO is considered to be one of the major cytotoxic mechanisms in host responses to protozoan infection. *L. major* actually invades the macrophage, a major source of NO, but the production of NO has been shown to be the main effector mechanism against *L. major* infection (Green *et al.*, 1990, Liew *et al.*, 1990). Control of *T. gondii* infection has been shown to involve NO during the chronic phase of infection (Gazzinelli *et al.*, 1993). Down-regulation of iNOS activity is thought to lead to re-activation of *T. gondii* infection. Killing of helminth parasites has been partially attributed to NO (James and Hibbs, 1990) possibly through inhibition of enzymes containing iron-sulphur complexes that are important in metabolic function. *Clostridium* and *Eschericha coli* have also been shown to be susceptible to NO mediated cytotoxicity (Kwon, Stuehr and Nathan, 1991, Green and Nacy, 1993).

NO production was identified to be one of the main effector mechanisms in the distruction of intra-hepatic malaria parasites (Nussler *et al.* 1991a). IL-6, IL-1, TNF and IFNγ have been shown to stimulate L-arginine dependent elimination of exo-erythrocytic stages of the parasite. Several cells in the liver have the potential to produce NO, such as endothelial cells, Kupffer cells and the hepatocytes themselves (Nussler *et al.,* 1991a). The protective role of NO against the exo-erythrocytic stage is thus well established but no definitive role for NO during the blood stage of infection has been obtained. The demonstration of killing of *P. falciparum in vitro* by NO derivatives (Rockett *et al.,* 1991) illustrated a possible direct, cytotoxic effect of NO against blood stage parasites. Exogenous TNFα, lymphotoxin and IL-1 can induce *in vivo* production of nitrite and nitrate in *P. vinckei* infected mice (Rockett *et al.,* 1992). The authors suggest that this indicates a possible role for inflammatory cytokines and NO production in the pathology associated with severe malaria. However, they do not record if the increase in nitrite and nitrate levels following administration of the cytokines, results in any alterations in the course of the *P. vinckei* infection.

The role of NO in cerebral malaria is controversial. Cerebral malaria caused by *P. falciparum* has conventionally been explained as resulting from blockage of the blood supply by parasitised erythrocytes which sequester in the cerebral microvasculature (Aikawa, 1988). The argument against this explanation is that those patients who recover from cerebral malaria but do not have the neurologic impairment associated with long term oxygen deprivation (Clark, Rockett and Cowden, 1992). Immunological analysis of the cause of coma during cerebral malaria infection has provided evidence for a NO-dependent pathology mechanism but also a protective role during the severe complications of malaria infection (see below).

In both experimental model and human infections, TNFα appears to play a pivotal role. Patients with cerebral malaria, demonstrate high levels of TNFα in plasma

(Kwiatkowski *et al.,* 1990, Grau *et al.,* 1989b). Individuals homozygous for the TNF2 allele, a variant of the TNFα gene promoter region, have a greatly increased risk for death or severe neurologic sequelae due to cerebral malaria (McGuire *et al.,* 1994). In the *P. berghei* ANKA mouse model of cerebral malaria, treatment with anti-IFNγ reduced the excessive overproduction of TNFα and prevented the development of cerebral lesions (Grau *et al.,* 1989a).

The mechanisms by which sequestered parasitised erythrocytes cause coma are unclear but the hypothesis is that the parasitised erythrocytes bind to specific receptors on endothelial cells via host adhesion molecules such as ICAM-1. Expression of adhesion molecules is increased during cerebral malaria as part of a systemic endothelial activation by inflammatory molecules. The adhesion of parasitised erythrocytes to endothelial cells in the microvasculature of the brain may stimulate local production of TNFα which in turn stimulates NO production. TNFα induced NO can then diffuse through the blood brain barrier and interfere with neurologic function possibly by stimulating guanylate cyclase in neurons. This would result in an increase in cyclic GMP levels leading to further stimulation of NO production, resulting in brain dysfunction (Asension *et al.,* 1993). This may be due to the toxic properties of NO (Snyder, 1992) or through inhibition of NO synthase activity by NO (Griscavage *et al.,* 1993) hence, disrupting local neurotransmission in the brain. NO may also be responsible for the increase in intracranial pressure observed in cerebral malaria patients through an increase in vasodilation (Newton *et al.,* 1991).

Further evidence supporting the pathological role attributed to TNF induced NO production and up-regulated ICAM-1 expression is supplied by *P. berghei* ANKA infection of TNFα/β double deficient mice. These mice have a complete disruption of TNF signalling pathways and hence no TNF production. *P. berghei* ANKA infection in intact mice induces fatal cerebral malaria with death occurring within 5 to 8 days. The TNFα/β deficient mice are completely resistant to *P. berghei* ANKA induced cerebral

malaria (Rudin *et al.,* 1997a). Furthermore, systemic release of NO and up-regulation of ICAM-1 expression on endothelial cells were only observed in the control mice. TNF also appears to be involved in the recruitment of mononuclear cells and microvascular damage because vascular leakage and perivascular haemorrhage plus mononuclear cell adhesion to endothelial cells were absent in the TNFα/β deficient mice (Rudin *et al.,* 1997a). The absence of adhesion by mononuclear cells could probably be attributed to the lack of ICAM-1 expression in the TNFα/β deficient mice during infection.

In contrast to these results the role of NO in the development of experimental cerebral malaria has been shown to be minimal. L-NMMA treatment had no influence on the course of murine cerebral malaria development (Kremsner *et al.,* 1993, Asension *et al.,* 1993). Other data suggests that the production of NO during cerebral malaria may be beneficial rather than pathological. *In vivo* inhibition of NO production results in an increased leukocyte adherence to endothelial cells via CD11/CD18 integrins (Kubes, Suzuki and Granger, 1991). Anti-CD11a treatment abrogated the development of experimental cerebral malaria indicating that CD11/CD18 mediated leukocyte adhesion is crucial in the pathogenesis of this experimental cerebral malaria (Falanga and Butcher 1991, Grau *et al.,* 1991). NO has been shown to inhibit leukocyte-endothelial interactions. Neutrophil adhesion to endothelial cells is inhibited by NO (Niu, Ibbotson and Kubes, 1996) and NO has also been shown to suppress ICAM-1 expression on endothelial cells (Biffl *et al.,* 1996). Hence, NO may suppress the recruitment of leukocytes by down-regulating CD11/CD18 mediated adhesion or the expression of other involved adhesion molecules.

The constriction of intracerebral arterioles occurs during human and experimental cerebral malaria (Polder, Jerusalem and Eling, 1991). Both NO and prostacyclin can induce vasodilation and inhibit platelet aggregation (Hyslop and De Nucci 1991). Administration of a prostacyclin analogue prevented the development of cerebral malaria suggesting that the vasodilatory property of NO may be protective during cerebral

malaria. There is, therefore, strong evidence from experimental cerebral malaria to support a protective role for NO during malaria infection. However, results obtained from the experimental model of cerebral malaria cannot be readily extrapolated to human cerebral malaria because the pathology observed in the model result from the accumulation of leukocytes, rather than parasitised erythrocytes in the cerebral blood vessels. The role of NO during human cerebral malaria has been investigated. There have been several studies which have analysed NO end products, reactive nitrogen intermediates (RNI), in plasma of patients. Contradictory results, supporting both a pathological and a protective role for NO have emerged.

RNI levels in Tanzanian children were inversely related to disease severity, with levels highest in subclinical infections and lowest in fatal cerebral malaria. IL-10, a cytokine which can suppress NO production (Cunha, Moncada and Liew, 1992), increased with severity (Anstey *et al.*, 1996). The suppression of NO synthesis in cerebral malaria may contribute to pathogenesis because NO may have a protective role during infection. Another study, proposing a protective role for NO was carried out on Gabonese patients. (Kremsner *et al.*, 1996). High plasma RNI levels during severe malaria could be correlated with an accelerated cure. In contrast with these two studies, a study performed on cerebral malaria patients from Papa New Guinea suggests a pathological role for NO (Al-Yaman *et al.*, 1996). The RNI levels measured were compared to disease severity and clinical outcome, and correlated with the depth and duration of coma. Higher RNI levels were observed in patients with deeper coma and with longer duration of coma. Fatal outcomes were correlated with significantly higher RNI levels. The results suggest that NO is involved in the development of coma during cerebral malaria.

Immunosuppression during malaria infection is well documented (reviewed in Weidanz, 1983). Malaria infected children have more severe gastro-intestinal and respiratory infections than normal children (Greenwood *et al.*, 1972). Malaria has also been

reported to impair the efficacy of childhood vaccination against tetanus, typhoid and meningococcal disease (Williamson and Greenwood 1978). A poor proliferative response of lymphocytes to Con A and other mitogens parallels the degree of immunosuppression in both human (MacDermott *et al.,* 1980) and rodent infections (Correa, Narayanon and Miller 1980). During trypanosome infection T cell responses are suppressed. This phenomenon has been shown to be mediated via suppressor macrophages which down-regulate T cell proliferative responses through NO and prostaglandin dependent mechanisms (Schleifer and Mansfield, 1993). The addition of L-NMMA to splenocyte cultures from malaria infected mice, restored the proliferative response of the splenocytes to similar levels as controls (Rockett *et al.,* 1994) suggesting that NO may mediate antigen-specific immunosuppression observed during malaria infection. NO mediated immunosuppression has also been observed in burn-injured rats (Bamberger *et al.,* 1992) and an increase in NO synthase in pregnant rats is thought to mediate the immunosuppression observed during pregnancy (Conrad *et al.,* 1993). There are several different mechanisms through which NO may induce immunosuppression. NO appears to suppress T cell clonal expansion but not T cell effector cell maturation and cytokine secretion (Schleifer and Mansfield, 1993). Hence, the suppression mediated by NO may occur as a consequence of discrete signalling events. This could be achieved by down-regulation of IL-2 receptor expression, modification of the CD3/TCR complex or modulation of accessory molecules which are important for the expression of T cells. NO could also inactivate components of the mitochondrial respiratory chain (Stuehr and Nathan, 1989) resulting in a reduction in cellular metabolic energy levels or it may inactivate ribonucleotide reductase which is required for DNA synthesis and hence cell proliferation (Kwon, Stuehr and Nathan, 1991).

The microbicidal activity of NO generated by a L-arginine dependent pathway has been extensively demonstrated during *T. gondii* (Adams *et al.,* 1990, Marletta *et al.,* 1988) and *L. major* infections (Green *et al.,* 1990, Liew *et al.,* 1990). As already mentioned

NO has been shown to inhibit the intrahepatic development of malaria parasites (Schofield *et al.,* 1987a, Nussler *et al.,* 1991a). The role of NO during the blood stage of infection, as discussed is unclear. In *P. chabaudi* infection, a NO peak coincides with peak parasitaemia (Taylor-Robinson *et al.,* 1994a). The increase in NO production may be necessary for the host to control the acute infection because L-NMMA given to mice results in an exacerbation of the parasitaemia (Taylor-Robinson *et al.,* 1993). During *P. chabaudi* infection, Th1 cells appear to control the primary parasitaemia with a switch to Th2 mediated immunity after the peak parasitaemia. It has been suggested that NO may be the molecule which induces the switch in immunity because it is capable of down-regulating Th1 cell functions (Taylor-Robinson and Phillips 1994b, Taylor-Robinson *et al.,* 1994a).

There is no substantial evidence for NO having a direct cytotoxic effect on parasitised erythrocytes. *In vitro* experiments performed by Rockett and colleagues (1991) demonstrated that derivatives of NO can inhibit parasite growth. However, the levels of the derivatives they used may not reflect physiological levels of NO produced during a malaria infection. This chapter describes the effect of NO, generated by s-nitroso-acetyl-penicillamine (SNAP), on malaria parasites *in vitro.* The effect on various stages of *P. falciparum* was examined and also the effect of SNAP on the rodent malarial parasites, *P. chabaudi* and *P. berghei.* The hypothesis that haemoglobin may quench NO in the bloodstream (Sternberg *et al.,* 1994) and prevent any direct effect against the parasite was also investigated.

Results

Generation of NO *in vitro* by SNAP

SNAP was freshly prepared in complete malaria medium (45-363μM, final concentration). Complete malaria medium alone was included as a control. All cultures were incubated at 37°C and supernatants were harvested at the time points indicated over a 72 hour period (Figure 8.1). Nitrite levels in the supernatant were measured by the Griess reaction as an indication of NO production by SNAP. The generation of NO by SNAP occurred over 24 hours for all the concentrations of SNAP analysed (Figure 8.1).

Inhibition of *P. falciparum* growth in the presence of SNAP

The effect of SNAP generated NO, on the growth of *P. falciparum* was determined by culturing the parasites in the presence of the range of concentrations of SNAP indicated and observing the effect on the uptake of tritiated hypoxanthine by the parasite. Controls contained either medium alone or equivalent concentrations of DL penicillamine or sodium nitrite. Neither of these two substances, at the concentrations used, inhibited parasite growth and therefore all the control values have been combined. In figure 8.2A and 8.2B, the *P. falciparum* cultures were initiated with late (trophozoite) stages. SNAP was added at the start of the experiment or after 24 hours when the majority of the parasites had re-invaded new erythrocytes and were now ring stages. The tritiated hypoxanthine was added at the same time as the SNAP and the cultures were harvested 24 hours later. SNAP at concentrations as low as 45μM could inhibit the growth of *P. falciparum* indicated by the reduction in the uptake of tritiated hypoxanthine compared with the controls. Cultures containing trophozoites (Figure 8.2A) appear to be more susceptible to the effect of SNAP than cultures containing mainly ring stages (Figure 8.2B). Examination of smears taken from the cultures

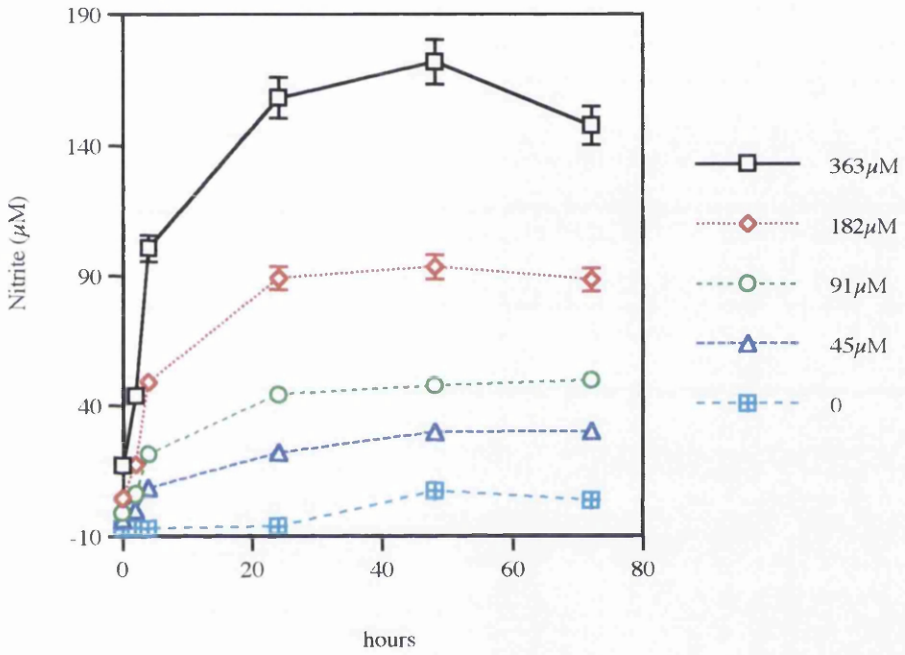


Figure 8.1. SNAP was freshly prepared and incubated at 37°C. Culture supernatants were harvested over a 72 hour period. Nitrite levels were measured by the Griess reaction as an indication of NO production by SNAP. Each data point is the mean ± SD combined from two replicate experiments.

indicated that the higher concentrations of SNAP were killing parasites but that the lower concentrations of SNAP, parasites appeared to be developing more slowly than parasites from control cultures. This suggests that SNAP at lower concentrations may be having a cytostatic effect on the growth of parasites.

Cytostatic effect of NO, generated by SNAP, on the growth of *P. falciparum*

To investigate the possible cytostatic effect of low concentrations of SNAP on the growth of *P. falciparum*, SNAP at a concentration of 91μM was added to cultures containing trophozoites. The tritiated hypoxanthine was then added at the times indicated on Figure 8.3. All the cultures were harvested at 31 hours after the addition of SNAP. As the amount of NO production declined (see Figure 8.1), the uptake of tritiated hypoxanthine increased to levels closer to controls. Smears from the cultures confirmed that during the period of NO generation by SNAP, the trophozoite stages were viable but not developing. However, at the end of the experiment, after NO production had ceased, the parasites had developed and some were undergoing division and re-invasion.

A different approach to investigating the cytostatic role of NO generated by SNAP was to add 91μM of SNAP to cultures containing trophozoites and add the tritiated hypoxanthine at the same time as the SNAP but harvest the cultures at the times indicated on Figure 8.4. SNAP had a greater inhibitory effect on the growth of the parasites during the early part of the experiment but as the duration of the experiment neared the cessation of NO generation by SNAP, a recovery in parasite growth was observed. This suggests a possible cytostatic role for NO rather than a cytotoxic effect on the growth of malaria parasites *in vitro*.

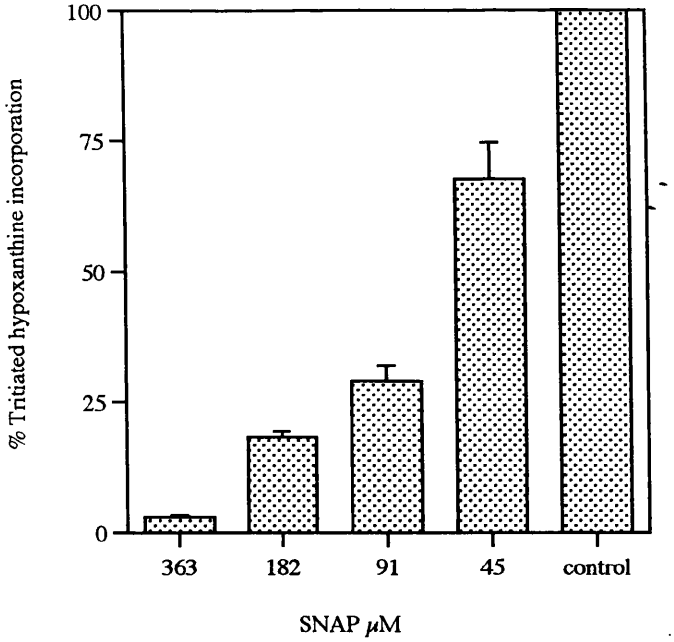


Figure 8.2A. The effect of SNAP on the growth of trophozoite stages of *P. falciparum in vitro* . Cultures were initiated with trophozoite stages. SNAP and tritiated hypoxanthine were added at the start (0 hours) and the cultures were harvested 24 hours later. Incorporation of tritiated hypoxanthine is shown relative to controls. Each point is the mean ± SD of an experiment performed in triplicate.

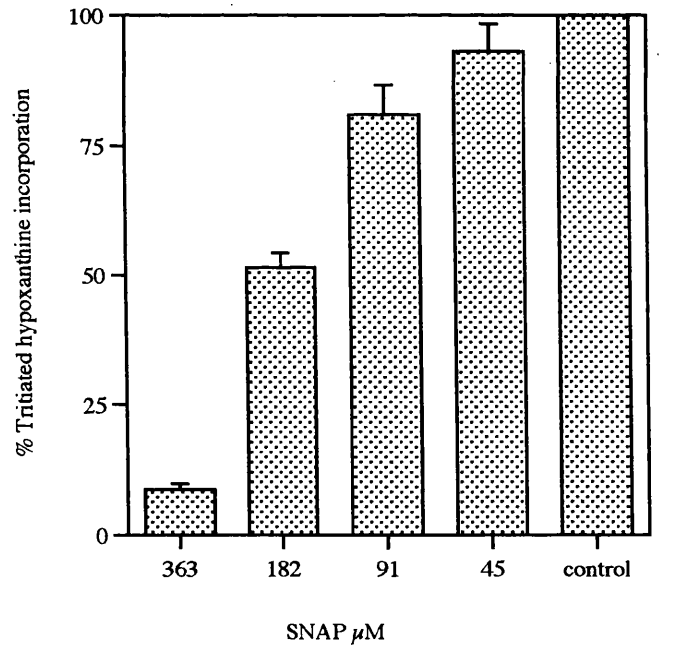


Figure 8.2B. The effect of SNAP on the growth of ring stages of *P. falciparum in vitro*. Cultures contained mainly ring stages. SNAP and tritiated hypoxanthine were added at 0 hours and the cultures were harvested 24 hours later. Incorporation of tritiated hypoxanthine is shown relative to controls. Each point is the mean ± SD of an experiment performed in triplicate.

The effect of NO generated by SNAP on the growth of the murine malaria parasites, *P. chabaudi* and *P. berghei in vitro*.

The effect of SNAP on the growth of *P. chabaudi* and *P. berghei* under *in vitro* culture conditions was investigated. Mice, infected with either parasite were bled on the day of the experiment. The parasitaemia of both parasites was adjusted to 1% by the addition of non-infected erythrocytes and cultures at a 5 % haematocrit. SNAP was added to the cultures at the concentrations indicated on Figure 8.5 and 8.6. Tritiated hypoxanthine was added at the same time as SNAP and the cultures were harvested 24 hours later. Figure 8.5 shows the effect SNAP had on the growth of *P. chabaudi in vitro*. SNAP was able to inhibit *P. chabaudi* growth at 91μM. The effect of the higher concentrations of SNAP, appears to be lessened when in culture with the murine parasite, *P. chabaudi*, compared to the results observed for the human parasite, *P. falciparum*. This observation is confirmed by the results obtained for the effect of SNAP on the growth of *P. berghei in vitro* (Figure 8.6). The protocol was designed exactly the same as the *P. chabaudi* experiment. SNAP had an inhibitory effect on the growth of *P. berghei in vitro* but only at 182μM and above, thus confirming the reduced effect of NO generated by SNAP on murine malaria parasites compared with the effect of NO on the growth of *P. falciparum*.

An increase in haemoglobin levels does not reduce the effect of NO generated by SNAP on the growth of malaria parasites *in vitro*.

NO can bind to the haeme group of haemoglobin (Hakim *et al.*, 1996) and it is possible that this may dilute any effect against the intra-erythrocytic malaria parasite. This hypothesis was investigated by using *P. chabaudi in vitro* at various parasitaemia obtained by the addition of non-infected erythrocytes. SNAP and the tritiated hypoxanthine were added at the same time and the cultures were harvested 24 hours

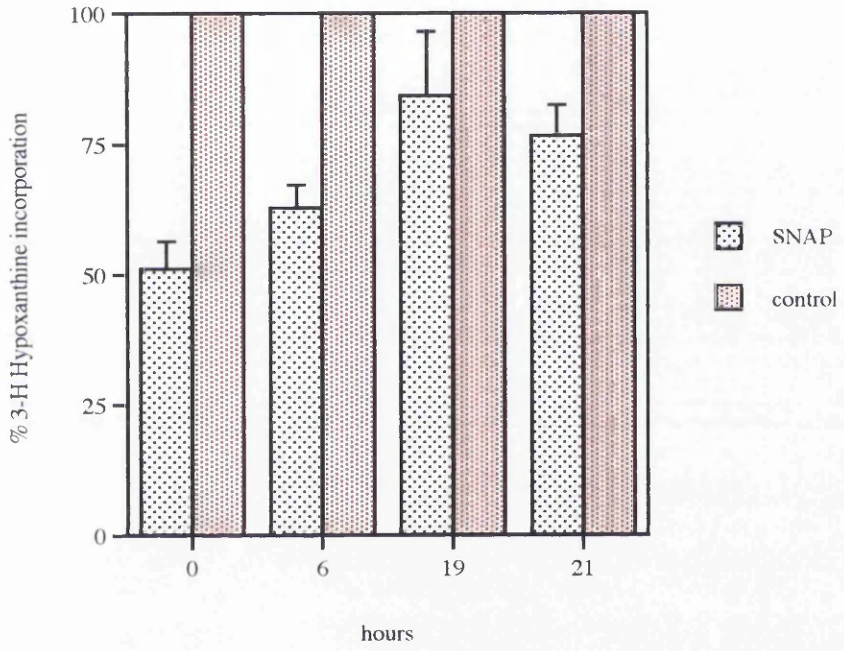


Figure 8.3. Cytostatic effect of NO on the growth of *P. falciparum in vitro*. SNAP at 91μM was added to trophozoite stages. Tritiated hypoxanthine was added at 0, 6, 19 and 21 hours. The cultures were harvested at 31 hours. Incorporation of tritiated hypoxanthine is shown relative to controls. Each point is the mean ± SD of an experiment performed in triplicate.

later. The parasites were cultured at 18%, 9%, 4.5% and 2.25% in the presence of SNAP. Inhibition of parasite growth was observed consistently at 91 μM SNAP and above with no decrease in the inhibitory effect of SNAP generated NO, as the number of non-infected erythrocytes increased (Figure 8.7). Therefore, the increase in the haemoglobin levels present in the cultures does not appear to suppress the inhibitory effect of NO, generated by SNAP, on the growth of malaria parasites.

Discussion

The *in vitro* experiments performed demonstrate that NO, generated by SNAP can inhibit the growth and development of malaria parasites. At concentrations of SNAP above 182μM, the growth of the asexual erythrocytic parasites was inhibited. However, at lower concentrations of SNAP, the trophozoite stage of the parasite life cycle, appeared more sensitive to the inhibitory effect of the NO donor, SNAP, than earlier ring stages. The effect of NO on the growth of malaria parasites *in vitro*, was observed not only on the human malaria parasite, *P. falciparum* but also the murine malaria species, *P. chabaudi* and *P. berghei.* Hence, these results suggest that NO may inhibit the growth and development of the malaria parasite within the erythrocyte.

NO, generated by SNAP, has been shown to kill *Trypanosoma cruzi* trypomastigotes *in vitro* (Vespa, Cunha and Silva, 1994) and *L. major* (Assreuy *et al.,* 1994), demonstrating the importance of NO during certain parasitic infections. Interestingly, NO does not have a protective role during *T. brucei* infection (Sternberg *et al.,* 1994). Indeed, the immunosuppression observed during *T. brucei* infection is mediated by NO and inhibition of NO production results in a reduced parasitaemia (Sternberg *et al.,* 1994). This illustrates how NO can have a varied influence on the immune response to infections. NO has been proposed to mediate the pathology observed during cerebral malaria (Clark, Rockett and Cowden, 1991) and also immunosuppression (Rockett *et al.,* 1994). The results presented in this study, propose a protective role for NO against the asexual erythrocytic stage of malaria. Previous work demonstrated killing of *P.*

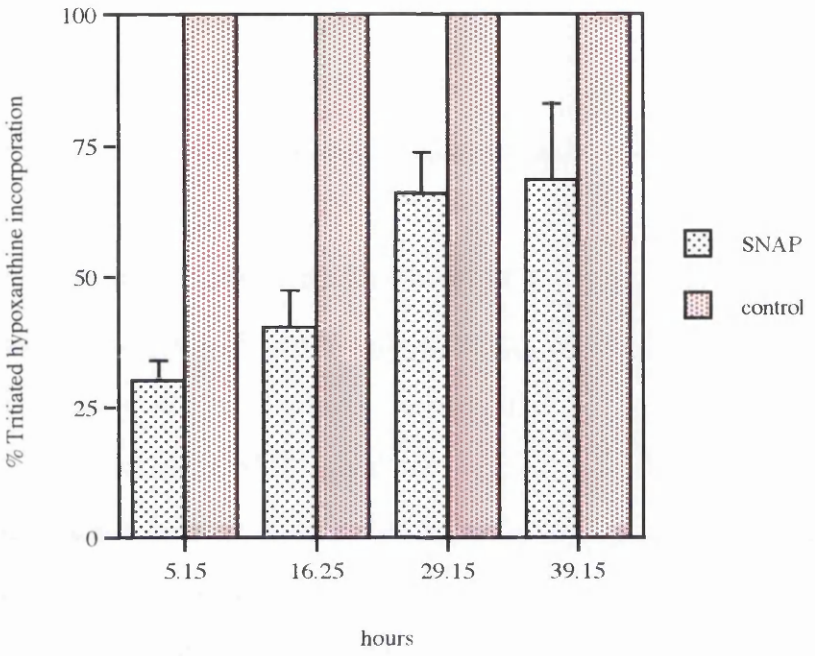


Figure 8.4. The recovery of parasite growth as the production of NO declines. SNAP at 91μM was added to trophozoite stages. Tritiated hypoaxanthine was added at 0 hours. The cultures were harvested at 5.15, 16.25, 29.15 and 39.15 hours. Incorporation of tritiated hypoxanthine is shown relative to controls. Each point is the mean ± SD of an experiment performed in triplicate.

falciparum in vitro by NO derivatives (Rockett *et al.,* 1991). Nitrosothiol derivatives of cysteine and glutathione were found to be the most effective compounds used. Nitrite was also reported to kill parasites *in vitro* (Rockett *et al.,* 1991), however, nitrite was found to have no inhibitory effect at levels equivalent to SNAP in the studies reported here. These contradictory results may be explained by the fact that Rockett and colleagues (1991) observed killing of *P. falciparum* by sodium nitrite at 30 times the concentration used in the experiments undertaken in this study.

In vivo studies have failed to define the role of NO during the asexual erythrocytic stage of malaria infection. *P. chabaudi* infected mice, receiving NMMA treatment, demonstrated a marked increase in parasitaemia (Taylor-Robinson *et al.,* 1993). Previous studies, using NMMA and aminoguanidine as NO synthase inhibitors, did not demonstrate any increase in the parasitaemia during *P. chabaudi* infection but did report an exacerbation in susceptibility to infection (Rockett *et al.,* 1994, Jacobs, Radzioch and Stevenson, 1995, Amante and Good, 1997). Different protocols may account for the conflicting results because Taylor-Robinson *et al.,* (1993) were using thymectomised mice and transfer of T cell clones while the other studies were using immunocompotent mice. Therefore, NO may be protective by a mechanism other than being parasiticidal. Mice pre-treated with NMMA, infected with *P. vinckei* and treated with chloroquine plus IFNγ exhibited higher mortality and more pronounced liver and kidney lesions (Kremnser *et al.,* 1992). This suggests that NO may act as a tissue-protective molecule during malaria infection either by inactivating tissue damage by oxygen radicals or inhibiting leukocyte adhesion to endothelium and preventing hypoxic tissue damage (Jacobs, Radzioch and Stevenson, 1995). Further evidence for this hypothesis comes from human malaria infection. Patients infected with *P. falciparum* or *P. vivax* have increased plasma levels of RNI (Nussler *et al.,* 1994). Also, the duration of coma due to cerebral malaria is shorter in children with relatively high NO plasma levels (Cot *et al.,* 1994).

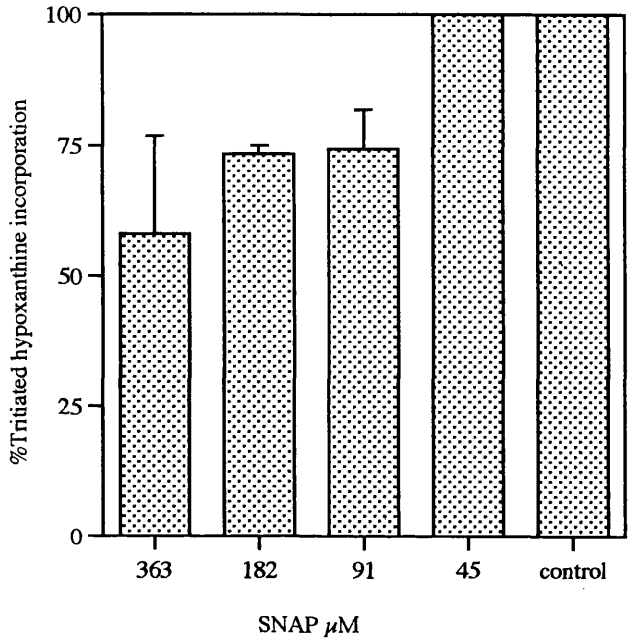


Figure 8.5. The effect of NO, generated by SNAP, on the growth of *P. chabaudi in vitro*. SNAP and tritiated hypoxanthine were added at 0 hours. The cultures were harvested 24 hours later. Incorporation of tritiated hypoxanthine is shown relative to controls. Each point is the mean ± SD of triplicates and is data combined from two replicate experiments.

Although, NO has been shown to be cytotoxic to *L. major* (Liew *et al.,* 1990) and *T. gondii* (Adams *et al.,* 1990) *in vivo*, there is insufficient evidence to confirm a direct cytotoxic effect of NO on malaria parasites *in vivo.* However, NO may act on the intraerythrocytic parasite in a cytostatic manner. As NO production by SNAP ceased, it was observed that the parasites resumed growth suggesting a cytostatic effect rather than cytotoxic. To elucidate how NO could be exerting a cytostatic effect on the growth of the malaria parasite, the mechanisms involved in the development of the intraerythrocytic phase of the complex life cycle have to be examined. An important process which is a likely candidate for interference by NO, is the reduction of ribonucleotide diphosphates to deoxyribonucleotide triphosphates which are needed for DNA synthesis. This reaction is catalysed by the enzyme, ribonucleotide reductase (RR) which is a target of macrophage derived NO cytotoxicity to tumour cells (Kwon, Stuehr and Nathan, 1991). RR is the rate-limiting step in DNA synthesis, depends on thiols and a non-haeme iron in its reaction centre (Stubbe, 1990) which maintain a key trosyl radical (Ochiai *et al.,* 1990). Thus, RR is a candidate for inactivation by NO. Activated macrophages and hydroxyurea, a pharmacological inhibitor of RR, were both shown to have a similar cytostatic effect on DNA synthesis in tumour cells (Kwon, Stuehr and Nathan, 1991, Lepoivre *et al.*, 1991). Iron chelation treatment of *P. falciparum* infected erythrocytes interferes with iron-dependent metabolism of malaria parasites and inhibits their development (Lytton *et al.,* 1994). The mechanism of this inhibition is to block DNA synthesis catalysed by RR. The iron chelators block integration of iron into newly synthesised R2 subunits rather than removal of iron from fully assembled RR protein. A similar inhibition of parasite growth was observed using hydroxyurea (Rubin *et al.,* 1993). Parasites were able to recover from iron chelation after removal of the drug depending upon the treatment regime and drug properties (Lytton *et al.,* 1994). These observations mirror the results obtained for NO mediated inhibition of malaria parasites *in vitro.* As the generation of NO by SNAP neared completion, the parasites were able to recover which is similar to the recovery of parasite growth following iron chelation treatment.

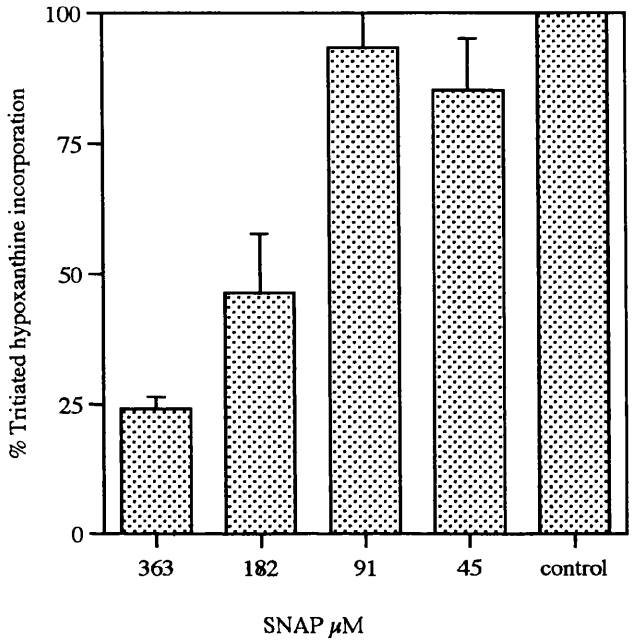


Figure 8.6. The effect of NO, generated by SNAP, on the growth of *P. berghei in vitro.* SNAP and tritiated hypoxanthine were added at 0 hours. The cultures were harvested 24 hours later. Incorporation of tritiated hypoxanthine is shown relative to controls. Each point is the mean ± SD of triplicates and is data combined from two replicate experiments.

Mature parasites, trophozoites and schizonts are more susceptible to both NO and iron chelators probably because peak RR activity is found at this stage of parasite development (Rubin *et al.,* 1993). Hence, lower RR activity in the ring stages may account for the increased resistance to NO and iron chelation treatment. Another factor influencing the effect of NO and iron chelators is access to the parasite. This is dependent upon the permeability of the erythrocyte membrane and the stage of the intraerythrocytic parasite. The parasite causes alterations in the erythrocytic membrane to facilitate the movement of nutrients and waste products. A tubovesicular membrane network extending from the parasite vacuole membrane probably has a central role in this process (Deitsch and Wellems, 1996). As the parasite matures, changes in the permeability of the erythrocyte membrane occur, allowing access to small molecular weight molecules to the parasite possibly via the parasitophorous duct which is an extension of the tubovesicular membrane and allows transfer of molecules between the parasite and extracellular environment (Deitsch and Wellems, 1996). Therefore, susceptibility of trophozoites to NO, compared with ring stages, is because NO has easier access to the parasite and that peak activity of RR, one of the main candidate targets, occurs during mature parasites. There are other possible targets for NO mediated inhibition of parasite growth such as mitochondria respiratory functions which are catalysed by iron containing enzymes. The cytostatic inhibition of parasite growth by NO may result in the asynchronous growth observed at peak parasitaemia during an infection.

An important observation made during these studies was that an increase in the levels of haemoglobin did not quench the effect of NO on the growth of the parasite. It had been proposed that erythrocytes would act as a sink for NO because of the scavenging activity of haemoglobin (Sternberg *et al.,* 1994), thus preventing any anti-parasite effect of NO in the bloodstream. However, increasing the levels of haemoglobin was found not

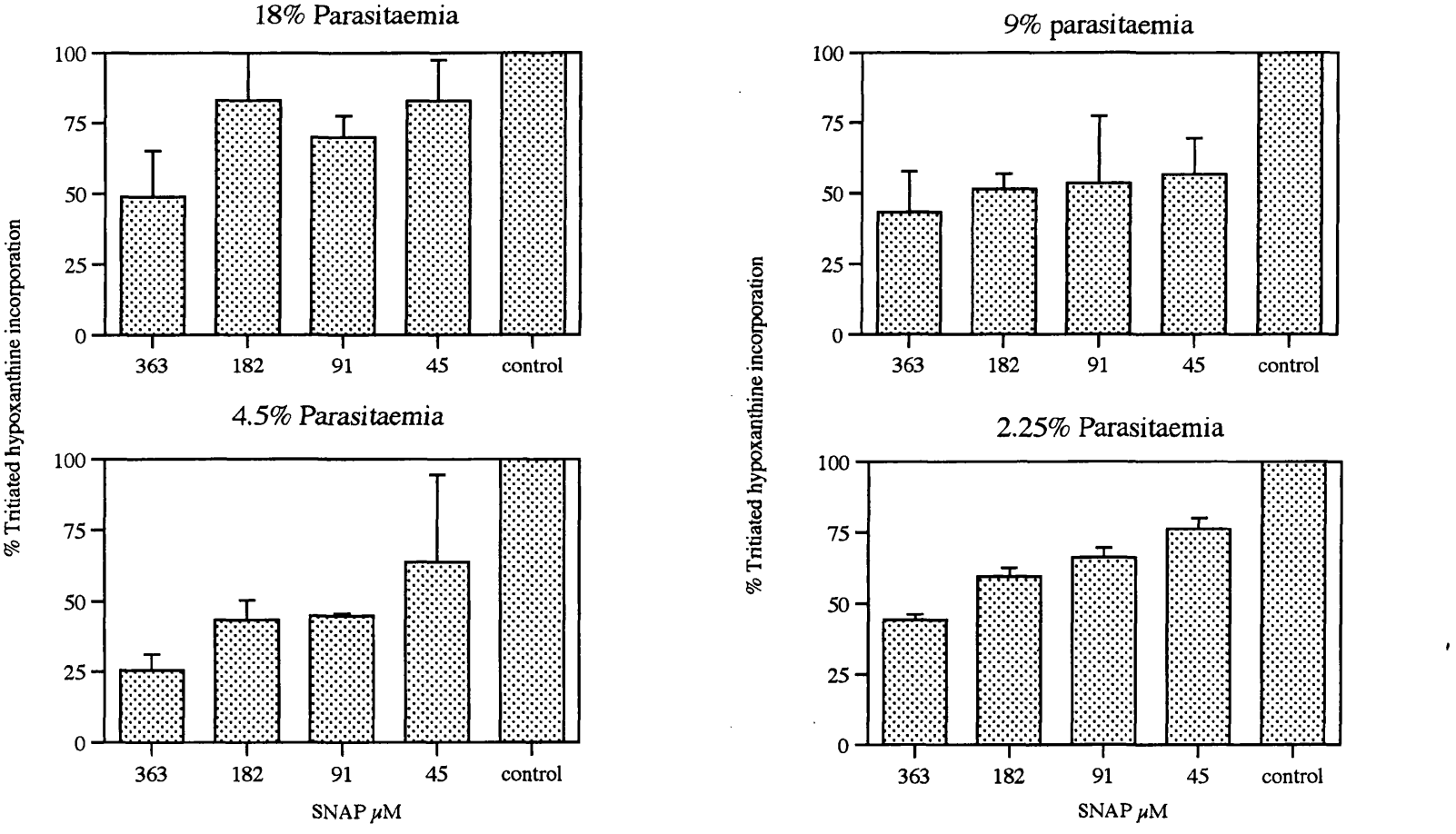


Figure 8.7. Increasing the level of haemoglobin does not prevent the inhibitory effect of NO generated by SNAP on the growth of malaria parasites *in vitro*. Blood from a *P. chabaudi* infected mouse was diluted with non-infected erythrocytes to give the parasitaemia required. Cultures containing a) 18%, b) 9%, c) 4.5% and d) 2.25% parasitaemia were used. SNAP and tritiated hypoxanthine were added at 0 hours. The cultures were harvested 24 hours later. The incorporation of tritiated hypoxanthine is shown relative to controls. Each point is the mean ± SD for an experiment performed in triplicate.

to diminish NO mediated inhibition of *P. chabaudi* growth *in vitro*. As the parasite develops within the erythrocyte, it degrades haemoglobin to yield amino acids which are a nutrient source for the developing parasite. The trophozoite stage digests the majority of the haemoglobin (Goldberg and Slater, 1992). Hence, NO may be able to inhibit the growth of the intraerythrocytic parasite because the scavenging capacity of haemoglobin is lost due to degradation by the parasite.

These results demonstrate that NO can inhibit the growth and development of malaria parasites in both a cytotoxic and cytostatic manner. Trophozoite stages are more susceptible to NO than ring stages possibly because of increased permeability of the erythrocyte membrane allowing increased access to the mature parasite for NO. The actual mechanism of inhibition mediated by NO is unclear. The potential of NO to inactivate RR and subsequently DNA synthesis offers one explanation of how NO may inhibit parasite growth and development. Analysis of various metabolic processes during parasite growth will confirm how NO inhibition is mediated. Measurement of DNA synthesis during the *in vitro* experiments in conjunction with observing the effect of the RR inhibitor, hydroxyurea would determine if this enzyme is susceptible to NO mediated inactivation. ATP levels would indicate if the mitochondria respiratory function was affected by the presence of NO.

The *in vitro* experiments suggest that *in vivo*, high localised concentrations of NO may be able to either kill intraerythrocytic parasites or more likely, inhibit their development. The mature stage parasites were more susceptible to NO, suggesting the parasite may be vulnerable *in vivo* during sequestration. This process involves the trophozoite/schizont infected erythrocytes adhering to endothelial linings of capillaries. The systemic inflammatory response induced by infection could stimulate a high, local production of NO by cells such as endothelial cells resulting in inhibition of parasite development. A cytotoxic effect would result in parasite death but a cytostatic effect may be important in

aiding the effector mechanism of the immune system to respond to the non-circulating parasitised erythrocyte.

Chapter Nine

The effect of chloroquine on nitric oxide production by macrophages

Introduction

The spread of *P. falciparum* resistance to current antimalarial drugs and the slow progress towards an effective vaccine have the emphasised the need for new antimalarials. Advances have been made in the understanding of the mode of action of some antimalarials. However, the interaction between chemotherapy and protective immune responses to malaria infection is unclear and a better understanding will be important in future control programmes which may combine vaccination with chemotherapy.

Some antimalarials are known to be immunosuppressive. Indeed, chloroquine is used in the treatment of autoimmune disorders because of its anti-inflammatory properties (Hurst *et al.*, 1986). The actual mechanism(s) of immunosuppression by antimalarials is unclear. Chloroquine has been shown to suppress the proliferation of lymphocytes (Trist and Weatherall, 1981, Salmeron and Lipsky, 1983) and this activity appears to be dose related, since only high concentrations of chloroquine profoundly suppressed the proliferation of mitogen and antigen stimulated lymphocytes (Bygberg and Flachs, 1986). Chloroquine, mefloquine and quinine, at high concentrations can inhibit IL-2 production (Bygberg *et al.*, 1987) which will affect subsequent T cell responses. Neutrophil chemotaxis can be inhibited by concentrations of the antimalarial achieved in the blood during malaria prophylaxis but no effect was observed on the bactericidal activity of these cells (Kharazmi *et al.*, 1983, Kharazmi and Eriksen, 1986). The immunosuppressive effect of chloroquine on leukocytes has only been demonstrated with high concentrations, suggesting that chemotherapy may not influence the immune response significantly. Furthermore, antimalarial prophylaxis does not appear to alter immune responses to commonly used vaccines (Bjorkman, 1988) but the effect on protective immune responses to malaria remains is still to be investigated.

Chloroquine has been shown to reduce antibody titres to rabies vaccination (Taylor, Wasi and Bernard, 1984, Pappaioanou *et al.*, 1986). However, a normal prophylactic dose of chloroquine, did not affect cellular responses to typhoid vaccination (Bygberg and Flachs, 1986) and chloroquine did not have any affect on antibody production in rabbits following typhoid vaccination (Thompson and Bartholomew, 1964).

The uptake or digestion of haemoglobin by the parasite is thought to be the metabolic process altered by chloroquine (Foley and Tilley, 1997). Chloroquine is a weak base which can traverse the membrane of the parasitised erythrocyte and moves down the pH gradient to accumulate in the acidic food vacuole (Fitch *et al.*, 1974). A high intracellular concentration of chloroquine inhibits haem polymerisation resulting in a build up of haem which is toxic to the parasite (Vander Jagt, Hunsaker and Campos, 1986). The immunomodulatory properties of chloroquine derive from its ability to influence antigenic presentation. This can come about because chloroquine can accumulate in vesicles such as lysosome and endosomes, causing an elevation in pH which influences the assembly of antigenic peptides with the class II MHC complex (Fox, 1995).

Macrophages are important antigen presenting cells and have a pivotal role in the immune system because they are involved in the induction of the immune response and subsequent effector mechanisms. Chloroquine has been shown to have suppressive effects on macrophages additional to its influence on antigen presentation. Production of TNFα and IL-6 by macrophages is inhibited by chloroquine (Picot *et al.*, 1993). Chloroquine has also been shown to inhibit IL-1 production by monocytes (Krogstad and Schlessinger, 1987). These observations indicate that chloroquine could have the potential to alter the production of inflammatory mediators by macrophages which could be important during a malarial infection. Overproduction of TNFα and NO, both macrophage products, has been implicated in the development of cerebral malaria (Clark, Rockett and Cowden, 1991). Mice infected with *P. chabaudi* produce a sharp peak of NO at peak parasitaemia (Taylor-Robinson *et al.*, 1993). However, in mice treated with

chloroquine 24-48 hours prior to peak parasitaemia, the production of NO was ablated (Phillips, Mathers and Taylor-Robinson, 1994). Investigations were carried out to examine if chloroquine inhibited NO production by macrophages.

Results

Chloroquine mediated inhibition of NO production by J774 cells

Cells of the murine macrophage-like cell line, J774, were cultured at $5x10^5$cells/ml and treated with chloroquine (1-1000μM final concentration) at 24 and 2 hours before, the same time as and 2 and 6 hours after stimulation with IFNγ (100U/ml) and LPS (25ng/ml). Nitrite in the culture supernatant was measured by the Griess reaction as an indication of NO production. 100 and 1000μM chloroquine inhibited NO production, both before and after stimulation (100μM: -24 hours, $p<0.04$; -2 hours, $p<0.004$; 0 hours, $p<0.005$, 1000mM: -24 hours, $p<0.05$; -2 hours, $p<0.002$; 0 hours, $p<0.006$; +2 hours, $p<0.01$; +6 hours, $p<0.003$). No inhibition of NO was observed for concentrations of chloroquine at 10μM or below. 1000μM chloroquine inhibited NO production by J774 cells at all the timepoints but the effect of 100μM chloroquine appeared to decrease when treatment was delayed until after stimulation.

Chloroquine induced inhibition of NO production by murine splenic macrophages

Splenic macrophages were obtained from of naive mice and cultured at $5x10^5$ cells/ml. Chloroquine treatment was identical to the protocol described above and the cells were again similarly stimulated with IFNγ and LPS. NO production by the splenic macrophages was inhibited by 100 and 1000μM chloroquine, similar to the result

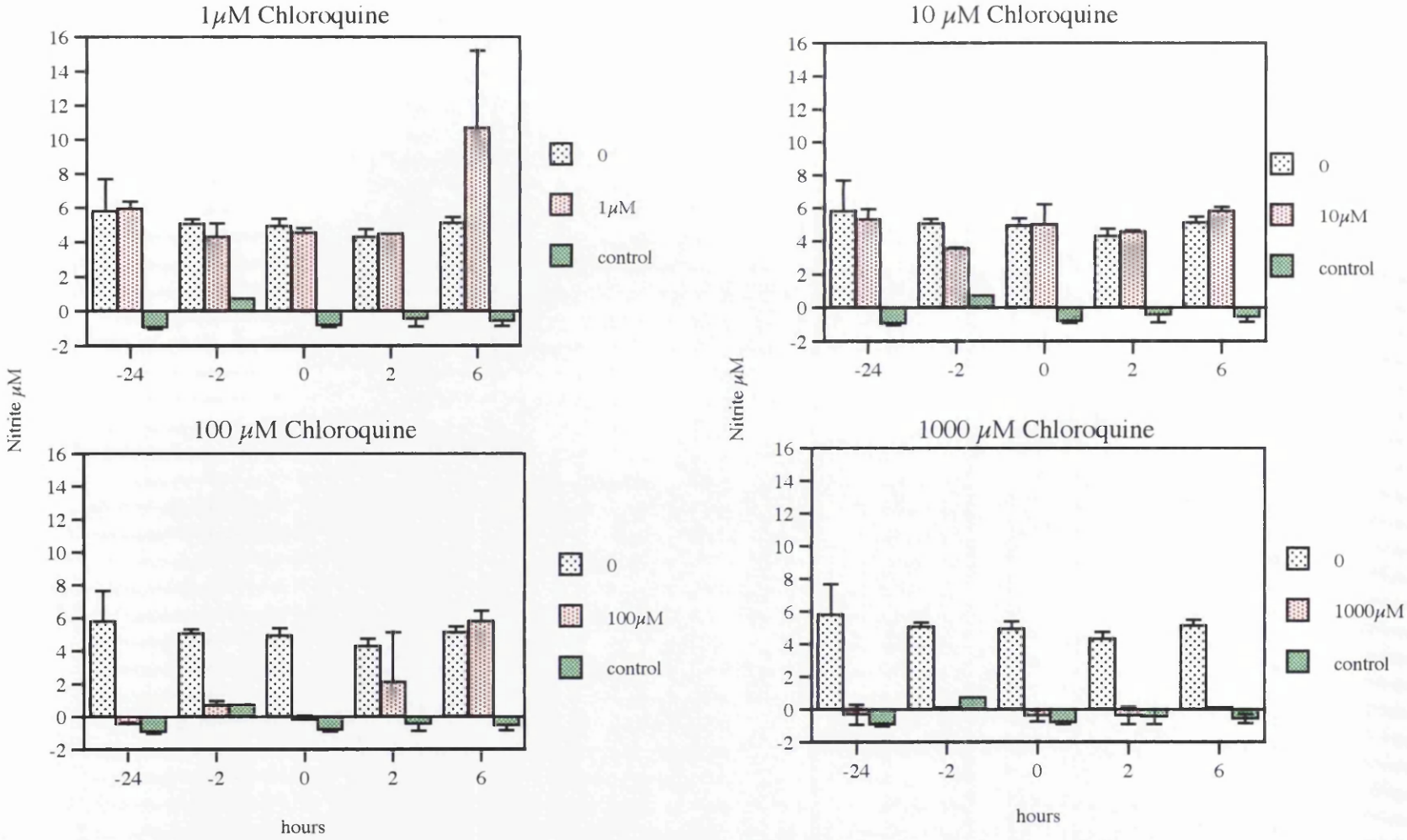


Figure 9.1. Chloroquine mediated inhibtion of NO production by J774 cells. J774 cells were cultured at $5x10^5$ cells/ml and treated with either 1, 10, 100 or 1000 μM chloroquine at the time points shown. The cells were stimulated with IFN γ and LPS at 0 hours. Control wells were cells plus medium alone. Nitrite was measured in the culture supernatants as an indication of NO production. Each point is the mean ± SD of triplicates combined from data obtained from two replicative experiments.

obtained for the J774 cells (100μM: -24 hours, $p<0.0001$; 0 hours, $p<0.0001$; +2 hours, $p<0.01$; 1000mM: -24 hours, $p<0.0002$; -2 hours, $p<0.00006$; 0 hours, $p<0.00006$: +2 hours, $p<0.00003$; +24 hours, $p<0.0001$). 10μM chloroquine and below did not inhibit NO production by splenic macrophages.

NO production by murine peritoneal was cells is inhibited by chloroquine.

Peritoneal wash cells were obtained from naive mice and cultured at $5x10^5$ cells/ml. The cells were treated with 100μM chloroquine 24 hours prior to and the same time as stimulation with IFNγ and LPS. NO production by the murine peritoneal wash cells was significantly inhibited at both timepoints (-24 hours, $p<0.00001$; 0 hours, $p<0.00002$).

Discussion

The results performed in this study, demonstrate that chloroquine at a concentration 100μM can alter the production of effector molecules by stimulated macrophages. In earlier studies, chloroquine mediated inhibition of IL-1 (Krogstad and Schlessinger, 1987), TNFα and IL-6 production (Picot *et al.*, 1993) by macrophages has been demonstrated and this has now been extended to include NO. Chloroquine treatment of three types of macrophages, J774, murine splenic macrophages and murine peritoneal (adherent) wash cells resulted in an inhibition of NO production following stimulation with IFNγ and LPS. Inhibition of NO production was observed with chloroquine treatment of the cells both prior to and after stimulation. However, with 100μM chloroquine treatment, a recovery of NO production was observed when treatment of the cells was after stimulation which was not observed in the cells treated with 1000μM chloroquine.

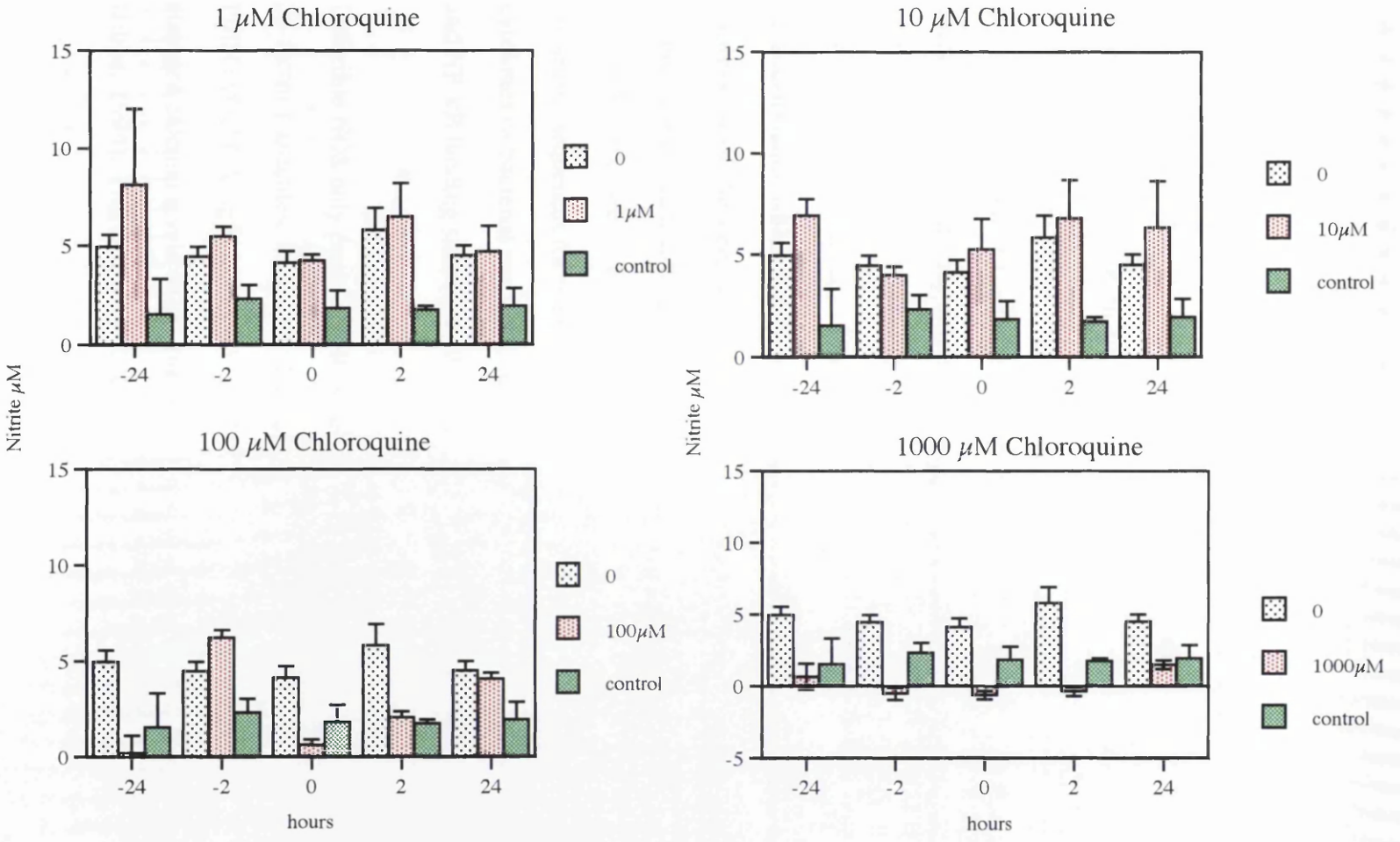


Figure 9.2. Chloroquine mediated inhibition of NO production by splenic macrophages. Splenic macrophages harvested from the spleens of naive mice were cultured at $5x10^5$ cells/ ml and stimulated with IFN γ and LPS. The cells were treated with chloroquine at the times shown (relative to stimulation). Control wells were cells plus medium alone. Nitrite was measured by the Griess Reaction, as an indicator of NO production. Each point is the mean ± SD of an experiment performed in triplicate.

The production of NO by macrophages is a complex process. Stimulation of the macrophage is required to induce NOS transcription and the signal transduction pathway involves several different processes. Transcription factors such as NF-κB and IRF-1 have binding sites in the promoter region of the iNOS gene (Martin, Nathan and Xie, 1994). LPS stimulation results in the binding of a NF-κB complex to the promoter region of iNOS (Xie, Kashiwabara and Nathan, 1994) but it may also enhance the production of transcription factors stimulated by IFNγ (Xie, Whisnant and Nathan, 1993). IFNγ stimulation induces IRF-1 to bind to the appropriate binding site on the promoter sequence of the iNOS gene (Martin, Nathan and Xie, 1994). Macrophages from IRF-1 deficient mice did not express iNOS mRNA upon stimulation with IFNγ and LPS (Kamijo *et al.*, 1994). The induction of IRF-1 by IFNγ stimulation may be mediated by gamma activated factor (GAF) undergoing IFNγ induced tyrosine phosphorylation which in turn induces translocation of GAF to the nucleus resulting in expression of the necessary transcription factors (Martin, Nathan and Xie, 1994). Approximately 22 transcription factor consensus sequences within the promoter region of iNOS have been identified (Xie, Whisnant and Nathan, 1993). These include consensus sequences for transcription factors involved in the induction of other genes by cytokines or bacterial products, such as the IFNγ response element, the γ-activated site and NF-κB binding sites (Xie, Whisnant and Nathan, 1993).

Inducible NOS only produces NO when homodimeric and attached to at least five other co-factor molecules: haem, tetrahydrobiopterin, calmodulin and the flavins, FAD and FMN (MacMicking *et al.*, 1997). The binding of calmodulin can occur in the absence of elevated calcium levels unlike the constitutively expressed forms of NOS (Bastian and Hibbs, 1994). This allows iNOS to sustain production of NO because there is no requirement for elevated calcium.

Inhibition of iNOS activity by glucocorticoids has been demonstrated at transcription and post-transcriptional levels (Kunz *et al.*, 1996). TGFβ1 destabilises iNOS mRNA

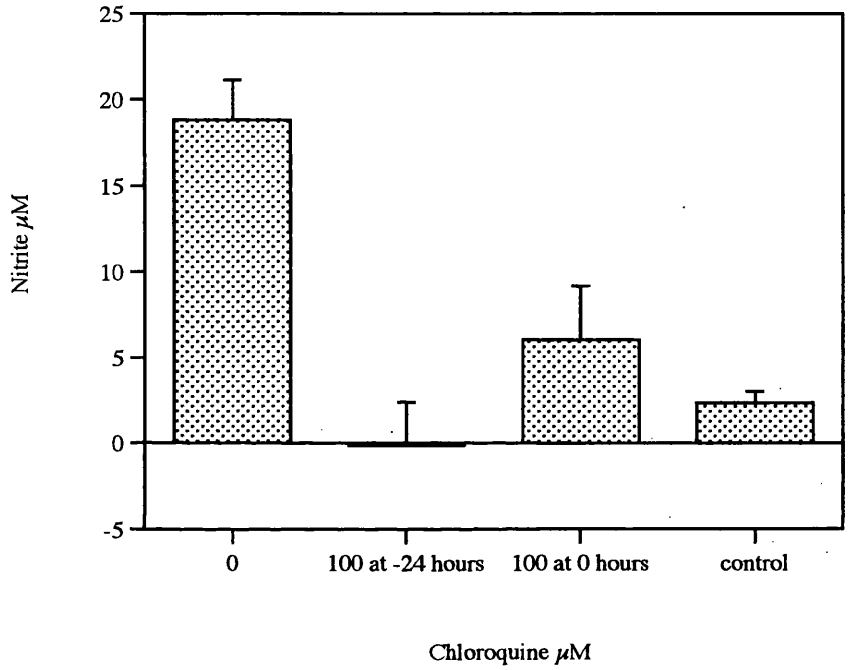


Figure 9.3. Chloroquine induced inhibition of NO production by murine (adherent) peritoneal wash cells. The cells were harvested from the peritoneal cavity of naive mice and cultured at $5x10^5$ cells/ml. The cells were treated with 100μM chloroquine 24 hours before or at the same time as stimulation with IFNγ and LPS. Control wells were cells plus medium alone. Each point is the mean ± SD for an experiment performed in triplicate.

and accelerated its degradation (Vodovotz and Bogdan, 1994). Cytokines such as IL-4 and IL-10 inhibit NO production by macrophages (Sands *et al.*, 1994, Cunha, Moncada and Liew, 1992). From the experiments performed, it is unclear if chloroquine mediated inhibition of NO production occurs at the transcriptional or post-transcriptional level. Extraction of iNOS mRNA from the macrophage cultures treated with chloroquine would determine if the inhibtion mediated by chloroquine occurs at the transcriptional level. The inhibition is dose-dependent and at 100μM the inhibition is not complete in relation to the treatment regime. TNF production by macrophages is also inhibited by choloroquine in a dose-dependent manner, through disruption of iron homeostasis (Picot *et al.*, 1993). Recently a regulatory loop between iNOS induction and iron metabolism has been proposed (Weiss *et al.*, 1994). An increase in ferric ion concentration, decreased iNOS activity in response to LPS stimulation whereas iron chelation treatment of macrophages resulted in an increase in iNOS activity (Weiss *et al.*, 1994). The changes in enzyme activity result from alterations in the transcription of iNOS mRNA. The stability of the mRNA is not significantly affected by the different treatments but the nuclear transcription of iNOS mRNA is increased following iron chelation and decreased by the presence of ferric ions (Weiss *et al.*, 1994). Iron metabolism, therefore, has a regulatory role during the production of NO by iNOS. A regulatory element in the iNOS promoter region, responsive to iron chelation has recently been elucidated confirming the link between iron metabolism and iNOS expression (Melillo *et al.* 1997). Chloroquine has been shown to inhibit LPS-induced TNFα production via disruption of iron homeostasis (Picot *et al.*, 1993), hence, chloroquine inhibition of IFNγ/LPS induced NO production by macrophages, may be mediated by disruption of the regulatory loop between iron metabolism and iNOS activity.

Chloroquine inhibition of cytokine secretion by macrophages has been shown to be not only related to disruption of iron metabolism (Picot *et al.*, 1993). Chloroquine has also been shown to inhibit the activity of phospholipase A2 (PLA2) (Zidovetzki, Sherman and O'Brien, 1993, Nosal, Jancinova and Petrikova, 1995). This has important

consequences on signal transduction pathways because PLA2 catalyses the production of arachadonic acid metabolites which can activate protein kinase C (PKC), which, through phosphorylation of proteins, initiates a cascade of kinase activation resulting in the induction of the required gene. Inhibitors of PKC activity has been shown to inhibit IFNγ/LPS induced NO production (Severn, Wakelam and Liew, 1992). IL-4 mediated inhibition of NO production may occur through inhibition of phospholipase activity, reducing the generation of diacylglycerols subsequently inhibiting PKC activity (Severn, Wakelam and Liew, 1992). Upon activation, PKC is translocated to the cell membrane (Severn, Wakelam and Liew, 1992), however, the reduction of diacylglycerol stimulation of PKC by IL-4, results in an inhibition of iNOS gene transcription (Sands *et al.*, 1994). Hence, chloroquine mediated inhibition of IFNγ/LPS induced NO production, could possibly result from chloroquine suppression of PKC activation by products generated by phospholipase cleavage of phospholipids. Measurement of PKC activity would determine if chloroquine treatment of macrophages prevents NO production through inhibition of this pathway.

TNFα production has been shown to be important, in an autocrine fashion, in the induction of NO production (Jun *et al.*, 1995). Anti-TNFα antibodies were shown to inhibit IFNγ and taxol (an anti-cancer agent) induced NO production (Jun *et al.* 1995). The suppressive effect of IL-10 on NO production is exerted indirectly via its inhibition of TNFα production (Oswald *et al.*, 1992). Chloroquine inhibits TNFα production (Picot *et al.*, 1993), hence, the inhibition of NO production observed in this study may result from an inhibition of the autocrine pathway of stimulation involving TNFα. Both TNFα and NO have been implicated in the immunopathological damage associated with cerebral malaria (Clark, Rockett and Cowden, 1991). The spread of *P. falciparum* resistance to chloroquine means that it is rarely used in some areas. However, chloroquine may provide an inexpensive method of suppressing the pathology mediated by TNFα and NO during cerebral malaria.

The effect of chloroquine (or other antimalarial) chemotherapy or prophylaxis treatment on the generation of protective immunity to malaria infection is an area which remains poorly understood. There are several factors which can influence the interaction between antimalarial compound and immune response. These include: the immunosuppressive potential of antimalarials; the effect of antimalarials on malaria-linked immunosuppression and immunopathology; the induction of immunity in relation to chemotherapy. The immunosuppressive effect of chemotherapy has already been briefly discussed but it is clear that antimalarials do have the potential to suppress the development of protective immune effector mechanisms although this may be dependent upon the dosage used in the chemotherapy (Bjorkman, 1988). Immunosuppressive effects tend to be observed with higher doses of antimalarial drugs. However, prophylactic doses of chloroquine (600mg base/week) have been shown to inhibit the phagocytosis of IgG coated sheep red blood cells by human monocytes (Osorio, Fonte and Finlay, 1992) demonstrating the immunosuppressive potential of antimalarials. The interaction between chemotherapy and the induction of immunity to malaria infection is an important issue. It is unclear if protection given by chemotherapy will reduce the level of acquired immunity gained by infection and re-infection. Semi-immune schoolchildren were given antimalarial treatment for one-two months in an endemic area of Tanzania (Pringle and Avery-Jones, 1966). Subsequent new clinical infections caused clinical symptoms and a parasitaemia that was significantly greater than before treatment. It was concluded that the short duration of drug treatment had reduced the immune status of the children. Hence, the difference between sub-curative and curative treatment of malaria may influence the immune status of an individual and interfere with the development of naturally acquired immunity to malaria infection. Conversely the immune status of an individual may influence the efficiency of antimalarial chemotherapy. Chloroquine was more effective against *P. berghei* after a degree of immunity had developed following exposure to several infections (Golenser *et al.*, 1978). The efficiency of chemotherapy treatment of *P. chabaudi* infection is reduced in T cell deficient mice compared to intact mice (Lwin *et al.*, 1979). The combination of passively transferred IgG antibodies, from

hyper-immune mice infected with *P. chabaudi*, and chemotherapy was more effective than either form of therapy used alone (Bjorkman, 1988). This demonstrates that chemotherapy may have differing effects on humoral and cell-mediated immunity which may have consequences on the development of protective immunity to infection.

Chapter Ten
General Discussion

The studies presented in this thesis cover two main areas: firstly, the role of cytokines in the development of protective immune responses to *P. chabaudi* infection in mice and secondly, the role of non-specific inflammatory mediators during a primary malaria infection in rodents. As already discussed the immune response to *P. chabaudi* infection in mice is mediated by a sequential involvement of Th1 and Th2 regulated immunity (Langhorne *et al.*, 1989, Taylor-Robinson *et al.*, 1993). The role of Th1 and Th2 cytokines during *P. chabaudi* infection was investigated using IFNγR gene deficient mice, IL-4 and IL-6 gene deficient mice. This allowed a degree of comparison to be drawn between the effect of Th1 (IFNγR deficient mice) and Th2 (IL-4 and IL-6 deficient mice) deficiencies during infection.

The development of protective immunity to *P. chabaudi* infection involves three distinct pathways (illustrated in Diagram 3). The first is a rapid inflammatory response initiated by malarial antigen or parasite derived toxins stimulating effector cells to produce inflammatory mediators such as NO, oxygen radicals, TNFα, IL-1 which have been implicated in various mechanisms of parasite killing (Phillips, 1994a, Stevenson *et al.*, 1995, Taylor-Robinson, 1995). IL-12 and TNFα are important macrophage products which can stimulate NK cells and result in further activation of effector cells through the production of IFNγ. The second pathway is initiated by antigen presentation to $CD4^+$ T cells. The presence of the correct co-stimulatory molecules and microenvironment will result in the expansion of Th1 type $CD4^+$ T cells which can activate effector cells through the production of cytokines such as IFNγ and TNFβ. IL-12 is a cytokine which has been shown to provide the correct microenvironment for the development of Th1 cells (Trinchieri, 1995). Essentially Th1 mediated responses amplify the rapid inflammatory response, resulting in an increase in parasite killing.

The third pathway is the development of Th2 mediated responses. Presentation of malarial antigen via, for example, B cells and in the presence of IL-4 will promote the

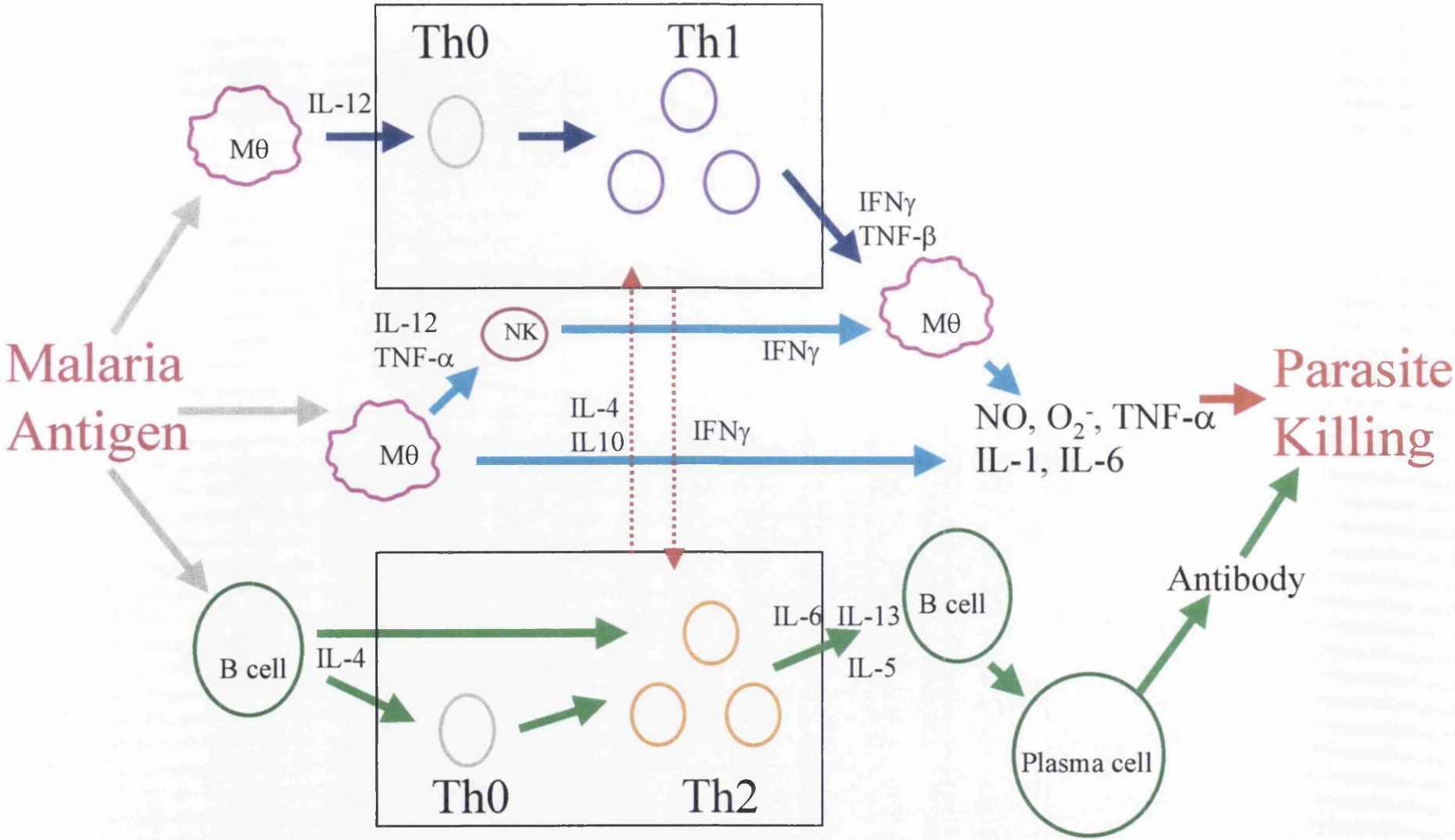


Diagram 3. Development of the $CD4^+$ T cell mediated immunity to *P. chabaudi* infection.

development of Th2 cell proliferation. This results in the production of several Th2 associated cytokines important for the development of humoral immunity and thereby, involved in parasite removal. This description of the protective immune response to *P. chabaudi* is a gross simplification. There are several crucial interactions between the Th1/Th2 associated cytokines which regulate the $CD4^+$ T cell response. The balance between IL-12 and IL-4/IL-10 may influence which subset, Th1 or Th2 will be preferentially induced (von der Weid *et al.*, 1994). IFNγ, a Th1 cytokine, can inhibit the expansion of Th2 cells (Gajewski and Fitch, 1988, Maggi *et al.*, 1992) whereas IL-10, a Th2 cytokine, can inhibit the induction of Th1 cell expansion (Fiorentino *et al.*, 1991). Furthermore, humoral immunity is not exclusively mediated by Th2 cells because IFNγ promotes the production of IgG2a (Snapper and Paul, 1987a).

In IFNγR deficient mice, the absence of a functional IFNγ receptor and subsequently a loss of IFNγ mediated responses, it would be expected that during *P. chabaudi* infection, a reduction in inflammatory responses (non-specific and Th1 mediated) would be observed. The interactions between Th1/Th2 responses may be altered due to an abrogation of IFNγ mediated down-regulation of Th2 development. The loss of IFNγ function would also be reflected in the level of IgG2a observed. Consistently, IFNγR deficient mice, compared to control mice had an exacerbated peak of parasitaemia following *P. chabaudi* infection (although this was not statistically significant), indicative of a reduced inflammatory response. Raised total IgE levels in the serum of the IFNγR deficient mice may indicate a strong Th2 response which could reflect a loss of IFNγ function in the preferential activation of Th1 cells and the inhibition of Th2 cell expansion. The total IgG2a response and the parasite-specific antibody level are both reduced in IFNγR deficient mice suggesting that IFNγ induced humoral responses are important during a primary *P. chabaudi* infection. IFNγR deficient mice, therefore, are more susceptible to *P. chabaudi* infection compared to intact control mice, exhibiting a high mortality rate. The IFNγR deficient mice, which survive the acute phase of *P.*

chabaudi infection, are unable to reduce the parasitaemia to sub-patent levels and consequently have a persistent, low grade parasitaemia.

The results observed for *P. chabaudi* infection of the mice deficient in IL-4 and IL-6 production are not as striking as the infection of IFNγR deficient mice. Both IL-4 and IL-6 deficient mice recover from *P. chabaudi* infection, no mortality is observed and the efficiency of parasite clearance is broadly similar to that of intact mice. In IL-4 deficient mice, reduction in the IL-4 mediated Th2 development would be expected. An alteration in the balance of Th1 and Th2 activation and expansion might occur. However, IL-10 is regarded as the main inhibitory cytokine to Th1 development (Fiorentino *et al.*, 1991). Furthermore, a reduction in the production of IL-4 associated Ig isotypes would be expected. An exacerbation of the peak parasitaemia in IL-4 deficient mice was consistently observed, coupled with a reduction in the total IgG1 response. The exacerbation of the peak parasitaemia may be attributed to an alteration in the balance between Th1/Th2 mediated immunity which results in a reduction of parasite killing. However, control of the peak parasitaemia may involve IgG1 mediated responses and hence, the reduction in total IgG1 levels may contribute to the exacerbated peak parasitaemia. The involvement of the humoral response in the control of the primary peak of parasitaemia is also demonstrated in IL-6 deficient mice. Infection of IL-6 deficient mice with *P. chabaudi* results in a significant delay in parasite clearance after the peak of the primary parasitaemia. An exacerbation of peak parasitaemia was consistently observed (although never statistically significant) using $1x10^5$ pRBCs as the infective dose in the IL-6 deficient mice compared to intact control mice. The differences in the course of infection in IL-6 deficient mice coincide with a reduction in total IgG2a and total IgG1 levels.

The studies using the gene deficient mice have produced some interesting results which merit further investigation. IFNγ has been shown to be a critical mediator in the development of protective immunity to a primary *P. chabaudi* infection, whereas the

absence of IL-4 or IL-6 function does not significantly alter the outcome of infection. IFNγR deficient mice have been shown to be as equally protected as control mice upon re-infection but it would be interesting to re-infect the IL-4 or IL-6 deficient mice because the protective response to re-infection is regarded as a Th2 mediated humoral response. The studies performed suggest that the Th2 associated cytokines, IL-4 and IL-6 are not required for the control of a primary *P. chabaudi* infection. However, it is possible that redundancies in the cytokine network compensate for the loss of either cytokine, resulting in only slight deficiencies in the immune response to *P. chabaudi* infection. Further investigations are required to elucidate why IFNγR deficient mice succumb to *P. chabaudi* infection and why mice deficient in the Th2 associated cytokines are capable of controlling a primary infection. Analysis of tissue-specific cytokine mRNA expression is planned as well as determination of the levels of various cytokines in the serum. Problems with the preparation of the parasite lysate antigen, prevented parasite-specific analysis of the proliferation and antibody studies which may have differentiated between general defects in the immune response due to the gene disruption and defective responses to infection.

Non-specific inflammatory mediators are an integral part of the immune response to malaria infection. Acute phase proteins are induced rapidly in response to various stimuli including malaria infection (Gillespie *et al.*, 1991). The role of the murine acute phase protein, SAP, was investigated during a *P. chabaudi* infection. *P. chabaudi* infection of mice stimulates a systemic acute phase response indicated by raised SAP levels which peak around the peak of the primary parasitaemia. The actual role of SAP during infection remains unclear. However, the *in vitro* studies suggest that SAP may have an anti-parasite effect but it is more likely to be an important immunomodulatory molecule. It would be interesting to perform infection studies where SAP has been depleted either by treatment with antibodies or gene disruption. The effect of the absence of SAP on the outcome of infection could be determined. However, it is most probable that SAP has only a minor role in the control of infection because IL-6 gene deficient mice do not

produce SAP during a *P. chabaudi* infection but are still able to clear the parasite with a similar efficiency to that of controls.

The liver is the major site of synthesis of acute phase proteins and preliminary studies have suggested that the liver may also be the site of a protective immune response during a *P. chabaudi* infection. An increase in the number of lymphomyeloid cells present in the liver during a *P. chabaudi* infection was observed. Adoptive transfer of the lymphomyeloid cells from the liver during a *P. chabaudi* infection gave some protection to recipient mice from a homologous challenge. These studies confirmed previous work suggesting that the liver is involved in a protective response to murine malaria parasites (Playfair *et al.*, 1979, Dockrell, De Souza and Playfair, 1980, Playfair and De Souza, 1982). *P. chabaudi* is known to sequester to the liver (Cox, Semoff and Hommel, 1987) during the latter stages of the asexual erythrocytic cycle of the parasite. Hence the parasite is present in the liver, at stages (late trophozoite/schizont and merozoites) which are the most likely to be vulnerable to the immune response. Hence, the liver is a potential site where parasite killing may occur. The studies using the gene deficient mice illustrated that immune responses in the liver may be involved in protective immunity because there was a reduction in lymphomyeloid cells present in the liver of these mice during a *P. chabaudi* infection. It is unclear if the increase in the number of LM cells present is due to an expansion of intrahepatic lymphomyeloid cells or if there is increased migration of cells to the liver during infection. Further investigations of the lymphomyeloid cells present in the liver are planned with the focus on identifying what these cells are and monitoring the change in the composition of these cells, particularly the lymphoid compartment, during the course of a *P. chabaudi* infection. It would be interesting to see if the sequestration of the parasite to the liver is required to induce the increase in the number of lymphomyeloid cells present. Non-sequestering murine malaria parasites induce a systemic acute phase protein response but it is unknown if there is an increase in the number of lymphomyeloid cells present in the liver of mice infected with non-sequestering species of murine malaria.

NO is a non-specific inflammatory molecule which has been shown to inhibit the development of intrahepatic malaria parasites (Nussler *et al.*, 1991). Hence, the liver may be a source of NO production during the pre-erythrocytic stage of infection but as already mentioned, during a *P. chabaudi* infection, the parasite may be vulnerable to immune attack and molecules such as NO, during sequestration in the liver. The studies performed here suggest that at high local concentrations, NO may have a cytotoxic effect on malaria parasites. However, a cytostatic effect is more probable. NO has been shown to have a direct effect on parasite development both *in vivo* (Taylor-Robinson *et al.*, 1993) and *in vitro* (Rockett *et al.*, 1991) but a protective role for NO which is not anti-parasitic, but host protective has also been suggested (Jacobs, Radzioch and Stevenson, 1996).

Hence the liver may be an important site of a protective immune response mediated by the lymphomyeloid cells in the liver during infection and through the production of non-specific inflammatory molecules such as SAP and NO. Furthermore, the liver may be a site where immunity induced to the pre-erythrocytic malaria infection may also be protective against an ongoing blood stage infection, because the inflammatory molecules produced are not stage specific and may be involved in protective immune responses to the different stages. The liver is one of several sites which *P. falciparum* sequesters to and this highlights a problem facing researchers investigating the immunology of malaria infection. Immune responses of infected individuals are analysed by re-stimulating PBMN cells. However, there may be tissue-specific responses which are important in the development protective immunity. Hence, the development of an effective vaccine is further complicated by this issue because observations made from in vitro studies of PBMN cells may not reflect the complete protective immune mechanisms. Therefore, if a vaccine is designed to induce the responses observed from the in vitro studies, the protective response induced will not include a protective mechanism which may be critical to the development of effective immunity.

To conclude, the studies reported in this thesis have revealed two areas of work which merit further investigation. The first is, to examine why a deficiency in IFNγ mediated cellular responses results in susceptibility to *P. chabaudi* infection. IFNγR deficient mice provide a model which can be utilised to investigate the failure to develop a protective immune response in the absence of IFNγ mediated responses. It will be of importance to expand the current studies by including analysis of parasite-specific cellular responses in the IFNγR deficient mice. Determination of cytokine production by splenocytes (re-stimulated *in vitro*) and measurement of cytokine levels in sera is planned. Tissue-specific expression of cytokine mRNA will also be investigated to observe if there is deficiency or alteration in the development of tissue-specific protective immune responses. The second area which merits further investigation is the analysis of the LM cells present in the livers of *P. chabaudi* infected mice. Identification of the cell types present is necessary and it would be interesting to perform a time-course experiment where the changes in the liver could be analysed, giving an indication of the mechanism of the immune response mediated by the LM cells at various times during the course of a *P. chabaudi* infection.

Appendix

Phosphate buffered saline (pH 7.2)

60g $Na_2HPO_4.12H_2O$

13.6g $Na_2HPO_4.2H_2O$

8.5g NaCl

Made up to 1 litre with de-ionised and distilled water.

RPMI 1640 stock medium

10.39g RPMI 1640 powdered medium (with L-glutamine) (Gibco)

5.94g N2-hydroxyethylpiperazine-N-2 ethane sulphonic acid (HEPES) (25mM)

Made up to 1 litre with de-ionised and distilled water, filter sterilised (Millipore/Gelman filter 0.22μm size) and pH adjusted to pH 7.2

Incomplete RPMI 1640 medium

To stock RPMI 1640 medium the following were added:

11 ml L-glutamine (Gibco)

5.5 ml $NaHCO_3$

0.55 ml 2-Mercaptoethanol (0.1M)

22 ml Fungizone (Gibco)

2.2 ml Gentamycin (Sigma)

Complete RPMI 1640 medium

Heat inactivated foetal calf serum (Gibco) was added to incomplete RPMI 1640 medium at a final concentration of 10%.

RPMI 1640 Malaria stock medium

10.39g	RPMI 1640 powdered medium (with L-glutamine) (Gibco)
5.94g	N2-hydroxyethylpiperazine-N-2 ethane sulphonic acid (HEPES) (25mM)

Made up to 960 ml with distilled water and filter sterilised.

Incomplete malaria medium

To 100 ml aliquots of RPMI 1640 malaria stock medium, the following were added:

4.2 ml	5% $NaHCO_3$
0.25 ml	Gentamycin (Sigma)

Complete malaria medium

Heat inactivated human AB serum was added to incomplete malaria medium at a final concentration of 10%.

Giemsa's Buffer

3g	Na_2HPO_4
0.6g	KH_2PO_4

Made up to 1 litre with distilled water and adjusted to pH 7.4

Giemsa's stain

Giemsa's stain (Gurr BDH Ltd) was diluted 1:10 in Giemsa's buffer

Tris Buffered Saline (TBS)

9g	NaCl
1.6g	Tris HCl

The pH was adjusted to pH 7.6 with HCl and made up to final volume of 1 litre with de-ionised and distilled water.

Carbonate/Bicarbonate Coating Buffer (0.05M)

1.59g Na_2CO_3

2.93g $NaHCO_3$

0.2g NaN_3

Made up to 1 litre with de-ionised and distilled water and adjusted to pH 9.6

Bicarbonate Coating Buffer

8.4g $NaHCO_3$

Made up to 1 litre with de-ionised and distilled water and pH adjusted to 8.2

PBS/Tween

0.5ml Tween 20 (polyoxyethylene sorbitan monolaurate)

Made up to 1 litre with PBS.

References

ADAMS, L.B., HIBBS, J.B. J r., TAINTOR, R.R. AND KRAHENBUHL, J. (1990)
Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii.*
Journal of Immunology **144** 2725-2729

ADERKA, D., LE, J. AND VILCEK, J. (1989)
IL-6 inhibits lipopolysaccharide-induced tumour necrosis factor production in cultured human monocytes, U937 cells and in mice.
Journal of Immunology **143** 3517-3523

AGGARWAL, A., KUMAR, S., JAFFE, R., HONE, D., GROSS, M. AND SADOFF, J. (1990)
Oral salmonella: Malaria circumsporozoite recombinants induce specific $CD8^+$ cytotoxic T cells.
Journal of Experimental Medicine **172** 1083-1090

AIDOO, M., LALVANI, A., ALLSOPP, C.E.M., PLEBANSKI, M., MEISNER, S.J., KRAUSA, P., BROWNING M., MORRIS-JONES, S., GOTCH, F., FIDOCK, D.A., TAKIGUCHI, M., ROBSON, K.J.H., GREENWOOD, B.M., DRUILHE, P., WHITTLE, H.C., HILL, A.V.S. AND GOOD, M.F. (1995)
Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria.
The Lancet **345** 999-1000 +1003-1007

AIKAWA, M. (1988)
Human cerebral malaria.
American Journal of Tropical Medicine and Hygiene **39** 3-10

AKANMORI, B.D., KAWAI, S. AND SUZUKI, M. (1996)
Recombinant mouse IL-6 boosts specific serum anti-plasmodial IgG subtype titres and suppresses parasitaemia in *Plasmodium chabaudi chabaudi* infection.
Parasite Immunology **18** 193-199

AKANMORI, B.D., WAKI, S. AND SUZUKI, M. (1994)
Immunoglobulin G(2a) isotype may have a protective role in *Plasmodium berghei* NH65 infection in immunised mice.
Parasitology Research **80** 638-641

AKIRA, M., TAGA, T. AND KASHIMOTO, T. (1993)
Interleukin-6 in biology and medicine.
Advances in Immunology **54** 1-78

ALLISON, A.C. (1954)
Protection afforded by sickle-cell trait against subtertian malarial infection.
British Medical Journal **i** 190

ALLISON, J.P. (1993)
$\gamma\delta$ cell development.
Current Opinion in Immunology **5** 241-246

ALONSO, P.L., SMITH, T., ARMSTRONG SCHELLENBERG, J.R.M., MASANJA, H., MWANKUSYE, S., URASSA, H., BASTOS de AZEVEDO, I., CHONGELA, J., KOBERO, S., MENENDEZ, C., HURT, N., THOMAS, M.C., LYIMO, E., WEISS, N.A., HAYES, R., KITUA, A.Y., LOPEZ, M.C., KILAMA, W.L., TEUSCHER, T. AND TANNER, M. (1994)
Randomised trial of efficacy of SPf66 vaccine against *Plasmodium falciparum* in children in Southern Tanzania.
The Lancet **344** 1175-1181

ALUOCH, J.R. (1997)
Higher resistance to *Plasmodium falciparum* infection in patients with homozygous sickle cell disease in Western Kenya.
Tropical Medicine and International Health **2** 568-571

AL-YAMAN, F.M., MOKELA, D., GENTON, B., ROCKETT, K.A., ALPERS, M.P. AND CLARK, I.A. (1996)
Association between serum levels of reactive nitrogen intermediates and coma in children with cerebral malaria in Papua New Guinea.
Transactions of the Royal Society of Tropical Medicine and Hygiene **90** 270-273

AMANTE, F.H. AND GOOD, M.F. (1997)
Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice.
Parasite Immunology **19** 111-126

AMBER, I.J., HIBBS, J.B. Jr., TAINTOR, R.R. AND VAVRIN, Z. (1988)
Cytokines induce an L-arginine-dependent effector system in non-macrophage cells.
Journal of Leukocyte Biology **44** 58-65

ANDUS, T., GEIGER, T., HIRANO, T., NORTHOFF, H. GANTER, U., KISHIMOTO, T. AND HEINRICH, P.C. (1987)
Recombinant human B cell stimulatory factor 2 (BSF-2/IFN-β2) regulates β-fibrinogen and albumin mRNA levels in Fao-9 cells.
FEBS Letters **221** 18-22

ANSTEY, N.M., WEINBERG, J.B., HASSANALI, M.Y., MWAIKAMBO, E.D., MANYENGA, D., MISUKONIS, M.A., ARNELLE, D.R. AND HOLLIS, D. (1996)
Nitric oxide in Tanzanian children with malaria: Inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression.
Journal of Experimental Medicine **184** 557-567

ARASE, H., ARASE, N. AND SAITO, T. (1996)
Interferon gamma production by natural killer (NK) cells and NK1.1$^+$ T cells upon NKR-P1 cross-linking.
Journal of Experimental Medicine **183** 2391-2396

ASENSION, V.C., OSHIMA, H. AND FALANGA, P.B. (1993)
Plasmodium berghei: Is nitric oxide involved in the pathogenesis of mouse cerebral malaria?
Experimental Parasitology **77** 111-117

ASSRUEY, J., CUNHA, F.Q., LIEW, F.Y. AND MONCADA, S. (1993)
Feedback inhibition of nitric oxide synthase activity by nitric oxide.
British Journal of Pharmacology **108** 833-837

BACH, E.A., AGUET, M. AND SCHREIBER, R.D. (1997)
The IFNgamma receptor: A paradigm for cytokine receptor signaling.
Annual Review of Immunology **15** 563-591

BAIRD, J.K., JONES, T.R., DANUDIRGO, E.W., ANNIS, B.A., BANGS, M.J., BASRI, H., PURNOMO AND MASBAR, S. (1991)
Age-dependent acquired protection against *Plasmodium falciparum* in people having two years exposure to hyperendemic malaria.
American Journal of Tropical Medicine and Hygiene **45** 65-76

BAIRD, J.K., PURNOMO, BASRI, H., BANGS, M.J., ANDERSEN, E.M., JONES, T.R., MASBAR, S., HARJOSUWARNO, S., SUBIANTO, B. AND ARBANI, P.R. (1993)
Age-specific prevalence of *Plasmodium falciparum* among six populations with limited histories of exposure to endemic malaria.
American Journal of Tropical Medicine and Hygiene **49** 707-719

BALLOU, W.R., SHERWOOD, J.A., NEVA, F.A., WIRTZ, R.A., WASSERMAN, G.F., DIGGS, C.L., HOFFMAN, S.L., HOLLINGDALE, M.R., HOCKMEYER, W.T., SCHNEIDER, I., YOUNG, J.F., REEVE, P. AND CHALAY, J.D. (1987)
Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine.
The Lancet **1** 1277-1281

BAMBERGER, T., MASSON, I., MATHIEU, J., CHAUVELOT-MOACHON, L., GIROUD, J.P. AND FLORENTIN, I. (1992)
Nitric oxide mediates the depression of lymphoproliferative responses following burn injury in rats.
Biomedicine and Pharmacotherapy **46** 495-500

BANCROFT, A.J., ELSE, K.J. AND GRENCIS, R.K. (1994)
Low-level infection with *Trichuris muris* significantly affects the polarization of the CD4 response.
European Journal of Immunology **24** 1726-1728

BARBASHOV, S.F., WANG, C. AND NICHOLSON-WELLER, A. (1997)
Serum amyloid P component forms a stable complex with human C5b6.
Journal of Immunology **158** 3830-3835

BARR, P.J., GREEN, K.M., GIBSON, H.L., BATHHURST, I.C., QUAKYI, I.A. AND KASLOW, D.C. (1991)
Recombinant Pfs25 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals.
Journal of Experimental Medicine **174** 1203-1208

BASTIAN, N.R. AND HIBBS JNR, J.B. (1994)
Assembly and regulation of NADPH Oxidase and nitric oxide synthase.
Current Opinion in Immunology **6** 131-139

BATE, C., TAVERNE, J., BOOTSMA, H., MASON, R., SKALKO, N., GREGORIADIS, G. AND PLAYFAIR, J. (1992)
Antibodies against phosphatidylinositol and inositol monophosphate specifically inhibit tumour necrosis factor induction by malaria exoantigens.
Immunology **76** 35-41

BATE, C.A.W., TAVERNE, J., DAVE, A. AND PLAYFAIR, J.H.L. (1990)
Malaria exoantigens induce T-independent antibody that blocks their ability to induce TNF.
Immunology **70** 315-320

BATE, C.A.W., TAVERNE, J. AND PLAYFAIR, J.H.L. (1988)
Malarial parasites induce TNF production by macrophages.
Immunology **64** 227-231

BEAGLEY, K.W., ELDRIDGE, J.H., LEE, F., KIYONO, H., EVERSON, M.P., KOOPMAN, W.J., HIRANO, T., KISHIMOTO, T. AND MCGHEE, J.R. (1989)
Interleukins and IgA synthesis: Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells.
Journal of Experimental Medicine **169** 2133-2148

BENSON, M.D., SKINNER, M., SHIRAHAMA,T. AND COHEN, A.S. (1976)
P-component of amyloid: isolation from human serum by affinity chromatography.
Arthritis Rheumatology. **19** 749-754

BERNADETTE, L., KASPER, L.H., SMITH, K. AND HAQUE, A. (1996)
In vivo treatment with interleukin 2 reduces parasitaemia and restores IFN-gamma gene expression and T-cell proliferation during acute murine malaria.
Comptes Rendus de l'Academie des Sciences-Serie1ii, Sciences de la Vie **319** 705-710

BIFFL, W.L., MOORE, E.E., MOORE, F.A. BARNETT, C.C., Jr. (1996)
Nitric oxide reduces endothelial expression of intercellular adhesion molecule (ICAM)-1.
Journal of Surgical Research **63** 328-332

BILLIAR, T.R., CURRAN, R.D., STUEHR, D.J., FERRARI, F.K. AND SIMMONS, R.L. (1989)
Evidence that activation of Kupffer cells results in production of L-arginine metabolites that release cell-associated iron and inhibit hepatocyte protein synthesis.
Surgery **106** 364-372

BJORKMAN, A. (1988)
Interactions between chemotherapy and immunity to malaria.
Progress in Allergy **41** 331-356

BOEHM, U., KLAMP, T., GROOT, M. AND HOWARD, J.C. (1997)
Cellular responses to Interferon-γ.
Annual Review of Immunology **15** 749-795

BORDESSOULE, D., GAULARD, P. AND MASON, D.J. (1990)
Preferential localisation of human lymphocytes bearing the gamma-delta T cell receptor to the red pulp of the spleen.
Journal of Clinical Pathology **43** 461-464

BORST, J., VAN DE GRIEND, R.J., VAN OOSTVEEN, J.W., AUG, S-L., MELIEF, C.J., SEIDMAN, J.G. AND BOLHUIS, R.L.H.
A T cell receptor γ/CD3 complex found on cloned functional lymphocytes.
Nature **325** 683-688

BOUHAROUN-TAYOUN, H., ATTANATH, P., SABCHAREEN, A., CHONGSUPHAJAISIDDHI, T. AND DRUILHE, P. (1990)
Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro*, but act in cooperation with monocytes.
Journal of Experimental Medicine **172** 1633-1641

BOUHAROUN-TAYOUN, H. AND DRUILHE, P. (1992)
Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity.
Infection and Immunity **60** 1473-1481

BOUHAROUN-TAYOUN, H., OEUVRAY, C., LUNEL, F. AND DRUILHE, P. (1995)
Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmoudium falciparum asexual blood stages.
Journal of Experimental Medicine **182** 409-418

BRADLEY, L.M., DALTON, D.K. AND CROFT, M. (1996)
A direct role for IFN-γ in regulation of Th1 cell development.
Journal of Immunology **157** 1350-1358

BREDT, D.S., FERRIS, C.D. AND SNYDER, S.H. (1992)
Nitric oxide synthase regulatory sites.
Journal of Biological Chemistry **267** 10976-10981

BRETSCHER, P., WEI, G. AND MENON, J. (1992)
Establishment of stable cell mediated immunity that makes susceptible mice resistant to *Leishmania major.*
Science **257** 539-542

BREWSTER, D., KWIATKOWSKI, D. AND WHITE N.J. (1990)
Neurological sequelae of cerebral malaria in childhood.
The Lancet **336** 1039-1043

BROWN, J., GREENWOOD, B.M. AND TERRY, R.J. (1986)
Cellular mechanisms involved in recovery from acute malaria in Gambian children.
Parasite Immunology **8** 551-564

BROWN, J. AND SMALLEY, M.E. (1980)
Specific antibody-dependent cellular cytotoxicity in human malaria.
Clinical and Experimental Immunology **41** 423-429

BRUCE-CHWATT, L.J. (1952)
Malaria in African infants and children in southern Nigeria.
Annals of Tropical Medicine and Parasitology **46** 173-200

BUCHTA, R., FRIDKIN, M., PONTET, M., CONTESSI, E., SCAGGIANTE, B. AND ROMEO, D. (1987)
Modulation of human neutrophil function by C-reactive protein.
European Journal of Biochemistry **163** 141-146

BUTCHER, G.A., MITCHELL, G.H. AND COHEN, S. (1973)
Mechanisms of host specificty in malarial infection.
Nature **244** 40-42

BYGBERG, I.C. AND FLACHS, H. (1986)
Effect of chloroquine on human lymphocyte proliferative proliferation.
Transaction of the Royal Society of Tropical Medicine and Hygiene **80** 231-235

BYGBERG, I.C., SVENSON, M., THEANDER, T.G. AND BENDTZEN, K. (1987)
Effect of antimalarial drugs on stimulation and interleukin 2 production of human lymphocytes.
International Journal of Immunopharmacology **9** 513-519

CARTER, R., GRAVES, P.M., KEISTER, D.B. AND QUAKYI, I.A. (1990)
Properties of epitopes of Pfs 48/45, a target of transmission-blocking monoclonal antibodies, on gametes of different isolates of *Plasmodium falciparum.*
Parasite Immunology **12** 587-603

CARTER, R., KUMAR, N., QUAKYI, I., GOOD, M., MENDIS, K., GRAVES, P. AND MILLER, L.H. (1988)
Immunity to asexual stages of malaria parasites.
Progress in Allergy **41** 193-214

CAVACINI, L.A., PARKE, L.A. AND WEIDANZ, W.P. (1990)
Resolution of acute malarial infections by T cell-dependent non-antibody-mediated mechanisms of immunity.
Infection and Immunity **58** 2946-2950

CELADA, A., CRUCHARD, A. AND PERRIN, L.H., (1984)
Independence of complement on *in vitro* phagocytosis of *Plasmodium falciparum* parasitised erythrocytes by human monocytes and polymorphonuclear leukocytes.
International Archives of Allergy and Applied Immunology **73** 363-366

CERAMI, C., KWAKYE-BERKO, F. AND NUSSENZWEIG, V. (1992)
Binding of malarial CS protein to sulfatides Gal(3-SO_4)b1-Cer and cholesterol-3-sulfate and its dependence on disulfide bond formation between cysteines in Region II.
Molecular and Biochemical Parasitology **54** 1-12

CHAROENVIT, Y., MELLOUK, S., COLE, C., BECHARA, R., LEEF, M.F., SEDEGAH, M., YUAN, L.F., ROBEY, F.A., BEAUDON, R.L. AND HOFFMAN, S.L. (1991)
Monoclonal, but not polyclonal, antibodies protect against *Plasmodium yoelii* sporozoites.
Journal of Immunology **146** 1020-1025

CHAROENVIT, Y., COLLINS, W.E., JONES, T.R., MILLET, P., YUAN, L., CAMPBELL, G.H., BEAUDON, R.L., BRODERSON, J.R. AND HOFFMAN, S.L. (1991)
Inability of malaria vaccine to induce antibodies to a protective epitope within its sequence.
Science **251** 668-671

CHAROENVIT, Y., MELLOUK, S., SEDEGAH, M., TOYOSHIMA, T., LEEF, M., DE LA VEGA, P., BEAUDOIN, R.L., AIKAWA, M., FALLARME, V. AND HOFFMAN, S.L. (1995)
Plasmodium yoelii: 17-kDa hepatic and erythrocytic stage protein is the target of an inhibitory monoclonal antibody.
Experimental Parasitology **80** 419-429

CHATTERJEE, S., FRANCOIS, G., DRUILHE, P., TIMPERMAN, G. AND WERY, M. (1996)
Immunity to *Plasmodium berghei* exoerythrocytic forms derived from irradiated sporozoites.
Parasitology Research **82** 297-303

CHATURVEDI, P., YU, Q., SOUTHWOOD, S., SETTE, A. AND SINGH, B. (1996)
Peptide analogs with different affinites for MHC alter the cytokine profile of T helper cells.
International Immunology **8** 745-755

CHEN, C. AND NABAVI, N. (1994)
In vitro induction of T cell anergy by blocking B7 and early T cell costimulatory molecul ETC-1/B7-2.
Immunity **1** 147-154

CHEN, D.H., TIGELAAR, R.E. AND WEINBAUM, F.I. (1977)
Immunity to sporozoite inducd malaria infection in mice.
Journal of Immunology **118** 1322-1327

CHER, D.J. AND MOSMANN, T.R. (1987)
Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by T(H)1 clones.
Journal of Immunology **138** 3688-3694

CLARK, I.A. (1987)
Cell-mediated immunity in protection and pathology of malaria.
Parasitology Today **3** 300-305

CLARK I.A., HUNT, N.H., BUTCHER, G.A. AND COWDEN, W.B. (1987)
Inhibition of murine malaria (*Plasmodium chabaudi*) *in vivo* by recombinant interferon-γ or tumour necrosis factor, and its enhancement by butylated hydroxyanisole.
Journal of Immunology **139** 3493-3496

CLARK, I.A., ROCKETT, K.A. AND COWDEN, W.B. (1991)
Proposed link between cytokines, nitric oxide and human cerebral malaria.
Parasitology Today **7** 205-207

CLARK, I.A., ROCKETT, K.A. AND COWDEN, W.B. (1992)
Possible central role of niric oxide in conditions clinically similar to cerebral malaria.
The Lancet **340** 894-896

CLARK, I.A., VIRELIZIER, J.L., CARSWELL, E.A. AND WOOD, P.R. (1981)
Possible importance of macrophage derived mediators in acute malaria.
Infection and Immunity **32** 1058-1066

CLAASSEN, E. AND VAN ROOIJEN, N. (1986)
Preparartion and characteristics of dichloromethylene-diphosphonate containing liposomes.
Journal of Microencapsulation **3** 109-114

CLYDE, D.F., McCARTHY, V.C., MILLER, R.M. AND HORNICK, R.B. (1973)
Specificity of protection of man immunised against sporozoite-induced falciparum malaria.
American Journal of Medical Science **266** 398-403

CLYDE, D.F., McCARTHY, V.C., MILLER, R.M. AND WOODWARD, W.E. (1975)
Immunisation of man against falciparum and vivax malaria by use of attenuated sporozoites.
American Journal of Tropical Medicine and Hygiene **24** 397-401

COCCIA, E.M., MARZIALI, G., STELLACI, E., PERROTI, E., ILARI, R., ORSATTI, R. AND BATTISTINI, A. (1995)
Cells resistant to interferon-beta respond to interferon-gamma via the Stat1-IRF-1 pathway.
Virology **211** 113-122

COFFMAN, R.L., O'HARA, J., BOND, M.W., CARTY, J., ZLOTNIK, A. AND PAUL, W.E. (1986)
B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells.
Journal of Immunology **136** 4538-4541

COHEN,S. AND BUTCHER, G.A. (1970)
Properties of protective malarial antibody.
Immunology **19** 369-383

COHEN, S., McGREGOR, L.A. AND CARRINGTON, S.S. (1961)
Gamma globulin and acquired immunity to human malaria.
Nature **192** 733-737

CONRAD, D.H., WALDSCHMIDT, T., LEE, W.T., RAO, M., KEEGAN, A.D., NOELLE, R.J., LYNCH, R.G. AND KEHRY, M.R. (1987)
Effect of B cell stimulatory factor-1 (interleukin-4) on Fcε and Fcγ receptor expression on murine B lymphocytes and B cell lines.
Journal of Immunology **139** 2290-2296

CONRAD, K.P., JOFFE, G.M., KRUSZYNA, H., KRUSZYNA, R., ROCHELLE, L.G., SMITH, R.P., CHAVEZ, J.E. AND MOSHER, M.D. (1993)
Identification of increased nitric oxide biosynthesis during pregnancy in rats.
FASEB **7** 566-571

CONSTANT, S.L. AND BOTTOMLY, K. (1997)
Induction of TH1 and TH2 $CD4^+$ T cell responses: The alternative approaches.
Annual Review of Immunology **15** 297-322

CORREA, M., NARAYANAN, P.R. AND MILLER, H.C. (1980)
Suppression activity of splenic adherent cells from *Plasmodium chabaudi* infected mice.
Journal of Immunology **125** 749-754

COT, S., RINGWALD, P., MULDER, B., MIAILHES, P., YAP-YAP, J., NUSSLER, A.K. AND ELING, W.M.C. (1994)
Nitric oxide in cerebral malaria.
Journal of Infectious Diseases **169** 1417-1418

COVELL, G. AND NICOL, W.D. (1951)
Clinical, chemotherapeutic and immunological studies on induced malaria.
British Medical Bulletin **8** 51-55

COX, J., SEMOFF, S. AND HOMMEL, M. (1987)
Plasmodium chabaudi: a rodent malaria model for *in vivo* and *in vitro* cytoadherence of malaria parasites in the absence of knobs.
Parasite Immunology **9** 543-561

CRISPE, I.N. AND MEHAL, W.Z. (1996)
Strange brew: T cells in the liver.
Immunology Today **17** 522-525

CROFTON, R.W., DIESSELHOFF-DEN DULK, M.M.C. AND VAN FURTH, R. (1978)
The origins, kinetics and characteristics of the Kupffer cells in the normal steady state.
Journal of Experimental Medicine **148** 1-17

CUNHA, F.Q., MOCADA, S. AND LIEW, F.Y. (1992)
Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-g in murine macrophages.
Biochemical and Biophysical Research Communications **182** 1155-1159

DALRYMPLE, S.A., LUCIAN, L.A., SLATTERY, R., McNEIL, T., AUD, D.M., FUCHINO, S., LEE, F. AND MURRAY, R. (1995)
Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia.
Infection and Immunity **63** 2262-2268

DALRYMPLE, S.A., SLATTERY, R., AUD, D.M., KRISHNA, M., LUCIAN, L.A. AND MURRAY, R. (1996)
Interleukin-6 is required for a protective immune response to systemic *Escherichia coli* infection.
Infection and Immunity **64** 3231-3235

DALY, T.M. AND LONF, C.A. (1995)
Humoral response to a carboxl-terminal region of the merozoite surface protein-1 plays a predominant role in controlling blood-stage infection in rodent malaria.
Journal of Immunology **155** 236-243

DAVID, P.H., HOMMEL, M., MILLER, L.H., UDEINYA, I.J. AND OLIGINO, L.D. (1983)
Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes.
Proceedings of the National Academy of Sciences USA **80** 5075-5079

DE BEER, F.C., BALTZ, M.L., HOLFORD, S., FEINSTEIN, A. AND PEPYS, M.B. (1981)
Fibronectin and C4-binding protein are selectively bound by aggregated amyloid P component.
Journal of Experimental Medicine **154** 1134-1149

DEITSCH, K.W. AND WELLEMS, T.E. (1996)
Membrane modifications in erythrocytes parasitized by *Plasmodium falciparum.*
Molecular and Biochemical Parasitology **76** 1-10

DE NAOTUNNE, S.T., KARUNAWEERA, N.D., DEL GUIDICE, G., KULARATNE, M.U., GRAU, G.E., CARTER, R. AND MENDIS, K.N. (1991)
Cytokines kill malaria parasites during infection crisis: Extracellular complemetary factors are essential.
Journal of Experimental Medicine **173** 523-529

DENKERS, E.Y., GAZZINELLI, R.T., MARTIN, D. AND SHER, A. (1993)
Emergence of NK1.1$^+$ cells as effectors of IFNγ-dependent immunity to *Toxoplasma gondii* in class I-deficient mice.
Journal of Experimental Medicine **178** 1465-1472

DENG, W., THIEL, B., TANNENBAUM, S., HAMILTON, T.A. AND STUEHR, D.J. (1993)
Synergistic cooperation between T cell lymphokines for induction of NOS gene in murine peritoneal macrophages.
Journal of Immunology **151** 322-329

DICK, S., WATERFALL, M., CURRIE, J., MADDY, A. AND RILEY, E. (1996)
Naive human alphabeta T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of interferon-γ.
Immunology **88** 412-420

DING, A.H., NATHAN, C.F. AND STUEHR, D.J. (1988)
Release of reactive intermediates and reactive oxygen intermediates from mouse peritoneal macrophages.
Journal of Immunology **141** 2407-2412

DING, A., NATHAN, C.F., GRAYCAR, J., DERYNCK, R., STUEHR, D.J. AND SRIMAL, S. (1990)
Macrophage deactivating factor and transforming growth factors-β_1,-β_2 and -β_3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ.
Journal of Immunology **145** 940-944

D'IMPERIO LIMA, M.R., ALVAREZ, J.M., FURTADO, G.C., KIPRIS, T.L., COUTINHO, A. AND MINOPRIO, P. (1996)
Ig-isotype patterns of primary and secondary B cell responses to *Plasmodium chabaudi chabaudi* correlate with IFN-γ and IL-4 cytokine production and with CD45RB expression by CD4$^+$ spleen cells.
Scandinavian Journal of Immunology **43** 263-270

DOCKRELL, H.M., DE SOUZA, J.B. AND PLAYFAIR, J.H.L. (1980)
The role of the liver in immunity to blood stage murine malaria.
Immunology **41** 421-430

DOOLAN, D.L., BECK, H-P. AND GOOD, M.F. (1994)
Evidence for limited activation of distinct $CD4^+$ T cell subsets in response to the *Plasmodium falciparum* circumsporozoite protein in Papua New Guinea.
Parasite Immunology **16** 129-136

DOOLAN, D.L., HOUGHTEN, R.A. AND GOOD, M.F. (1991)
Location of human cytotoxic T cell epitopes within a polymorphic domain of the *Plasmodium falciparum* circumsporozoite protein.
International Immunology **3** 511-516

DRAPIER, J.C., WIETZERBIN, J. AND HIBBS, J.B. Jr. (1988)
IFN-γ and TNF induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages.
European Journal of Immunology **18** 1587-1592

DVORAK, J.A., MILLER, L.H., WHITEHOUSE, W.C. AND SHIROISHI, T. (1975)
Invasion of erythrocytes by malaria merozoites.
Science **187** 748-750

EDIRISINGHE, J.S., FERN, E.B. AND TARGETT, G.A.T. (1981)
The influence of dietary protein on the development of malaria.
Annals of Tropical Medicine **1** 87-91

EGAN, J.E., WEBER, J.L., BALLOU, W., MAJARIAN, W., GORDON, D.M., HOFFMAN, S.L., WIRTZ, R.A., SCHNEIDER, I., WOOLLETT, G., HOLLINGDALE, M.R., YOUNG, J.F. AND HOCKMEYER, W.T. (1987)
Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development.
Science **236** 453-456

ELLOSO, M.M., VAN DER HEYDE, H.C., VANDE WAA, J.A., MANNING, D.D. AND WEIDANZ, W.P. (1994)
Inhibition of *Plasmodium falciparum in vitro* by human γδ T cells.
Journal of Immunology **153** 1187-1194

ELSE, K.J., FINKELMAN, F.D., MALISZEWSKI, C.R. AND GRENCIS, R.K. (1994)
Cytokine-mediated regulation of chronic intestinal helminth infection.
Journal of Experimental Medicine **179** 347-351

ELSE, K.J. AND GRENCIS, R.K. (1991)
Cellular immune responses to the murine nematode parasite *Trichuris muris*. I. Differential cytokine production during acute or chronic infection.
Immunology **72** 508-513

FAIRLEY, N.H. (1947)
Sidelights on malaria obtained by subinoculation experiments.
Transactions of the Royal Society of Tropical Medicineand Hygiene **40** 621-676

FALANGA, P.B. AND BUTCHER, E.C. (1991)
Late treatment with anti-LFA-1 (CD11a) antibody prevents cerebral malaria in a mouse model.
European Journal of Immunology **21** 2259-2263

FALANGA, P.B., D'IMPERIO LIMA, M.R., COUTINHO, A. AND PEREIRA DA SILVA, L. (1987)
Isotypic pattern of the polyclonal B cell response during primary infection by *Plasmodium chabaudi* and in immune-protected mice.
European Journal of Immunology **17** 599-603

FALINI, B., FLENGHI, L., PILERI, S., PELICCI, P., FAGIOLI, M., MARTELLI, M.F., MORETTA, L. AND CICCONE, E. (1989)
Distribution of T cells bearing different forms of the T cell receptor γδ in normal and pathological human tissues.
Journal of Immunology **143** 2480-2488

FARRAR, M.A. AND SCHREIBER, R.D. (1993)
The molecular cell biology of interferon-gamma and its receptor.
Annual Review of Immunology **11** 571-611

FAURE, P., HULIER, E., MILTGEN, F., RENIA, L. AND MAZIER, D. (1995)
Protective immunity against malaria: cellular changes in the liver vary according to the method of immunization.
Parasite Immunology **17** 469-477

FAURE, P., MARUSSIG, M., GOOSSENS, P., MILTGEN, F. AND MAZIER, D. (1994)
Characterization of liver lymphomyeloid cells in mice infected with *Plasmodium yoelii* sporozoites.
Immunology **82** 645-650

FAVRE, N., RYFFEL, B., BORDMANN, G. AND RUDIN, W. (1997)
The course of *Plasmodium chabaudi chabaudi* infection in interferon-gamma receptor deficient mice.
Parasite Immunology **19** 375-383

FENG, Z., HOFFMAN, R., NUSSENZWEIG, R.S., TSUJI, M., FUJIOKA, H., AIKAWA, M., LENSEN, T.H., PONNUDURAI, T. AND POLOGE, L.G. (1993)
Pfs2400 can mediate antibody-dependent malaria transmission inhibition and may be the *Plasmodium falciparum* 11.1 gene product.
Journal of Experimental Medicine **177** 273-281

FERNANDEZ-BOTRAN, R., SANDERS, V.M., MOSMANN, T.R. AND VITETTA, E.S. (1988)
Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells.
Journal of Experimental Medicine **168** 543-558

FERREIRA, A., SCHOFIELD, L., ENEA, V., SCHELLEKENS, H., VAN DER MEIDE, P., COLLINS, W.E., NUSSENZWEIG, R.S. AND NUSSENZWEIG, V. (1986)
Inhibition of development of exoerythrocytic forms of malaria parasites by γ-Interferon.
Science **232** 881-884

FERRINI, S., PRIGIONE, I. AND BOTTINO, C. (1989)
Monoclonal antibodies which react with the T cell receptor γδ rcognize different subsets of $CD3^+$ $WT31^-$ T lymphocytes.
European Journal of Immunology **19** 57-61

FEVANG, P., BJORKMANN, A. AND HOSTMERK, A. (1992)
Suppression of *Plasmodium falciparum in vitro* by polyunsaturated fatty acids.
In *Abstarcts of the XIII International Congress for Tropical Medicine and Malaria* 268

FINKLEMANN, F.D., HOLMES, J., KATONA, I.M., URBAN, J.F.J., BECHMANN, M.P., PARL, L.S., SCHOOLEY, K.A., COFFMAN, R.L., MOSMANN, T.R. AND PAUL, W.E. (1990)
Lymphokine control of *in vivo* immunoglobulin isotype selection.
Annual Review of Immunology **8** 303-333

FIORENTINO, D.F., ZLOTNIK, A., VIEIRA, P., MOSMANN, T.R., HOWARD, M., MOORE, K.M. AND O'GARRA, A. (1991)
IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells.
Journal of Immunology **146** 3444-3451

FITCH, C.D., YUNIS, N.G., CHEVLI, R. AND GONZALES, Y. (1974)
High-affinity accumulation of chloroquine by mouse erythrocytes infected with *Plasmodium berghei*.
Journal of Clinical Investigations **54** 24-33

FOLEY, M. AND TILLEY, L. (1997)
Quinoline Antimalarials: Mechanisms of action and resistance.
International Journal for Parasitology **27** 231-240

FOUNTOULAKIS, M., ZULAUF, M., LUSTIG, A. AND GAROTTA, G. (1992)
Stoichiometry of interaction between interferon gamma and its receptor.
European Journal of Biochemistry **208** 781-787

FOX, R. (1995)
Antimalarial drugs: Mechanisms of action in autoimmune disease and prospects for drug development.
Clinical Immunotherapeutics **4** 219-234

FRANCO, A., BARNABA, V., NATALI, P., BALSANO, C., MUSCA, A. AND BALSANO, F. (1988)
Expression of Class I and Class II major histocompatibility complex antigens on human hepatocytes.
Hepatology **8** 449-454

FREEDMAN, A.S., FREEMAN, G.J., RHYNHART, K. AND NADLER, L.M. (1991)
Selective induction of B7/BB-1 on interferon-γ stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway.
Cellular Immunology **137** 429-437

FREEMAN, G.J., BOUSSIOTIS, V.A., ANUMATHAN, A., BERNSTEIN, G.M., KE, X-Y., RENNERT, P.D., GRAY, G.S., GRIBBEN, J.G. AND NADLER, L.M. (1995)
B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 costimulates the initial production of IL-4.
Immunity **2** 523-532

FREIDMAN, M.J. (1978)
Erythrocytic mechanisms of sickle cell resistance to malaria,
Proceedings of the National Academy of Sciences USA **75** 1994-1997

FREIDMAN, M.J., (1979)
Oxidant damage mediates variant red cell resistance to malaria.
Nature **280** 245-247

FRIES, L.F., GORDON, D.M., SCHNEIDER, I., BEIER, J.C., LONG, G.W., GROSS, M., QUE, J.U., CRYZ, S.J. AND SADOFF, J.C. (1992)
Safety, immunogenecity, and efficacy of a *Plasmodium falciparum* vaccine comprising a circumsporozoite protein repeat region peptide conjugated to *Pseudomonas aeruginosa* toxin A.
Infection and Immunity **60** 1834-1839

FUJITA, T., REIS, L.F., WATANABE, N., KIMURA, Y., TANIGUCHI, T. AND VILCEK, J. (1989)
Induction of the transcription factor IRF-1 and interferon-β mRNAs by cytokines and activators of second-messenger pathways.
Proceedings of the National Academy of Sciences USA **86** 9936-9940

GAJEWSKI, T.F. AND FITCH, F.W. (1988)
Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones.
Journal of Immunology **140** 4245-4252

GAJEWSKI, T.F., PINNAS, M., WONG, T. AND FITCH, F.W. (1991)
Murine T_H1 and T_H2 clones proliferate optimally in response to distinct antigen presenting cell populations.
Journal of Immunology **146** 1750-1758

GARMEN, R.D., JACOBS, K.A., CLARK, S.C. AND RAULTET, D.H. (1987)
B-cell-stimulatory factor 2 (β2 interferon) functions as a second signal for interleukin 2 production by mature murine T cells.
Proceedings of the National Academy of Sciences. USA **84** 7629-7633

GARNHAM, P.C.C. (1966)
Malaria parasites and other haemosporidia.
Blackwell Scientific Publications, Oxford

GAULDIE,J., SAUDER, D.N., MCADAM, K.P.W.J. AND DINARELLO, C.A. (1987)
Purified interleukin-1 (IL-1) from human monocytes stimulates acute phase protein synthesis by rodent hepatocytes *in vitro*.
Immunology **60** 203-207

GAZZINELLI, R.T., ELTOUM, I., WYNN, T.A. AND SHER, A. (1993)
Acute cerebral toxoplasmosis is induced by *in vivo* neutralisation of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation.
Journal of Immunology **151** 3672-3681

GAZZINELLI, R.T., OSWALD, I.P., JAMES, S.L. AND SHER, A. (1991)
IL-10 inhibits parasite killing and nitrogen production by IFN-γ activated macrophages.
Journal of Immunology **148** 1792-1796

GIARDINA, B., MESSANA, I., SCATENA, R. AND CASTAGNOLA, M. (1995)
The multiple functions of hemoglobin.
Critical Reviews in Biochemistry and Molecular Biology **30** 165-196

GILKS, C.F., WALLIKER, D. AND NEWBOLD, C.I. (1990)
Relationships between sequestration, antigenic variation and chronic parasitism in *Plasmodium chabaudi chabaudi*- A rodent malaria model.
Parasite Immunology **12** 45-64

GILLESPIE, S.H., DOW, C., RAYNES, J.G., BEHRENS, R.H., CHIODINI, P.L. AND McADAM, K.P.W.J. (1991)
Measurement of acute phase proteins for assessing the severity of *Plasmodium falciparum* malaria.
Journal of Clinical Pathology **44** 228-231

GODFREY, D.G. (1957)
Antiparasite action of dietary cod liver oil upon *Plasmodium bergehi* and its reversal by vitamin E.
Experimental Parasitology **6** 555-565

GOLDBERG, D.E. AND SLATER, A.F.G. (1992)
The pathway of hemoglobin degradation in malaria parasites.
Parasitology Today **8** 280-283

GOLENSER, J., PEELS, L.G., LEEUWENBERG, A.D.E.M., VERHAVE, J.P. AND MEUWISSEN, J.H.E.T. (1978)
The influence of chloroquine on establishment of immunity against *P. berghei*.
Journal of Protozoology **25** 14B

GOOD, M.F., QUAKYI, I.A., SAUL, A., BERZOFSKY, J.A., CARTER, R. AND MILLER, L.H. (1987)
Human T clones reactive to the sexual stages of *Plasmodium falciparum* malaria: high frequency of gamete-reactive T cells in peripheral blood from non-exposed donors.
Journal of Immunology **138** 306-311

GOOSSENS, P., JOUIN, H. AND MILON, G. (1991)
Dynamics of lymphocytes and inflammatory cells recruited in liver during murine listeriosis.
Journal of Immunology **147** 3514-3520

GRANGER, D.L., PERFECT, J.R. AND DURACK, D.J. (1986)
Macrophage-mediated fungistasis: Requirement for a macromolecular component in serum.
Journal of Immunology **137** 693-701

GRAU, G.E. AND BEHR, C. (1994)
T cells and malaria: Is Th1 cell activation a prerequisite for pathology?
Research in Immunology **145** 441-454

GRAU, G.E., FAJARDO, L.F., PIGUET, P.F., ALLET, B., LAMBERT, P-H. AND VASSALLI, P. (1987)
Tumour necrosis factor (cachectin) as an essential mediator in murine malaria.
Science **237** 1210-1212

GRAU, G.E., HEREMANS, H., PIQUET, P-F., POINTAIRE, P., LAMBERT, P-H., BILLIAU, A. AND VASSALLI, P. (1989a)
Monoclonal antibody against interferon-γ can prevent experimental cerebral malaria and its associated overproduction of tumour necrosis factor.
Proceedings of the National Academy of Sciences USA **86** 5572-5574

GRAU, G.E., POINTAIRE, P., PIGUET, P-F., VESIN, C., ROSEN, H., STAMENKOVIC, I., TAKEI, F. AND VASSALLI, P. (1991)
Late administration of monoclonal antibody to leukocyte function antigen 1 abrogates incipient murine cerebral malaria.
European Journal of Immunology **21** 2265-2267

GRAU, G.E., TAYLOR, T.E., MOLYNEUX, M.E., WIRIMA, J.J., VASSALLI, P., HOMMEL, M. AND LAMBERT, P-H. (1989b)
Tumour necrosis factor and disease severity in children with falciparum malaria.
New England Journal of Medicine **320** 1586-1591

GRAY, C.D. AND PHILLIPS, R.S. (1981)
Use of sorbitol in the cryopreservation of *Babesia*.
Research in Veterinary Science **30** 388-389

GREEN, S.J., MELTZER, M.S., HIBBS, J.B. Jr. AND NACY, C.A. (1990)
Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine dependent killing mechanism.
Journal of Immunology **144** 278-283

GREEN, S.J. AND NACY, C.A. (1993)
Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthesis.
Current Opinion in Infectious Diseases **6** 384-396

GREENLUND, A.C., FARRAR, M.A., VIVIANO, B.L. AND SCHREIBER, R.D. (1994)
Ligand-induced IFN(gamma) receptor tyrosine phosphorylation couples the receptor to its signal transduction system.
EMBO Journal **13** 1591-1600

GREENLUND, A.C., MORALES, M.O., VIVIANO, B.L., YAN, H., KROLEWSKI, J. AND SCHREIBER, R.D. (1995)
Stat recruitment by tyrosine phosphorylated cytokine receptors: An ordered reversible affinity-driven process.
Immunity **2** 677-687

GREENLUND, A.C., WENNER, C.A. AND SCHREIBER, R.D. (1992)
Analysis of IFN-γ receptor binding to IFN-γ.
Journal of Interferon Research 12 103

GREENWOOD, B.M., BRADLEY, A.K., GREENWOOD, A.M., BYASS, P., JAMMEH, K., MARSH, K., TULLOCH, S., OLDFIELD, F.S.J. AND HAYES, R. (1987)
Mortality and morbidity from malaria among children in a rural area of The Gambia, West Africa.
Transactions of the Royal Society of Tropical Medicine and Hygiene **81** 478-486

GREENWOOD, B.M., BRADLEY-MOORE, A.M., BRYCESON, A.D.M. AND PALIT, A. (1972)
Immunosuppression in children with malaria.
The Lancet **i** 169-172

GREENWOOD, B.M. AND BRUETON, M.J. (1974)
Complement activation in children with acute malaria.
Clinical and Experimental Immunology **18** 267-272

GRISCAVAGE, J.M., ROGERS, N.E., SHERMAN, M.P. AND IGNARRO, L.J. (1993)
Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide.
Journal of Immunology **151** 6329-6337

GROUX, H. AND GYSIN, J. (1990)
Opsonisation as an effector mechanism in human protection against asexual blood stages of *Plasmodium falciparum*: Functional role of IgG subclasses.
Research in Immunology **141** 529-542

GRUN, J.L. AND WEIDANZ, W.P. (1981)
Immunity to *Plasmodium chabaudi adami* in the B-cell deficient mice.
Nature **290** 143-145

GRUN, J.L. AND WEIDANZ, W.P. (1983)
Antibody-independent immunity to reinfection malaria in B-cell deficient mice.
Infection and Immunity **41** 1197-1204

HAAS, W., PEREIRA, P. AND TONEGAWA, S. (1993)
Gamma/delta cells.
Annual Review of Immunology **11** 637-685

HAKIM, T.S., SUGIMORI, K., CAMPORESI, E.M. AND ANDERSON, G. (1996)
Half-life of nitric oxide in aqueous solutions with and without haemoglobin.
Physiological Measurement **17** 267-277

HARADA, H., FUJITA, T., MIYAMOTO, M., KIMURA, Y., MARUYAMA, M., FURIA, A., MIYATA, T. AND TANIGUCHI, T. (1989)
Structurally similar but functionally distinct factors, IRF-1 and IRF-2 bind to the same regulatory elements of IFN and IFN inducible genes.
Cell **58** 729-739

HARADA, H., TAKAHASHI, E-I, ITOJ, S., HARADA, K., HORI, T-A AND TANIGUCHI, T. (1994)
Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system.
Molecular and Cellular Biology **14** 1500-1509

HARDY, K.J. AND SAWADA, T. (1989)
Human gamma interferon strongly upregulates its own gene expression in peripheral blood lymphocytes.
Journal of Experimental Medicine **170** 1021-1026

HARPAZ, R., EDELMAN, R., WASSERMAN, S.S., LEVINE, M.M., DAVIS, J.R. AND SZTEIN, M.B. (1992)
Serum cytokine profiles in experimental human malaria.
Journal of Clinical Investigation **90** 515-523

HATHCOCK, K.S., LASZLO, G., PUCILLO, C., LINSLEY, P. AND HODES, R.J. (1994)
Comparative analysis of B7-1 and B7-2 costimulatory ligands: Expression and function.
Journal of Experimental Medicine **180** 631-640

HAWKING, F. (1954)
Milk, PABA and malaria in rats and monkeys.
British Medical Journal **i** 425-429

HAYGLASS, K.T., NAIDES, S.J., SCOTT, C.F., BENACERRAF, B. AND SY, M.S. (1986)
T cell development in B cell deficient mice. IV. The role of B cells as antigen-presenting cells *in vivo*.
Journal of Immunology **136** 823-829

HEALER, J., McGUINESS, D., HOPCROFT, P., HALEY, S., CARTER, R. AND RILEY, E. (1997)
Complement-mediated lysis of *Plasmodium falciparum* gamets by malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs230.
Infection and Immunity **65** 3017-3023

HEIM, M.H., KERR, I.M., STARK, G.R. AND DARNELL, J.E. Jr. (1995)
Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway.
Science **267** 1347-1349

HEINRICH, P.C., CASTELL, J.V. AND ANDUS, T. (1990)
Interleukin 6 and the acute phase response.
Biochemistry **265** 621-636

HEINZEL, F.P., SADICK, M.D., MUTHA, S.S. AND LOCKSLEY, R.M. (1991)
Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by $CD4^+$ lymphocytes *in vivo* during healing and progressive leishmaniasis.
Proceedings of the National Academy of Sciences USA **88** 7011-7015

HELFGOTT, D.C., MAY, L.T., STHOEGER, Z., TAMM, I. AND SEHGAL, P.B. (1987)
Bacterial lipopolysaccharide (endotoxin) enhances expression and secretion of beta2 interferon by human fibroblasts.
Journal of Experimental Medicine **166** 1300-1309

HELMBY, H., PERLMANN, H., TROYE-BLOMBERG, M. AND PERLMANN, P. (1996)
Immunoglobulin E elevation in *Plasmodium chabaudi* malaria.
Infection and Immunity **64** 1432-1433

HERMSEN, C., VAN DE WIEL, T., MOMMERS, E., SAUERWEIN, R. AND ELING, W. (1997)
Depletion of CD4+ or CD8+ T-cells prevents *Plasmodium berghei* induced cerebral malaria in end-stage disease.
Parasitology **114** 7-12

HERRINGTON, D.A., CLYDE, D.F., LOSONSKY, G., CORTESIA, M., MURPHY, J.R., DAVIS, J., BAQAR, S., FELIX, A.M., HEIMER, E.P., GILLESSEN, D., NARDIN, E., NUSSENZWEIG, R.S., NUSSENZWEIG, V., HOLLINGDALE, M.R. AND LEVINE, M.M. (1987)
Safety and immunogenecity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites.
Nature **328** 257-259

HIBBS, J.B. Jr., TAINTOR, R.R., VAVRIN, Z. AND RACHLIN, E.M. (1988)
Nitric Oxide: A cytotoxic activated macrophage effector molecule.
Biochemical and Biophysical Research Communications **157** 84-94

HILL, A.V.S., ALLSOPP, C.E.M., KWIATKOWSKI, D., ANSTEY, N.M, TWUMASI, P., ROWE, P.A., BENNETT, S., BREWSTER, D., McMICHAEL, A.J. AND GREENWOOD, B.M. (1991)
Common West African HLA antigens are associated with protection from severe malaria.
Nature **352** 595-600

HILL, A.V.S., ELVIN, J., WILLIS, A., AIDOO, M., ALLSOPP, C.E.M., GOTCH, F.M., GAO, X.M., TAKIGUCHI, M., GREENWOOD, B.M., TOWNSEND, A.R.M., McMICHAEL, A.J. AND WHITTLE, H.C. (1992)
Molecular analysis of the association of HLA-B53 and resistance to severe malaria.
Nature **360** 434-439

HILL, M.R., STITH, R.D. AND MCCALLUM, R.E. (1990)
Mechanisms of action of interferon-β/interleukin-6 on induction of hepatic liver enzymes.
Annals of the New York Academy of Sciences **557** 502-505

HIRAMATSU, K., YOSHIKAI, Y., MATSUZAKI, G., OHGA, S., MURAMOIR, K., MATSUMOTO, K., BLUESTONE, J.A. AND NOMOT, K. (1992)
A protective role of γδ T cells in primary infection with *Listeria monocytogenes* in mice.
Journal of Experimental Medicine **175** 49

HOLLINGDALE, M.R., MAZIER, D., APPIAH, A., LELAND, P., DERKS, T., YAP, S.H., BALLOU, W.R., HERRINGTON, D. AND VERHAVE, J.P. (1988)
Plasmodium falciparum CS vaccines elicit antibodies in human volunteers that inhibit sporozoite invasion of hepatoma cells but enhance invasion of human hepatocytes.
American Society of Tropical Medicine Meeting

HO, M. AND WEBSTER, H.K. (1990)
Immunology of human malaria. A cellular perspective.
Parasite Immunology **11** 105-116

HO, M., WEBSTER, H.K., GREEN, B., LOOAREESUWAN, S., KONGCHAREON, S. AND WHITE, N.J. (1988)
Defective production of and response to IL-2 in acute human falciparum malaria.
Journal of Immunology **141** 2755-2759

HO, M., WEBSTER, H.K., TONGTAWE, P., PATTANAPANYASAT, K. AND WEIDANZ, W.P. (1990)
Increased γδ T cells in acute *Plasmodium falciparum* malaria.
Immunology Letters **25** 139-142

HOFFMAN, S.L. AND FRANKE, E.D. (1994)
Inducing protective immune responses against the sporozoite and liver stages of *Plasmodium.*
Immunology Letters **41** 89-94

HOFFMAN, S.L., ISENBERGER, D., LONG, G.W., SEDEGAH, M., SZARFMEN, A., WATERS, L., HOLLINGDALE, M.R., VAN DER MEIDE, P.H., FINBLOOM, D.S. AND BALLOU, W.R. (1989)
Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes.
Science **244** 1078-1080

HOFFMAN, S.L., OSTER, C.N., PLOWE, C.V., WOOLLETT, G.R., BEIER, J.C., CHULAY, J.D., WIRTZ, R.A., HOLLINGDALE, M.R. AND MUGAMBI, M. (1987)
Naturally acquired antibodies to sporozoites do not prevent malaria: Vaccine development implications.
Science **237** 639-642

HOFFMAN, S.L., PIESSENS, W.F., RATINAYANTO, S., HUSSEIN, P.R., KURNIAWAN, L. AND PIESSENS, P.W. (1984)
Reduction of suppressor T lymphocytes in the tropical splenomegaly syndrome.
New England Journal of Medicine **310** 337-341

HOOD, A.T., FABRY, M.E., COSTANTINI, F., NAGEL, R.L. AND SHEAR, H.L. (1996)
Protection from lethal malaria in transgenic mice expressing sickle hemoglobin.
Blood **87** 1600-1603

HOUSSIAU, F.A., COULIE, P.G., OLIVE, D. AND VAN SNICK, J. (1988)
Synergistic activation of human T cells by interleukin 1 and interleukin 6.
European Journal of Immunology **18** 653-656

HOWARD, M., FARRAR, J., HILFIKER, M., JOHNSON, B., TAKATSU, K., HAMAOKA, T. AND PAUL, W.E. (1982)
Identification of a T-cell derived B-cell growth factor distinct from interleukin 2.
Journal of Experimental Medicine **155** 914-923

HUANG, S., HENDRIKS, W., ALTHAGE, A., HEMMI, S., BLUETHMANN, H., KAMIJO, R., VILCEK, J., ZINKERNAGEL, R.M. AND AGUET, M. (1993)
Immune response in mice that lack the interferon-gamma receptor.
Science **259** 1742-1745

HUNT, N.H., MANDUCI, N. AND THUMWOOD, C.M. (1993)
Amelioration of murine cerebral malaria by dietary restriction.
Parasitology **107** 471-476

HURST, N.P., FRENCH, J.K., BELL, A.L., NUKI, G., O'DONNELL, M.L., BETTS, W.H. AND CLELAND, L.G. (1986)
Differential effects of mepacrine, chloroquine and hydroxychloroquine on superoxide anion generation, phospholipid methylation and arachidonic acid release by human blood monocytes.
Biochemical Pharmacology **35** 3083-3089

HUTCHCRAFT, C.L., GEWURZ, H., HANSEN, B., DYCK, R.F. AND PEPYS, M.B. (1981)
Agglutination of complement-coated erythrocytes by serum amyloid P-component.
Journal of Immunology **126** 1217-1219

HVIID, L., REIMERT, C.M., THEANDER, T.G., JEPSEN, S. AND BENDTZEN, K. (1988)
Recombinant human tumour necrosis factor is not inhibitory to *Plasmodium falciparum in vitro.*
Transactions of the Royal Society of Tropical Medicine and Hygiene **82** 48-49

HVIID, L., THEANDER, T.G., ABDULHADI, N.H., ABU-ZEID, Y.A., BAYOUMI, R.A. AND JENSEN, J.B. (1991)
Transient depletion of T cells with high LFA-1 expression from peripheral circulation during acute *Plasmodium falciparum* malaria.
European Journal of Immunology **21** 1249-1253

HVIID, L., THEANDER, T.G., ELHASSAN, I.M. AND JENSEN, J.B. (1993)
Increased plasma levels of soluble ICAM-1 and ELAM-1 (E-selectin) during acute *Plasmodium falciparum* malaria.
Immunology Letters **36** 51-58

HYSLOP, S. AND De NUCCI, G. (1991)
The mechanisms and significance of the coupled release of endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI2) from endothelial cells.
Wiener Klinische Wochenschrift **103** 422-434

IBEKWE, C.A.C. AND UGWUNNA, S.C. (1990)
The effects of host diet on *Plasmodium yoelii nigeriensis.*
Journal of Parasitology **76** 903-912

IDZERDA, R.L., MARCH, C.J., MOSELY, B., LYMAN, S.D., VANDENBOS, T., GIMPEL, S.D., DIN, W.S., GRABSTEIN, K.H., WIDMER, M.B., PARK, L.S., COSMAN, D. AND BECKMANN, M.P. (1990)
Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily.
Journal of Experimental Medicine **171** 861-873

IGARASHI, K., GAROTTA, G., OZMEN, L., ZIEMIECKI, A., WILKS, A.F., HARPUR, A.G., LARNER, A.C. AND FINBLOOM, D.S. (1994)
Interferon-γ induces tyrosine phosphorylation of interferon-γ receptor and regulated association of protein tyrosine kinases, JAK 1 and JAK 2 with its receptor.
Journal of Biological Chemistry **269** 14333-14336

INABA, K., WITMER-PACK, M., INABA, M., HATHCOCK, K.S., SAKUTA, H., AZUMA, M., YAGITA, H., OKUMURA, K., LINSLEY, P.S., IKEHARA, S., MURAMATSU, S., HODES, R.J. AND STEINMAN, R.M. (1994)
The tissue distribution of the B7-2 costimulator in mice: abundnant expression on dendritic cells *in situ* and during maturation *in vitro.*
Journal of Experimental Medicine **180** 1849-1860

JACOBS, P., RADZIOCH, D. AND STEVENSON, M.M. (1995)
Nitric oxide expression in the spleen, but not in the liver, correlates with resistance to blood-stage malaria in mice.
Journal of Immunology **155** 5306-5313

JACOBS, P., RADZIOCH, D. AND STEVENSON, M.M. (1996)
In vivo regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice.
Infection and Immunity **64** 44-49

JAKOBSEN, P.H., MCKAY, V., MORRIS-JONES, D., MCGUIRE, W., BOELE VAN HENSBROEK, M., MEISNER, S., BENDTZEN, K., SCHOUSBOE, I., BYGBJERG, I.C. AND GREENWOOD, B.M. (1994)
Increased concentrations of interleukin-6 and interleukin-1 receptor antagonist and decreased concentrations of beta-2-glycoprotein I in Gambian children with cerebral malaria.
Infection and Immunity **62** 4374-4379

JAKOBSEN, P.H., MOON, R., RIDLEY, R.G., BATE, C.A.W., TAVERNE, J., HANSEN, M.B., TAKACS, B., PLAYFAIR, J.H.L. AND MCBRIDE, J.S. (1993)
Tumour necrosis factor and interleukin-6 production induced by components associated with merozoite proteins of *Plasmodium falciparum*.
Parasite Immunology **15** 229-237

JAMES, S.L. AND HIBBS JNR, J.B. (1990)
The role of nitrogen oxides as effector molecules of parasite killing.
Parasitology Today **6** 303-305

JAYAWARDENA, A.N., MURPHY, D.B., JANEWAY, C.A. AND GERSHON, R.K. (1982)
T cell mediated immunity in malaria. The Ly phenotype of T cells mediating resistance to *Plasmodium yoelii*.
Journal of Immunology **129** 377-381

JUN, C-D., CHOI, B-M., KIM,S-U., LEE, S.Y., KIM, H-M. AND CHUNG, H-T. (1995)
Down-regulation of transforming growth factor beta gene expression by antisense oligodeoxynucleotides increases recombinant interferon-gamma-induced nitric oxide synthesis in murine peritoneal macrophages.
Immunology **85** 114-119

KABELITZ, D., PECHOLD, K. AND BENDER, A. (1991)
Activation and activation-driven of human γδ T cells.
Immunological Reviews **120** 72-88

KAMIJO, R., HARADA, H., MATSUYAMA, T., BOSLAND, M., GERECITANO, J., SHAPIRO, D., LE, J., KOH, S.I., KIMURA, T., GREEN, S.J., MAK, T.W., TANIGUCHI, T. AND VILCEK, J. (1994)
Requirement for transcription factor IRF-1 in NO synthase induction in macrophages.
Science **263** 1612-1615

KAMIJO, R., LE, J., SHAPIRO, D., HAVELL, E.A., HUANG, S., AGUET, M., BOSLAND, M. AND VILCEK, J. (1993)
Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with Bacillus Calmette-Guerin and subsequent challenge with lipopolysaccharide.
Journal of Experimental Medicine **178** 1435-1440

KAPLAN, M.H., SCHINDLR, U., SMILEY, S.T. AND GRUSBY, M.J. (1996)
Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells.
Immunity **4** 313-319

KARUNWEERA, N., CARTER, R., GRAU, G., KWIATKOSKI, D., DEL GIUDICE, G. AND MENDIS, K. (1992)
Tumour necrosis factor-dependent parasite-killing effects during paroxysms in non-immune *Plasmodium vivax* malaria patients.
Clinical and Experimental Immunology **88** 499-505

KASAHARA, Y., MIYAWAKI, T., KATO, K., KANEGANE, H., YACHIE, A., YOKOI, T. AND TANIGUCHI, N. (1990)
Role of interleukin 6 for differential responsiveness of naive and memory $CD4^+$ T cells in CD2-mediated activation.
Journal of Experimental Medicine **172** 1419-1424

KASLOW, D.C. (1993)
Transmission-blocking immunity against malaria and other vector-borne diseases.
Current Opinion in Immunology **5** 557-565

KASLOW, D.C., ISAACS, S.N., QUAKYI, I.A., GWADZ, R.W., MOSS, B. AND KEISTER, D.B. (1991)
Induction of *Plasmodium falciparum* transmission-blocking antibodies by recombinant vaccinia.
Science **252** 1310

KASPER, L.H. AND ALGER, N.E. (1973)
Adoptive transfer of immunity to *Plasmodium berghei* by spleen and lymph node cells from young and old mice.
Journal of Protozoology **20** 445-449

KEEGAN, A.D., NELMS, K., WHITE, M., WANG, L-M., PIERCE, J.H. AND PAUL, W.E. (1994)
An IL-4 receptor region containing an insulin receptor motif is important for IL-4 mediated IRS-1 phosphorylation and cell growth.
Cell **76** 811-820

KERN, P., HEMMER, C.J., VAN DAMME, J., GRUSS, H-J. AND DIETRICH, M. (1989)
Elevated tumour necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria.
American Journal of Medicine **87** 139-143

KHAN, Z.M., NG, C. AND VANDERBERG, J.P. (1992)
Early hepatic stages of *Plasmodium berghei*: Release of circumsporozoite protein and host cellular inflammatory response.
Infection and Immunity **60** 264-270

KHAN, Z.M. AND VANDERBERG, J.P. (1992)
Specific inflammatory cell infiltration of hepatic schizonts in BALB/c mice immunised with attenuated *Plasmodium yoelii* sporozoites.
International Immunology **4** 711-718

KHARAZMI, A. AND ERIKSEN, H.O. (1986)
Phagocytosis and bactericidal activity of human leucoytes under influence of antimalrial drugs.
Transactions of the Royal Society of Tropical Medicine and Hygiene **80** 758-760

KHARAZMI, A., VALERIOUS, N.H. AND HIBY, N. (1983)
Effect of antimalarial drugs on human neutrophil chemotaxis *in vitro*.
Acta Pathological and Microbiological and Immunology **91** 293-298

KHUSMITH, S., CHAROENVIT, Y., KUMAR, S., SEDEGAH, M., BEAUDOIN, R.L. AND HOFFMAN, S.L. (1991)
Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein.
Science **252** 715-718

KHUSMITH, S., SEDEGAH, M. AND HOFFMAN, S.L. (1994)
Complete protection against *Plasmodium yoelii* by adoptive transfer of a $CD8^+$ cytotoxic T-cell clone recognizing sporozoite surface protein 2.
Infection and Immunity **62** 2979-2983

KLOTZ, F.W., SCHELLER, L.F., SEGUIN, M.C., KUMAR, N., MARLETTA, M.A., GREEN, S.J. AND AZAD, A.F. (1995)
Co-localization of inducible-nitric oxide synthase and *Plasmodium berghei* in hepatocytes from rats immunized with irradiated sporozoites.
Journal of Immunology **154** 3391-3395

KOBAYASHI, F., MORII, T., MATSUI, T., FUJINO, T., WATANABE, Y., WEIDANZ, W.P. AND TSUJI, M. (1996)
Production of interleukin 10 during malaria caused by lethal and nonlethal variants of *Plasmodium yoelii yoelii*.
Parasitology Research **82** 385-391

KOHASE, M., HENRIKSEN-DESTEFANO, D., MAY, L.T., VILCEK, J. AND SEHGAL, P.B. (1986)
Induction of β2-interferon by tumour necrosis factor: A homeostatic mechanism in the control of cell proliferation.
Cell **45** 659-666

KOJ, A.(1974)
Acute phase reactants: their synthesis, turnover and biological significance.
In *Structure and function of the plasma proteins*, Vol 1 Plenum Press, New York P73

KOPF, M., BAUMANN, H., FREER, G., FREUDENBERG, M., LAMERS, M., KISHMOTO, T., ZINKERNAGEL, R., BLUETHMANN, H. AND KOHLER, G. (1994)
Impaired immune and acute-phase responses in interleukin-6-deficient mice.
Nature **368** 339-342

KOPF, M., BROMBACHER, F., KOHLER, G., KIENZLE, G., WIDMANN, K-H., LEFRANG, K., HUMBORG, C., LEDERMANN, B. AND SOLBACH, W. (1996)
IL-4-deficient Balb/c mice are resistant to infection with *Leishmania major.*
Journal of Experimental Medicine **184** 1127-1136

KOPF, M., LE GROS, G., BACHMANN, M., LAMERS, M.C., BLUETHMANN, H. AND KOHLER, G. (1993).
Disruption of the murine IL-4 gene blocks T_H2 cytokine responses.
Nature **362** 245-248

KOPF, M., GROS, G.L., COYLE, A.J., KOSCO-VILBOIS, M. AND BROMBACHER, F. (1995)
Immune responses of IL-4, IL-5, IL-6 deficient mice.
Immunological Reviews **148** 45-69

KOYASU, S. (1994)
$CD3^+CD16^+NK1.1^+B220^+$ large granular lymphocytes arise from both alpha-beta $TCR^+CD4^-CD8^-$ and gamma-delta $TCR^+CD4^-CD8^-$ cells.
Journal of Experimental Medicine **179** 1957-1972

KOZIEL, M.J., DUDLEY, D., WONG, J.T., DIENSTAG, J., HOUGHTON, M., RALSTON, R. AND WALKER, B.D. (1992)
Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis.
Journal of Immunology **149** 3339-3344

KREMSNER, P.G., NEIFER, S., CHAVES, M.F., RUDOLPH, R. AND BIENZLE, U. (1992).
IFNγ induced lethality in the late phase of *Plasmodium vinckei* malaria despite effective parasite clearance by chloroquine.
European Journal of Immunology **22** 2873-2878

KREMSNER, P.G., NUSSLER, A., NEIFER, S., CHAVES, M.F., BIENZLE, U., SENALDI, G. AND GRAU, G.E. (1993)
Malaria antigen and cytokine-induced production of reactive nitrogen intermediates by murine macrophages: no relevance to the development of experimental cerebral malaria.
Immunology **78** 286-290

KREMSNER, P.G., WINKLER, S., WILDLING, E., PRADA, J., BIENZLE, U., GRANINGER, W. AND NUSSLER, A.K. (1996)
High plasma levels of nitrogen oxides are associated with severe disease and correlate with rapid parasitological and clinical cure in *Plasmodium falciparum* malaria.
Transactions of the Royal Society of Tropical Medicine and Hygiene **90** 44-47

KROGSTAD, D.J. AND SCHLESSINGER, P.H. (1987)
The basis of anti-malarial action: non weak base effects of chloroquine on acid vesicle pH.
American Journal of Tropical Medicine and Hygiene **36** 213-220

KROTOSKI, W.A., COLLINS, W.E., BRAY, R.S., GARNHAM, P.C.C., COGSWELL, F.B., GWADZ, R.W., KILLICK-KENDRICK, R., WOLF, R., SINDEN, R.S., KOONTZ, L.C. AND STANFILL, P.S. (1982)
Demonstration of hypnozoites in sporozoite transmitted *Plasmodium vivax* infection.
American Journal of Tropical Medicine and Hygiene **31** 1291-1293

KUBES, P., SUZUKI, M. AND GRANGER, D.N. (1991)
NO: An endogenous modulator of leukocyte adhesion.
Proceedings of the Natlional Academy of Sciences USA. **88** 4651-4655

KUCHROO, V.K., DAS, M.P., BROWN, J.A., RANGER, A.M., ZAMVIL, S.S., SOBEL, R.A., WEINER, H.L., NABAWI, N. AND GLIMCHER, L.H. (1995)
B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: Application to autoimmune disease therapy.
Cell **80** 707-718

KUHN, R., RAJEWSKY, K. AND MULLER, W. (1991)
Generation and analysis of interleukin-4 deficient mice.
Science **254** 707-710

KUMARARATNE, D.S., PHILLIPS, R.S., SINCLAIR, D., PARROTT, M.V.D. & FORRESTER, J.B. (1987)
Lymphocyte migration in murine malaria during the primary patent parasitaemia of *Plasmodium chabaudi* infections.
Clinical and Experimental Immunology **68** 65-77

KUMARATILAKE, L.M., ROBINSON, B.S., FERRANTE, A. AND POULOS, A. (1992)
Antimalarial properties of n-3 and n-6 polyunsaturated fatty acids: *in vitro* effects on *Plasmodium falciparum* and *in vivo* effect on *P. berghei*.
Journal of Clinical Investigation **89** 961-967

KUMAR, N. AND WIZEL, B. (1992)
Further characterization of interactions between gamete surface antigens of *Plasmodium falciparum*.
Molecular and Biochemical Parasitology **53** 113-120

KUMAR, S., GOOD, M.F., DONTFRAID, F., VINETZ, J.M. AND MILLER, L.H. (1989)
Interdependence of $CD4^+$ T cells and malarial spleen in immunity to *Plasmodium vinckei vinckei*. Relevance to vaccine develpoment.
Journal of Immunology **143** 2017-2023

KUMAR, S., MILLER, L.H., QUAKYI, I.A., KEISTER, D.B., HOUGHTEN, R.A., MALOY, W.L., MUSS, B., BERZOFSKY, J.A. AND GOOD, M.F. (1988)
Cytotoxic T cells specific for the circumsporozoite protein of *Plasmodium falciparum*.
Nature **334** 258-260

KUNZ, D., WALKER, G., EBERHARDT, W. AND PFEILSCHIFTER, J. (1996)
Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin 1β-stimulated mesangial cells: evidence for the involvement of transcriptional and posttranscriptional regulation.
Proceedings of the National Academy of Sciences USA **93** 255-259

KWIATKOWSKI, D. (1989)
Febrile temperatures can synchronise the growth of *Plasmodium falciparum in vitro*.
Journal of Experimental Medicine **169** 357-361

KWIATKOWSKI, D. (1995)
The biology of malarial fever.
Baillieres Clinical Infectious Diseases **2** 371-388

KWIATOKOWSKI, D., CANNON, J.G., MANOGUE, K.R., CERAMI, A., DINARELLO, C.A. AND GREENWOOD, B.M. (1989)
Tumour necrosis factor production in falciparum malaria and its association with schizont rupture.
Clinical and Experimental Immunology **77** 361-366

KWIATKOWSKI, D., HILL, A.V.S., SAMBOU, I., TWUMASI, P., CASTRACANE, J., MANOGUE, K.R., CERAMI, A., BREWSTER, D.R. AND GREENWOOD, B.M. (1990)
TNF concentrations in fatal cerebral, non-fatal cerebral and uncomplicated *Plasmodium falciparum* malaria.
The Lancet **326** 1201-1204

KWON, N.S., STUEHR, D.J. AND NATHAN, C.F. (1991)
Inhibition of tumour cell ribonucleotide reductase by macrophage-derived nitric oxide.
Journal of Experimental Medicine **174** 761-767

LAMONTAGUE, L.R., GAULDIE, J., BEFUS, A.D., McADAM, K.P.W.J., BALTZ, M.L. AND PEPYS, M.B. (1984)
The acute phase response in parasitic infection. *Nippostrongylus brasiliensis* in the mouse.
Immunology **52** 733-741

LANAR, D.E., TINE, J.A., DE TAISNE, C., SEGUIN, M.C., COX, W.I., WINSLOW, J.P., WARE, L.A., KAUFFMAN, E.B., GORDON, D., BALLOU, W.R., PAOLETTI, E. AND SADOFF, J.C. (1996)
Attenuated vaccinia virus-circumsporozoite protein recombinants confer protection against rodent malaria.
Infection and Immunity **64** 1666-1671

LANGHORNE, J. (1989)
The role of $CD4^+$ T cells in the immune response to *Plasmodium chabaudi.*
Parasitology Today **5** 362-364

LANGHORNE, J., GILLARD, S., SIMON, B., SLADE, S. AND EICHMANN, K. (1989)
Frequencies of $CD4^+$ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with T_H1 and T_H2 characteristics during infection.
International Immunology **1** 416-424

LANGHORNE, J., KIM, K-J. AND ASOFSKY, R. (1985)
Distribution of immunoglobulin isotypes in the non-specific B-cell response induced by infection with *Plasmodium chabaudi adami* and *Plasmodium yoelii.*
Cellular Immunology **90** 251

LANGHORNE, J., MOMBAERTS AND TONEGAWA, S. (1995)
$\alpha\beta$ and $\gamma\delta$ T cells in the immune response to the erythrocytic stages of malaria in mice.
International Immunology **7** 1005-1011

LANGHORNE, J., PELLS, S. AND EICHMANN, K. (1993)
Phenotypic characterization of splenic T cells from mice infected with *Plasmodium chabaudi chabaudi.*
Scandinavian Journal of Immunology **38** 521-528

LANIER, L.L., RUITENBERG, J.J. AND PHILLIPS, J.H. (1986)
Human $CD3^+$ T lymphocytes that express neither CD4 nor CD8 antigens.
Journal of Experimental Medicine **164** 339-344

LE, J., FREDRICKSON, G., REIS, L., DIAMANSTEIN, T., HIRANO, T., KISHIMOTO, T. AND VILCEK, J. (1988)
Interleukin-2-dependent and interleukin-2-independent pathways of regulation of thymocyte function by interleukin-6.
Proceedings of the National Academy of Sciences USA **85** 509-514

LE, P.T., MULLER, M. AND MORTENSEN, R.F. (1982)
Acute phase reactants of mice. I. Isolation of serum amyloid P-component (SAP) and its induction by a monokine.
The Journal of Immunology **129** 665-672

LEEUWENBERG, J. F. M., VON ASMUTH, E.J.U, JEUHOMME, T.M.A. AND BUURMAN, W.A. (1990)
IFNγ regulates the expression of the adhesion molecule ELAM-1 and IL-6 production by human endothelial cells *in vitro*.
Journal of Immunology **145** 2110-2114

LENSCHOW, D.J., WALUNAS, T.L. AND BLUESTONE, J.A. (1996)
CD28/B7 system of T cell costimulation.
Annual Review of Immunology **14** 233-258

LEPOIVRE, M., FIESCHI, F., COVES, J., THELANDER, L. AND FONTECAVE, M. (1991)
Inactivation of ribunucleotide reductase by nitric oxide.
Biochemical and Biophysical Research Communications **179** 442-448

LEUNG, K.H. AND MIHICH, E. (1980)
Prostaglandin modulation of development of cell-mediated immunity in culture.
Nature **288** 597-600

LEVANDER, O.A., FU, S., CAI, X., REN, L. AND MORRIS, V. (1994)
Effect of menhaden oil ethyl esters (MOEE) on the recrudescence of malaria in patients previously treated with quinghauso (QHS).
FASEB **8** A438

LEVO, Y., FRANGIONE, B. AND FRANKLIN, E.C. (1977)
Amino acid sequence similarities between amyloid P component, C1q, and CRP.
Nature **268** 56-57

LEVO, Y. AND WOLLNER, S. (1985)
Effects of serum amyloid P component on human lymphocytes.
International Archives of Allergy and applied Immunology **77** 322-325

LI, J.J., PEREIRA, M.E.A., DELELLIS, R.A. AND McADAM, K.P.W.J. (1984)
Human amyloid P component: A circulating lectin that modulates immunological responses.
Scandinavian Journal of Immunology **19** 227-236

LIEW, F.Y., MILLOTT, S., PARKINSON, C., PALMER, R.M.J. AND MONCADA, S. (1990)
Macrophage killing of *Leishmania* parasite *in vivo* is mediated by nitric oxide from L-arginine.
Journal of Immunology **144** 4794-4797

LIN, B-F., KU, N-O., ZAHEDI, K., WHITEHEAD, A.S. AND MORTENSEN, R.F. (1990)
IL-1 and IL-6 mediate increased production and synthesis by hepatocytes of acute-phase reactant mouse serum amyloid P-component (SAP).
Inflammation **14** 297-313

LINKE, A., KUHN, R., MULLER, W., HONARVAR, N., LI, C. AND LANGHORNE, J. (1996)
Plasmodium chabaudi chabaudi: Differential susceptibility of gene-targeted mice deficient in IL-10 to an erythrocytic stage infection.
Experimental Parasitology **84** 253-263

LINKE, A., LI, C., HONARVAR, N., KUHN, R., MULLER, W. AND LANGHORNE, J. (1995)
Response to *Plasmodium chabaudi* infection in IL-10-deficient mice.
Immunology **86** Supplement 1 138

LOCKSLEY, R.M., REINER, S.L., HATAM, F., LITTMAN, D.R. AND KILLEEN, N. (1993)
Helper T cells without CD4: control of leishmaniasis in Cd4-deficient mice.
Science **261** 1448-1451

LORRE, K., VAN DAMME, J., VERWILGHEN, J., BAROJA, M. L. AND CEUPPENS, J.L. (1990)
IL-6 is an accessory signal in the alternative CD2-mediated pathway of T cell activation.
Journal of Immunology **144** 4691-4687

LOTEM, J., SHABO, Y. AND SACHS, L. (1989)
Clonal variation in susceptibility to differentiation by different protein inducers in the myeloid leukaemia cell line M1.
Leukaemia **3** 804-807

LUCAS, B., ENGEL, A., CAMUS, D. AND HAQUE, A. (1993)
Plasmodium yoelii in mice: antigen reactivity of CD4- and CD8-bearing T cells.
Cellular Immunology **150** 59-71

LUGER, T.A., KRUTMAN, J., KIRNBAUER, R., URBANSKI, A., SCHWARZ, T., KLAPPACHER, G., KOCK, A., MICKSCHE, M., MALEJCZY, K.J., SCHAUER, E., MAY, L.T. AND SEHGAL, P.B. (1989)
IFNβ/IL-6 augments the activity of human natural killer cells.
Journal of Immunology **143** 1206-1209

LUZ, M.R.M.P., VAN LEUVEN, F. AND ARAUJO-JORGE, T.C. (1994)
Heterogeneity in the plasma levels of two acute-phase proteins in mice from inbred strains infected with *Trypanosoma cruzi*.
Parasitology Research **80** 439-441

LWIN, M., LISTON, A.J., DOENHOFF, M.J. AND TARGETT, G.A.T. (1979)
Chemotherapy of malaria (*P. chabaudi*) infections in immunosuppressed mice.
Transactions of the Royal Society of Tropical Medicine and Hygiene **73** 103

LYTTON, S.D., LOYEVSKY, M., LIBMAN, J., MESTER, B., SHANZER, A. AND CABANTCHIK, Z.I. (1994)
The biochemical basis for the selective antimalarial action of iron chelators on *Plasmodium falciparum* parasitised cells.
Advances in Experimental Medicine and Biology **356** 385-397

MACDERMOTT, R.P., WELLS, R.A., ZOLYOMI, S., PAVANAD, K., PHISPHUMVIDHI, P., PERMPANICH, B. AND GILBREATH, M. (1980)
Examination of peripheral blood mononuclear cells and sera from Thai adults naturally infected with malaria in assays of blastogenic responsiveness to mitogenic lectins and allogenic cell surface antigens.
Infection and Immunity **30** 781-785

MacMICKING, J., XIE, Q.W. AND NATHAN, C. (1997)
Nitric oxide and macrophage function.
Annual Review of Immunology **15** 323-350

MacPHERSON, G.G., WARRELL, M.J., WHITE, N.J., LOOAREESUWAN, S. AND WARRELL, D.A. (1985)
Human cerebral malaria: A quantitative ultrastructural analysis of parasitised erythrocyte sequestration.
American Journal of Pathology **119** 385-401

MAEGRAITH, B.G., DEEGAN, T. AND SHERWOOD JONES, E. (1952)
Suppression of malaria (*P. berghei*) by milk.
British Medical Journal **ii** 1382-1384

MAGGI, E., PARRONCHI, P., MANETTI, R., SIMONELLI, C., PICCINI, M.P., RUGIU, F.S., DE CARLI, M., RICCI, M. AND ROMAGNANI, S. (1992)
Reciprocal regulatory effects of IFN-gamma and IL-4 on the *in vitro* development of Th1 and Th2 clones.
Journal of Immunology **148** 2142-2147

MAHESHWARI, R.L., CZARNIECKI, C.W., DUTTA, G.P., PURI, S.K., DHAWAN, B.N.. AND FRIEDMAN, R.M. (1986)
Recombinant human gamma interferon inhibits Simian malaria.
Infection and Immunity **53** 628-630

MALIK, A., EGAN, J.E., HOUGHTEN, A., SADOFF, J.C. AND HOFFMAN, S.L. (1991)
Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein.
Proceedings of the National Academy of Sciences USA 88 3300-3304

MALIK, A., HOUGHTEN, R., CORRADIN, G., BUUS, S., BERZOFSKY, J.A. AND HOFFMAN, S.L. (1995)
Identification of nonameric H-2K(k)-restricted $CD8^+$ cytotoxic T lymphocyte epitope on the *Plasmodium falciparum* circumsporozoite protein.
Infection and Immunity **63** 1955-1959

MAMULA, M.J. AND JANEWAY, C.A. (1993)
Do B cells drive the diversification of immune responses?
Immunology Today **14** 151-152

MANAWADU, B.R. AND VOLLER, A. (1978)
Standardisation of the indirect fluorescent antibody test for malaria.
Transactions of the Royal Society of Tropical Medicine and Hygiene **72** 456-462

MANETTI, R., PARRONCHI, P., GIUDIZI, M.G., PICCINI, M-P., MAGGI, E., TRINCHIERI, G. AND ROMAGNANI, S. (1993)
Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4 producing Th cells.
Journal of Experimental Medicine **177** 1199-1204

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF,C.D. AND WISHNOK, J.S. (1988)
Macrophage oxidation of L-arginine to nitrite and nitrate: NO is an intermediate.
Biochemistry **27** 8706-8711

MARTIN, E., NATHAN, C. AND XIE, Q. (1994)
Role of interferon regulatory factor 1 in the induction of nitric oxide synthase.
Journal of Experimental Medicine **180** 977-984

MAZIER, D., COLLINS, W.E., MELLOUK, S., PROCELL, P.M., BERBIGUIER, N., CAMPBELL, G.H., MILTGEN, F., BERTOLOTTI, R., LANGLOIS, P. AND GENTILINI, M. (1987)
Plasmodium ovale: *in vitro* development of hepatic stages.
Experimental Parasitology **64** 393-400

MAZIER, D., MELLOUK, S., BEUDOIN, R.L., TEXIER, B., DRUILHE, P., HOCKMEYER, W., TROSPER, J., PAUL, C., CHAROENVIT, Y., YOUNG, J., MILTGEN, F., CHEDID, L., CHIGOT, J., GALLEY, B., BRANDICOURT, O. AND GENTILINI, M. (1986)
Effect of antibodies to recombinant and synthetic peptides on development of *P. falciparum* sporozoites *in vitro*.
Science **231** 156-159

MAZIER, D., MILTGEN, F., NUDELMAN, S., NUSSLER, A., RENIA, L., PIED, S., GOMA, J. AND GENTILINI, M. (1988)
Pre-erythrocytic stages of plasmodia. Role of specific and nonspecific factors.
Biology of the Cell **64** 165-172

MAZIER, D., RENIA, L., NUSSLER, A., PIED, S., MARISSIY, M., GOMA, J., GRILLOT, D., MILTGEN, F., DRAPIER, J-C., CORRADIA, G., Del GIUDICE, G. AND GRAU, G.E. (1990)
Hepatic phase of malaria is the target of cellular mechanisms induced by the previous and subsequent stages: A crucial role for liver nonparenchymal cells.
Immunology Letters **25** 65-70

McDONALD, V. AND PHILLIPS, R.S. (1978a)
Increase in non-specific antibody mediated cytotoxicity in malarious mice.
Cinical and Experimental Immunology **34** 159-163

McDONALD, V. AND PHILLIPS, R.S. (1978b)
Plasmodium chabaudi in mice. Adoptive transfer of immunity with enriched populations of spleen T and B lymphocytes.
Immunology **34** 821-830

McDONALD, V. AND PHILLIPS, R.S. (1980)
Plasmodium chabaudi: Adoptive transfer of immunity with different spleen cell populations and development of protective activity in serum of lethally irradiated recipient mice.
Experimental Parasitology **49** 26-33

McGREGOR, I.A. (1964)
Studies in the acquisition of immunity to *Plasmodium falciparum* infections in Africa.
Transactions of the Royal Society of Tropical Medicine and Hygiene **58** 80-87

McGUIRE, W., HILL, A.V.S., ALLSOPP, C.E.M., GREENWOOD, B.M. AND KWIATKOWSKI, D. (1994)
Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria.
Nature **371** 508-511

McLEAN, S.A., PEARSON, C.D. AND PHILLIPS, R.S. (1982)
Plasmodium chabaudi: evidence of antigenic variation during recrudescent parasitaemias in mice.
Experimental Parasitology **54** 296-302

McNALLY, J., O'DONOVAN, S.M. AND DALTON, J.P. (1992)
Plasmodium berghei and *Plasmodium chabaudi chabaudi*: development of simple *in vitro* erythrocyte invasion assays.
Parasitology **105** 355-362

MEDING, S.J., CHENG, S.C., SIMON-HAARHAUS, B. AND LANGHORNE, J. (1990)
Role of interferon-γ during infection with *Plasmodium chabaudi chabaudi.*
Infection and Immunity **58** 3671-3678

MEDING, S.J. AND LANGHORNE, J. (1991)
$CD4^+$ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi chabaudi.*
European Journal of Immunology **21** 1433-1438

MELILLO, G., TAYLOR, L.S., BROOKS, A., MUSSO, T., COX, G.W. AND VARESIO, L. (1997)
Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine.
Journal of Biological Chemistry **272** 12236-12243

MELLOUK, S., GREEN, S.J., NACY, C.A. AND HOFFMAN, S.L. (1991)
IFN gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism.
Journal of Immunology **146** 3971-3976

MELLOUK, S., MAHESHWARI, R.M., RHODES-FEUILLETTE, A., BEAUDOIN, R.L., BERBIGUIER, N., MATILE, H., MILTGEN, F., LANDAU, I., PIED, S., CHIGOT, J.P., FRIEDMANN, R.M. AND MAZIER, D. (1987)
Inhibitory activity of interferons and interleukin 1 on the development of *Plasmodium falciparum* in human hepatocyte cultures.
Journal of Immunology **139** 4192-4195

MELLOUK, S., MAZIER, D., DRUILHE, P., BERBIGUIER, N. AND DANIS, M. (1986a)
In vitro and in vivo results suggests that antisporozoite antibodies do not totally block *Plasmodium falciparum* sporozoite infectivity.
New England Journal of Medicine **315** 648

MELLOUK, S., MAZIER, D., DRUILHE, P., CHAROENVIT, Y., BEAUDOIN, R.L., BERBIGUIER, N., MILTGEN, F.,GALLEY, B., LANGLOIS, P. AND GENTILINI, M. (1986b)
Absence of total inhibition of the preerythrocytic stages of *Plasmodium falciparum* by human antibodies and monoclonal antibodies directed to sporozoite surface antigens.
Sixth International Congress of Parasitology Brisbane, Australia

MIGLIORINI, P., CORRADIN, G. AND CORRADIN, S.B. (1991)
Macrophage NO2-production as a sensitive and rapid assay for the quantitation of murine IFN-gamma.
Journal of Immunological Methods **139** 107-114

MILLER, L.H., DVORAK, J.A., SHIROSHI, T. AND DUROCHER, J.R. (1973)
Influence of erythrocyte membrane components on malaria merozoite invasion.
Journal of Experimental Medicine **138** 1597-1601

MILLER, L.H., MASON, S.J., CLYDE, D.F. AND McGINNIS, M.H. (1976)
The resistance factor to *Plasmodium vivax* in Blacks: the Duffy blood-group genotype, FyFy.
New England Journal of Medicine **295** 302-304

MILLER, L.H., MASON, S.J., DVORAK, J.A., McGINNIS, H. AND RATHMAN, I.K. (1975)
Erythrocyte receptors from (*Plasmodium knowlesi*) malaria: Duffy blood group determinants.
Science **189** 561-563

MINOPRIO, P.S., ITOHARA, S., HEUSSER, C., TONEGAWA, S. AND COUTINHOU, A. (1989)
Immunobiology of murine *Trypanosoma cruzi* infection: the predominance of parasite non specific responses and the activation of TCR1 T cells.
Immunological Reviews **112** 182-207

MODLIN, R.L., PIRMEZ, C., HOFMAN, F.M., TORIGIAN, V., UYEMURA, K., REA, T.H., BLOOM, B.R. AND BRENNER, M.B. (1989)
Lymphocytes bearing antigen-specific γδ T cell recptors accumulate in human infectious disease lesions.
Nature **338** 544-548

MOGIL, R.J., PATTON, C.L. AND GREEN, D.R. (1987)
Cellular subsets involved in cell-mediated immunity to murine *Plasmodium yoelii* 17X malaria.
Journal of Immunology **138** 1933-1939

MOHAMED, A.O., ELBASHIR, M.I., IBRAHIM, G., ISMAIL, M. AND VENGE, P. (1996)
Neutrophil leucocyte activation in severe malaria.
Transactions of the Royal Society of Tropical Medicine and Hygiene **90** 277

MOLD, C., NAKAYAMA, S., HOLZER, T.J., GEWURZ, H. AND DU CLOS, T.W. (1981)
C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice.
Journal of Experimental Medicine **154** 1703-1708

MOLYNEUX, M.E. (1995)
The clinical manifestations and diagnosis of malaria.
Baillieres Clinical Infectious Diseases **2** 271-292

MONCADA, S., PALMER, M.J. AND HIGGS, E.A. (1991)
Nitric Oxide: Physiology, pathophysiology and pharmacology.
Pharmacological Reviews **43** 109-142

MOORE, K.W., O'GARRA, A., DE WAAL MALEFYT, R., VIEIRA, P. AND MOSMANN, T.R. (1993)
Interleukin-10.
Annual Review of Immunology **11** 165-190

MOR, G., KLINMAN, D.M., SHAPIRO, S., HAGIWARA, E., SEDEGAH, M., NORMAN, J.A., HOFFMAN, S.L. AND STEINBERG, A.D. (1995)
Complexity of the cytokine and antibody response elicited by immunizing mice with *Plasmodium yoelii* circumsporozoite protein plasmid DNA.
Journal of Immunology **155** 2039-2046

MORTENSEN, R.F. AND DUSCKIEWICZ, J.A. (1977)
Mediation of CRP-dependent phagocytosis through mouse macrophage Fc receptors.
Journal of Immunology **119** 1611-1616

MORTENSEN, R.F., SHAPIRO, J., LIN, B-F., DOUCHES, S. AND NETA, R. (1988)
Interaction of recombinant IL-1 and recombinant tumor necrosis factor in the induction of mouse acute phase proteins.
Journal of Immunology **140** 2260-2266

MOSLEY, B., BECKMANN, M.P., MARCH, C.J., IDZERDA, R.L., GIMPEL, S.D., VANDENBOS, T., FRIEND, D., ALPERT, A., ANDERSON, D., JACKSON, J., WIGNALL, J., SMITH, C., GALLIS, B., SIMS, J.E., URDAL, D.L., WIDMER, M.B., COSMAN, D. AND PARK, L.S. (1989)
The murine interleukin-4 receptor: Molecular cloning and characterization of secreted and membrane forms.
Cell **59** 335-348

MOSKOWITZ, N.H., BROWN, D.R. AND REINER, S.L. (1997)
Efficient immunity against *Leishmania major* in the absence of interleukin-6.
Infection and Immunity **65** 2448-2450

MOSMANN, T.R., CHERWINSKI, H. BOND, M.W., GIELDIN, M.A. AND COFFMAN, R.L. (1986)
Two Types of murine helper T cell clone.
Journal of Immunology **136** 2348-2357

MOSMANN, T.R. AND COFFMAN, R.L. (1987)
Two types of mouse helper T-cell clone: implications for immune regulation.
Immunology Today **8** 223-227

MOSMANN, T.R. AND COFFMAN, R.L. (1989)
Different patterns of lymphokine secretion lead to different functional properties.
Annual Review of Immunology **7** 145-173

MOSMANN, T.R., LI, L. AND SAD, S. (1997)
Functions of CD8 T-cell subsets secreting different cytokine pattterns.
Seminars in Immunology **9** 87-92

MOSMANN, T.R. AND SAD, S. (1996)
The expanding universe of T-cell subsets: Th1, Th2 and more.
Immunology Today **17** 138-146

MOTARD, A., LANDAU, I., NUSSLER, A., GRAU, G., BACCAM, D., MAZIER, D. AND TARGETT, G.A.T. (1993)
The role of reactive nitrogen intermediates in modulation of gametocyte infectivity of rodent malaria parasites.
Parasite Immunology **15** 21-26

MSHANA, R.N., BOULANDI, J., MSHANA, N.M. (1991)
Cytokines in the pathogenesis of malaria: levels of IL-1β, IL-4, IL-6, TNF-α and IFN-γ in plasma of healthy individuals and malaria patients in holoendemic area.
Journal of Clinical and Laboratory Immunology **34** 131-139

MURAGACHI, A., HIRANO, T., TANG, B., MATSUDA, T., HORII, Y., NAKAJIMA, K. AND KISHIMOTO, T. (1988)
The essential role of B cell stimulatory factor 2 (BSF/IL-6) for the terminal differentiation of B cells.
Journal of Experimental Medicine **167** 332-344

NAGEL, R.L. AND ROTH, E.F., Jr. (1989)
Malaria and red cell genetic defects.
Blood **74** 1213-1221

NARDIN, E.H., NUSSENZWEIG, R.S., McGREGOR, I.A. AND BRYAN, J.H. (1979)
Antibodies to sporozoites: their frequent occurrence in individuals living in an area of hyperendemic malaria.
Science **206** 597-599

NEWBOLD, C.T., BOYLE, D.B., SMITH, C.C. AND BROWN, K.N. (1982)
Stage specific protein and nucleic acid synthesis during asexual cycle of the rodent malaria *Plasmodium chabaudi.*
Molecular and Biochemical Parasitology **5** 33-44

NEWTON, C.R.J.C., KIRKHAM, F.J., WINSTANLEY, P.A., PASVOL, G., PESHU, N., WARRELL, D.A. AND MARSH, K. (1991)
Intracranial pressure in African children with cerebral malaria.
The Lancet **337** 573-576

NIU, X-F., IBBOTSON, G. AND KUBES, P. (1996)
A balance between nitric oxide and oxidants regulates mast cell-dependent neutrophil-endothelial cell interactions.
Circulation Research **79** 992-999

NOELLE, R., KRAMMER, P.H., OHARA, J., UHR, J.W. AND VITETTA, E.S. (1984)
Increased expression of Ia antigens on resting B cells: A new role for B cell growth factor.
Proceedings of the National Academy of Sciences USA **81** 6149-6153

NOMA, T., MIZUTA, T., ROSEN, A., HIRANO, T., KISHIMOTO, T. AND HONJO, T. (1987)
Enhancement of the interleukin-2 receptor expression on T cells by multiple B-lymphotropic lymphokines.
Immunology Letters **15** 249-253

NORTH, R.J. (1970)
The relative importance of blood monocytes and fixed macrophages to the expression of cell-mediated immunity to infection.
Journal of Experimental Medicine **132** 521-534

NOSAL, R., JANCINOVA, V. AND PETRIKOVA, M. (1995)
Chloroquine inhibits stimulated platelets at the arachadonic acid pathway.
Thrombosis Research **77** 531-542

NOSTEN, F., LUXEMBURGER, C., KYLE, D.E., BALLOU, W.R., WITTES, J., WAH, E., CHONGSUPHAJAISIDDHI, T., GORDON, D.M., WHITE, N.J., SADOFF, J.C., HEPPNER, D.G., BATHE, K., BLOOD, J., BROCKMAN, A., COBLEY, U.T., HACKING, D., HOGG, D., KYAW, H.U., MAELANKIRI, L., CHUANAK, N., PERMPANICH, B., PRICE, R., RAIMOND, D., SCHABENBERGER, O., SINGHARAJ, P., SINGHASIVANON, P., SLIGHT, T., TULAYON, S., TWAY, K.L., YNINT, T., VINCENTI-DELMAS, DE VRIES, A. AND WEBSTER, H.K. (1996)
Randomised double-blind placebo-controlled trial of Spf66 malaria vaccine in children in northwestern Thailand.
The Lancet **348** 701-707

NUDELMANN, S., RENIA, L., CHAROENVIT, Y., MILTGEN, F., BEAUDOIN R.L. AND MAZIER, D. (1988)
Dual action of anti-sporozoite antibodies *in vitro.*
Journal of Immunology **143** 996-1000

NUSSENZWEIG, R.S., VANDERBERG, J., MOST, H AND ORTON, C. (1967)
Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei.*
Nature **216** 160-162

NUSSLER, A., DRAPIER, J-C., RENIA, L., PIED, S., MILTGEN, F., GENTILLI, M. AND MAZIER, D. (1991a)
L-arginine-dependent destruction of intrahepatic malaria parasites in response to tumour necrosis factor and/or interleukin-6 stimulation.
European Journal of Immunology **21** 227-230

NUSSLER, A.K., ELING, W. AND KREMSNER, P.G. (1994)
Patients with *Plasmodium vivax* malaria show increased nitrite and nitrate plasma levels.
Journal of Infectious Diseases **169** 1418-1419

NUSSLER, A.K., PIED, S., GOMA, J., RENIA, L., MILTGEN, F., GRAU, G.E. AND MAZIER, D. (1991b)
TNF inhibits malaria hepatic stages *in vitro* via IL-6 liver synthesis.
International Immunology **3** 317

NUSSLER, A., PIED, S., PONTET, M., MILTGEN, F., RENIA, L., GENTILINI, M. AND MAZIER, D. (1991c)
Inflammatory status and preerythrocytic stages of malaria: Role of the C-reactive protein.
Experimental Parasitology **72** 1-7

OCHIAI, E-I., MANN, G.J., GRASLUND, A. AND THELANDER, L. (1990)
Tyrosyl free radical formation in the small subunit of mouse ribonucleotide reductase.
Journal of Biological Chemistry **265** 15758-15761

OCKENHOUSE, C.F., SCHULMAN, S. AND SHEAR, H.L. (1984)
Induction of crisis forms in the human malaria parasite *Plasmodium falciparum* by gamma-interferon-activated, monocyte-derived macrophages.
Journal of Immunology **133** 1601-1608

OHARA, J. AND PAUL, W.E. (1988)
Up-regulation of interleukin 4 / B cell stimulatory factor 1 receptor expression.
Proceedings of the National Academy of Sciences USA **85** 8221-8225

OHTEKI, T. AND MACDONALD, H.R. (1994)
Major histocompatibility complex class I related molecules control the development of $CD4^+8^-$ and $CD4^-8^-$ subsets of natural killer 1.1^+ T cell receptor-alpha/beta$^+$ cells in the liver of mice.
Journal of Experimental Medicine **180** 699-704

OKADA, M., KITAHARA, M., KISIMOTO, S., MATSUDA, T., HIRANO, T. AND KISHIMOTO, T. (1988)
IL-6/BSF-2 functions as a killer helper factor in the *in vitro* induction of cytotoxic T cells.
Journal of Immunology **141** 1543-1549

O'NEILL, P. ADN JOHNSON, G.D. (1970)
Multispot immunofluorescence: a simple method of processing large numbers of test.
Journal of Clinical Pathology **23** 185-187

OO, M., TIN-SHWE, THAN, M. AND O'SULLIVAN, W.J. (1995)
Genetic red cell disorders and severity of falciparum malaria in Myanmar.
Bulletin of the World Health Organization **73** 659-665

OSMAND, A.P., FRIEDENSON, B., GEWURZ, H., PAINTER, R.H., HOFTMAN, E. AND SHELTON, E. (1977)
The characterization of C-reactive protein and the C1t subcomponent of C1 as pentraxins: homologous proteins displaying cyclic pentameric symmetry.
Proceedings of the National Academy of Sciences USA **74** 739-743

OSMAND, A.P., MORTENSEN, R.F., SIEGEL, J. AND GEWURZ, H. (1975)
Interactions of C-reactive protein with the complement system. III. Complement-dependent passive hemolysis initiated by CRP.
The Journal of Experimental Medicine **142** 1065-1077

OSORIO, L.M., FONTE, L. AND FINALY, C.M. (1992)
Inhibition of human monocyte function by prophylactic doses of chloroquine.
American Journal of Tropical Medicine and Hygiene **46** 165-168

OSWALD, I.P., GAZZINELLI, R.T., SHER, P. AND JAMES, S.L. (1992)
IL-10 synergises with IL-4 and transforming growth factor-b to inhibit macrophage cytotoxic activity.
Journal of Immunology **148** 3578-3582

PANDITA, R., POCSIK, E. AND AGGARWAL, B.B. (1992)
Interferon-gamma induces cell surface expression for both type of tumour necrosis factor receptors.
FEBS Letters **312** 87-90

PAPPAIOANOU, M., FISHBEIN, D.B., DREESEN, D.W., SCHWARTZ, T.K., CAMPBELL, G.H., SUMNER, J.W., PATCHEN, L.C. AND BROWN, W.J. (1986)
Antibody response to preexpose human diploid-cell rabies vaccine given concurrently with chloroquine.
New England Journal of Medicine **314** 280-264

PATEL, S.S., WACHOLTZ, M.C. AND DUBY, A.D. (1989)
Analysis of the functional capabilities of $CD3^+$, $CD4^-$, $CD8^-$ and $CD3^+$, $CD4^+$, $CD8^+$ human T cell clones.
Journal of Immunology **143** 1108-1117

PAUL, W.E. (1991)
Interleukin-4: A prototypic immunoregulatory lymphokine.
Blood **77** 1859-1870

PEPYS, M.B., BALTZ, M.L., GOMER, K., DAVIES, A.S. AND DOENHOFF, M. (1979)
Serum amyloid P-component is an acute phase reactant in the mouse.
Nature **278** 259-261

PEPYS, M.B., DASH, A.C., FLETCHER, T.C., RICHARDSON, N., MUNN, E.A. AND FEINSTEIN, A. (1978)
Analogues in other mammals and in fish of human plasma proteins: C-reactive protein and amyloid P-component.
Nature **273** 168-170

PERLMANN, H., HELMBY, H., HAGSTEDT, M., CARLSON, J., LARSSON, P.H., TROYE-BLOMBERG, M. AND PERLMANN, P. (1994)
IgE elevation and IgE anti-malarial antibodies in *Plasmodium falciparum* malaria: Association of high IgE levels with cerebral malaria.
Clinical and Experimental Immunology **97** 284-292

PERLMANN, P., PERLMANN, H., FLYG, B.W., HAGSTEDT, M., ELGHAZALI, G., WORKU, S., FERNANDEZ, V., RUTTA, A.S.M. AND TROYE-BLOMBERG, M. (1997)
Immunoglobulin E, a pathogenic factor in *Plasmodium falciparum* malaria.
Infection and Immunity **65** 116-121

PERUSSIA, B. (1991)
Lymphokine-activated cells, natural killer cells and cytokines.
Current Opinion in Immunology **3** 49-55

PEYRON, F., BURDIN, N., RINGWALD, P., VUILLEZ, J.P., ROUSSET, F. AND BANCHEREAU, J. (1994)
High levels of circulating IL-10 in human malaria.
Clinical and Experimental Immunology **95** 300-303

PHILLIPS, R.S. (1970)
Plasmodium berghei: passive transfer of immunity by antisera and cells.
Experimental Parasitology **27** 479-495

PHILLIPS, R.S. (1994a)
Malarial vaccines-a problem solved or simply a promising start?
Protozoological Abstracts **18** 459-486

PHILLIPS, S. (1994b)
Effector mechanisms against erythrocytic stages of *Plasmodium.*
Immunology Letters **41** 109-114

PHILLIPS, R.S. AND JONES, V.E. (1972)
Immunity to *Plasmodium berghei* in rats: maximum levels of protective antibody are associated with eradication of infection.
Parasitology **64** 117-127

PHILLIPS, R.S., MATHERS, K.E. AND TAYLOR-ROBINSON, A.W. (1994)
T cells in immunity to *Plasmodium chabaudi chabaudi*: Operation and regulation of different pathways of protection.
Research in Immunology **145** 406-412

PHILLIPS, R.S., TRIGG, P.I., BARTHOLOMEW, R.K. AND SCOTT-FINNEAGAN, T.J. (1972)
Culture of *Plasmodium falciparum in vitro*: A subculture technique used for demonstrating antiplasmodial activity in serum from Gambians, resident in an endemic area.
Parasitology **65** 525-535

PICOT, S., PEYRON, F., DONADILLE, A., VUILLEZ, J-P., BARBE, G. AND AMBROISE-THOMAS, P. (1993)
Chloroquine-induced inhibition of the production of TNF, but not of IL-6 , is affected by disruption of iron metabolism.
Immunology **80** 127-133

PIED, S., CIVAS, A., BERLOT-PICCIRD, F., RENIA, L., MILTGEN, F., GENTILINI, M., DOLY, J. AND MAZIER, D. (1992)
IL-6 induced by IL-1 inhibits malaria pre-erythrocytic stages but its secretion is down-regulated by the parasite.
Journal of Immunology **148** 197-201

PIED, S., NUSSLER, A., PONTET, M., MILTGEN, F., MATILE, H., LAMBERT, P-H. AND MAZIER, D. (1989)
C-reactive protein protects against preerythrocytic stages of malaria.
Infection and Immunity **57** 278-282

PLAYFAIR, J.H.L.AND DE SOUZA, J.B. (1982)
Lymphocyte traffic and lymphocyte destruction in murine malaria.
Immunology **46** 125-133

PLAYFAIR, J.H.L., DE SOUZA, B., DOCKRELL, H.M., AGOMO, P.U. AND TAVERNE, J. (1979)
Cell-mediated immunity in the liver of mice vaccinated against malaria.
Nature **282** 731-734

PLAYFAIR, J.H.L., DE SOUZA, J.B. AND TAVERNE, J. (1982)
Endotoxin induced tumour-necrosis serum kills a subpopulation of normal lymphocytes *in vitro*.
Clinical and Experimental Immunology **47** 753-755

PLAYFAIR, J.H.L., TAVERNE, J., BATE, C.A.W. AND DE SOUZA, J.B. (1990)
The malaria vaccine: anti-parasite or anti-disease?
Immunology Today **11** 25-27

PODOBA, J.E. AND STEVENSON, M.M. (1991)
$CD4^+$ and $CD8^+$ T lymphocytes both contribute to acquired immunity to blood stage *Plasmodium chabaudi* AS.
Infection and Immunity **59** 51-58

POLDER, T.W., JERUSALEM, C.R. AND ELING, M.C. (1991)
Morphological characteristics of intracerebral arterioles in clinical (*Plasmodium falciparum*) and experimental (*Plasmodium berghei*) cerebral malaria.
Journal of the Neurological Sciences **101** 35-46

POTOCNJACK, P., YOSHIDA, N., NUSSENZWEIG, R.S. AND NUSSENZWEIG, V. (1980)
Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malaria infection.
Journal of Experimental Medicine **151** 1504-1513

PRINGLE, G. AND AVERY-JONES, S. (1966)
Observations on the early course of untreated falciparum malaria in semi-immune African children following a short period of protection.
Bulletin of the World Health Organsiation **34** 269-272

QUAKYI, I.A., CARTER, R., RENER, J., KUMAR, N., GOOD, M.F. AND MILLER, L.H. (1987)
The 230 kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmission-blocking antibodies.
Journal of Immunology **139** 4213-4217

RAMADORI, G., SIPE, J.D., DINARELLO, C.A., MIZEL, B. AND COLTEN, H.R. (1985)
Pretranslational modulation of acute phase hepatic protein synthesis by murine recombinant interleukin 1 (IL-1) and purified human IL-1.
Journal of Experimental Medicine **162** 930-942

RAMSEY, J.M., BEAUDOIN, R.L. AND HOLLINGDALE, M.R. (1982)
Infection of tissue-culture cells with Co gamma-irradiated malaria sporozoites.
Nuclear techniques in the study of parasitic infections. Vienna, Austria: International Atomic Energy Agency 19-25

RAWLINGS, D.J. AND KASLOW, D.C. (1992)
Adjuvant-dependent immune response to malarial transmission-blocking vaccine candidate antigens.
Journal of Experimental Medicine **176** 1483-1487

REINER, S.L. AND LOCKSLEY, R.M. (1995)
The regulation of immunity to *Leishmania major.*
Annual Review of Immunology **13** 151-177

RENAULD, J-C., VINK, A. AND VAN SNICK, J. (1989)
Accessory signals in murine cytolytic T cell responses: dual requirement for IL-1 and IL-6.
Journal of Immunology **143** 1894-1898

RENER, J., GRAVES, P.M., CARTER, R., WILLIAMS, J.L. AND BURKOT, T.R. (1983)
Target antigens of transmission blocking immunity on gametes of *Plasmodium falciparum.*
Journal of Experimental Medicine **158** 976-981

RENGGLI, J., VALMORI, D., ROMERO, J.F., EBERL, G., ROMERO, P., BETSCHART, B. AND CORRADIN, G. (1995)
$CD8^+$ T-cell protective immunity induced by immunization with *Plasmodium berghei* CS protein-derived synthetic peptides: Evidence that localization of peptide-specific CTLs is crucial for protection against malaria.
Immunology Letters **46** 199-205

RENIA, L., GRILLOT, D., MARUSSIG, M., CORRADIN, G., MILTGEN, F., LAMBERT, P.H. AND del GIUDICE, G. (1993)
Effector functions of circumsporozoite peptide-primed $CD4^+$ T cell clones against *Plasmodium yoelii* liver stages.
Journal of Immunology **150** 1471-1475

RENIA, L., RODRIGUES, M.M. AND NUSSENZWEIG, V. (1994)
Intrasplenic immunization with infected hepatocytes: A mouse model for studying protective immunity against malaria pre-erythrocytic stage.
Immunology **82** 164-168

RHODES-FEUILLETTE, A., BELLOSGUARDO, M., DRUILHE, P., BALLET, J.J., CHOUSTERMAN, S., CANIVET, F. AND PERIES, J. (1985)
The interferon compartment of the immune response in human malaria. II Presence of serum-interferon gamma following the acute attack.
Journal of Interferon Research **5** 169-178

RILEY, E.M, ANDERSSON, G., OTOO, L.N., JEPSEN, S. AND GREENWOOD, B.M. (1988)
Cell-mediated immune responses to *Plasmodium falciparum* antigens in adult Gambians.
Clinical and Experimental Immunology **73** 17

RILEY, E. AND GREENWOOD, B. (1990)
Measuring cellular immune responses to malaria antigens in endemic populations: epidemiological, parasitological and physiological factors which influence in vitro assays.
Immunology Letters **25** 221-229

RILEY, E.M., JOBE, D. AND WHITTLE, H.C. (1989)
$CD8^+$ T cells inhibit *Plasmodium falciparum*-induced lymphoproliferation and gamma interferon production in cell preparations from some malaria-immune individuals.
Infection and Immunity **57** 1281-1284

RILEY, E.M., MacLENNAN, C., KWIATKOWSKI, D.K. AND GREENWOOD, B.M. (1989)
Suppression of *in vitro* lymphoproliferative response in acute malaria patients can be partially reversed by indomethacin.
Parasite Immunology **11** 509-517

RINCON, M., ANGUITA, J., NAKAMURA, T., FIKRIG, E. AND FLAVELL, R.A. (1997)
Interleukin (IL)-6 directs the differentiation of IL-4 producing $CD4^+$ T cells.
Journal of Experimental **185** 461-469

ROBERTS, C.W., FERGUSON, D.J.P., JEBBARI, H., SATOSKAR, A., BLUETHMANN, H. AND ALEXANDER, J. (1996)
Different roles for interleukin-4 during the course of *Toxoplasma gondii* infection.
Infection and Immunity **64** 897-904

ROBERTS, J.A. AND TRACEY-PATTE, P. (1969)
Adoptive transfer of immunity to *Plasmodium berghei.*
Journal of Protozoology **16** 728-730

ROCKETT, K.A., AWBURN, M.M., AGGARWAL, B.B., COWDEN, W.B. AND CLARK, I.A. (1992)
In vivo induction of nitrite and nitrate by tumour necrosis factor, lymphotoxin and IL-1: Possible roles in Malaria.
Infection and Immunity **60** 3725-3730

ROCKETT, K.A., AWBURN, M.M., COWDEN, W.B. AND CLARK, I.A. (1991)
Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives.
Infection and Immunity **59** 3280-3283

ROCKETT, K.A., AWBURN, M.M., ROCKETT, E.J., COWDEN, W.B. AND CLARK, I.A. (1994)
Possible role of nitric oxide in malarial immunosuppression.
Parasite Immunology **16** 243-249

RODRIGUES, M., NUSSENZWEIG, R.S., ROMERO, P. AND ZAVALA, F. (1992)
The *in vivo* cytotoxic activity of $CD8^+$ T cell clones correlates with their levels of expression of adhesion molecules.
Journal of Experimental Medicine **175** 895-905

ROMAGNANI, S. (1996)
TH1 and TH2 in human diseases.
Clinical Immunology and Immunopathology **80** 225-235

ROMAGNANI, S. (1997)
The Th1/Th2 paradigm.
Immunology Today **18** 263-266

ROMANI, L., MENCACCI, A., CENCI, E., SPACCAPELO, R., TONIATTI, C., PUCCETTI, P., BISTONI, F. AND POLI, V. (1996)
Impaired neutrophil response and $CD4^+$ T helper cell 1 development in interleukin-6-deficient mice infected with *Candida albicans.*
Journal of Experimental Medicine **183** 1345-1355

ROMERO, P., MARYANSKI, J.L, CORRADIN, G., NUSSENZWEIG, R.S., NUSSENZWEIG, V. AND ZAVALA, F. (1989)
Cloned cytotoxic T cells recognise an epitope in the circumsporozoite protein and protect against malaria.
Nature **341** 323-326

RON, Y. AND SPRENT, J. (1987)
T cell priming in vivo: a major role for B cells in presenting antigen to T cells in lymph nodes.
Journal of Immunology **138** 2848-2856

ROSENBERG, R., WIRTZ, R.A., SCHNEIDER, I. AND BURGE, R. (1990)
An estimation of the number of malaria sporozoites ejected by a feeding mosquito.
Transactions of the Royal Society of Tropical Medicine and Hygeine **84** 209-212

ROUSILHON, C., AGARPART, M., BALLET, J-J. AND BENSUSSAN, A. (1990)
T lymphocytes bearing the γδ T cell receptor in patients with acute *Plasmodium falciparum* malaria.
Journal of Infectious Diseases **162** 283

RUBIN, H., SALEM, J.S., LI, L-S., YANG, F-D., MAMA, S., WANG, Z-M., FISHER, A., HAMMAN, C.S. AND COOPERMAN, B.S. (1993)
Cloning, sequence determination, and regulation of the ribonucleotide reductase subunits from *Plasmodium falciparum*: A target for antimalarial therapy.
Proceedings of the National Academy of Sciences USA **90** 9280-9284

RUDIN, W., EUGSTER, H-P., BORDMANN, G., BONATO, J., MULLER, M., YAMAGE, M. AND RYFFEL, B. (1997a)
Resistance to cerebral malaria in tumour necrosis factor-alpha/beta deficient mice is associated with a reduction of intracellular adhesion molecule-1 up-regulation and T helper type 1 response.
American Journal of Pathology **150** 257-266

RUDIN, W., FAVRE, N., BORDMANN, G. AND RYFFEL, B. (1997b)
Interferon-gamma is essential for the development of cerebral malaria.
European Journal of Immunology **27** 810-815

RUSSO, D.M., ARMITAGE, R.J., BARRAL-NETTO, M., BARRAL, A., GRABSTEIN, K.H. AND REED, S.G. (1993)
Antigen-reactive γδ T cells in human Leishmaniasis.
Journal of Immunology **151** 3712-3718

RZEPCZYK., C.M., STAMATIOU, S., ANDERSON, K., STOWERS, A., CHENG, Q., SAUL, A., ALLWORTH, A., McCORMACK, J., WHITBY, M., OLIVE, C. AND LAWRENCE, G. (1996)
Experimental human *Plasmodium falciparum* infections: longitudinal analysis of lymphocyte responses with particular reference to γδ T cells.
Scandanavian Journal of Immunology **43** 219-227

SABCHAREON, A., BURNOUF, T., OUATTARA, D., ATTANATH, P., BOUHAROUN-TAYOUN, H., CHANTAVANICH, P., FOUCAULT, C., CHONGSUPHAJAISIDDHI, T AND DRUILHE, P.
Parasitlogic and clinical human response to immunoglobulin administration in falciparum malaria.
American Journal of Tropical Medicine and Hygiene **45** 297-308

SAD, S., MARCOTTE, R. AND MOSMANN, T.R. (1995)
Cytokine-induced differentiation of precursor mouse $CD8^+$ T cells into cytotoxic $CD8^+$ T cells secreting Th1 or Th2 cytokines.
Immunity **2** 271-279

SADICK, M.D., HEINZEL, F.P., HOLADAY, B.J., PU, R.T., DAWKINS, R.S. AND LOCKSLEY, R.M. (1990)
Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism.
Journal of Experimental Medicine **171** 115-127

SALMERON, G. AND LIPSKY, P.E. (1983)
Immunosuppressive potential of antimalarials.
American Journal of Medicine **75** 19-24

SALMON, D., VILDE, J.L., ANDRIEU, B., SIMONOVIC, R. AND LEBRAS, J. (1986)
Role of immune serum and complement in stimulation of the meatbolic burst of human neutrophils by *Plasmodium falciparum.*
Infection and Immunity **51** 801-806

SANDS, W.A., BULUT, V., SEVERN, A., XU, D. AND LIEW, F.Y. (1994)
Inhibition of nitric oxide synthesis by interleukin-4 may involve inhibiting the activation of protein kinase C epsilon.
European Journal of Immunology **24** 2345-2350

SARLO, K.T. AND MORTENSEN, R.F. (1985)
Enhanced interleukin 1 (IL-1) production mediated by mouse serum amyloid P component.
Cellular Immunology **93** 398-405

SARLO, K.T. AND MORTENSEN, R.F. (1987)
Regulation of the antibody response by the acute phase reactant: Mouse serum amyloid P-component (SAP).
Cellular Immunology **106** 273-286

SATOSKAR, A., BLUETHMANN, H. AND ALEXANDER, J. (1995)
Disruption of the murine interleukin-4 gene inhibits disease progression duing Leishmania mexicana infection but does not increase control of Leishmania donovani infection.
Infection and Immunity **63** 4894-4899

SCHERF, A., CARTER, R., PETERSEN, C., ALANO, P., NELSON, R., AIKAWA, M., MATTEI, D., PEREIRA DA SILVA, LEECH, J. (1992)
Gene inactivation of Pf11-1 of *Plasmodium falciparum* by chromosome breakage and healing: Identification of gametocyte-specific protein with a potential role in gametogenesis.
EMBO **11** 2293-2301

SCHINDLER, R., MANCILLA, J., GHORBANI, R., CLARK, S.C. AND DINARELLO, C.A. (1990)
Correlations and interactions in the production of interleukin-6 (IL-6), IL-1 and tumour necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF.
Blood **75** 40-47

SCHLEIFER, K.W. AND MANSFIELD, J.M. (1993)
Suppressor macrophages in African Trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins.
Journal of Immunology **151** 5492-5503

SCHOFIELD, L. (1989)
T cell immunity to malaria sporozoites.
Experimental Parasitology **68** 357-364

SCHOFIELD, L. AND HACKETT, F. (1993)
Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites.
Journal of Experimental Medicine **177** 145-153

SCHOFEILD, L., FERREIRA, A., ALTSZULER, R., NUSSENZWEIG, V. AND NUSSENZWEIG, R.S. (1987a)
Interferon-γ inhibits the intrahepatocytic development of malaria parasites *in vitro.*
Journal of Immunology **139** 2020-2025

SCHOFIELD, L., NOVAKOVIC, S., GEROLD, P., SCHWARZ, R.T., McCONVILLE, M.J. AND TACHADO, S.D. (1996)
Glycosylphosphatidylinositol toxin of *Plasmodium* up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction.
Journal of Immunology **156** 1886-1896

SCHOFIELD, L., NUSSENZWEIG, R.S. AND NUSSENZWEIG, V. (1988)
Report of the tenth meeting of the scientific working group on the immunology of malaria, WHO 17

SCHOFEILD, L., VILLAQUIRSN, J., FERRIERA, A., SCHELLEKENS, H., NUSSENZWEIG, R. AND NUSSENWEIG, V. (1987b)
γ Interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites.
Nature **330** 664-666

SCHWARZER, E., TURRININ, F., ULLIERS, D., GIRIBALDI, G., GINSBURG, H. AND ARESE, P. (1992)
Impairment of macrophage functions after the ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment.
Journal of Experimental Medicine **176** 1033-1041

SCUDERI, P., LAM, K.S., RYAN, K.J., PETERSON, E., STERLING, K.E., FINLEY, P.R., RAY, C.G., SLYMEN, D.J. AND SOLOMON, S.E. (1986)
Raised serum levels of tumour necrosis factor in parasite infections.
The Lancet **ii** 1364-1365

SEDEGAH, M., FINKLEMANN, F. AND HOFFMAN, S.L. (1994)
Interleukin 12 induction of interferon-gamma-dependent protection against malaria.
Proceedings of the National Academy of Sciences USA **91** 9866-9870

SEDEGAH, M., SIM, B.K.L., MASON, C., NUTMAN, T., MALIK, A., ROBERTS, C., JOHNSON, A., OCHOLA, J., KOECH, D., WERE, B. AND HOFFMAN, S.L. (1992)
Naturally acquired $CD8^+$ cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein,
Journal of Immunology **149** 966-971

SEDER, R.A. AND PAUL, W.E. (1994)
Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells.
Annual Review of Immunology **12** 635-673

SEGUIN, M.C., BALLOU, W.R. AND NACY, C.A. (1989)
Interactions of *Plasmodium berghei* sporozoites and murine Kupffer cells *in vitro*.
Journal of Immunology **143** 1716-1722

SEHGAL, P.B., WALTHER, Z. AND TAMM, I. (1987)
Rapid enhancement of β2-IFN/BSF-2 gene expression in human fibroblasts by diacylglycerols and the calcium inophore A23187.
Proceedings of the National Academy of Sciences USA **84** 3633-3637

SEIFERT, W. AND RUDLAND, P.S. (1974)
Possible involvement of cGMP in growth control of cultured mouse cells.
Nature **248** 138-140

SENOK, A.C., LI,K., NELSON, E.A.S., YU, L.M., TIAN, L.P. AND OPPENHEIMER, S.J. (1997)
Invasion and growth of *Plasmodium falciparum* is inhibited in fractionated thalassaemic erythrocytes.
Transactions of the Royal Society of Tropical Medicine and Hygiene **9** 138-143

SEVERN, A., WAKELAM, M.J.O AND LIEW, F.Y. (1992)
The role of Protein Kinase C in the induction of nitric oxide synthesis by murine macrophages.
Biochemical and Biophysical Research Communications **188** 997-1002

SHABO, Y., LOTEM, J., RUBINSTEIN, M., REVEL, M., CLARK, S.C., WOLF, S.F., KAMEN, R. AND SACHS, L. (1988)
The myeloid blood cell differentiation-inducing protein MGI-2A is interleukin-6.
Blood **72** 2070-2073

SHEAR, H.L., NUSSENZWEIG, R.S. AND BIANCO, C. (1979)
Immune phagocytosis in murine malaria.
Journal of Experimental Medicine **149** 1288-1298

SHEAR, H.L., SRINIVASAN, R., NOLAN, T. AND NG, C. (1989)
Role of IFNγ in lethal and non-lethal malaria in susceptible and resistant murine hosts.
Journal of Immunology **143** 2038-2044

SHUTE, P.G. (1952)
Malarial fever therapy.
The Lancet **263** 333-336

SIMS, S.H., CHA, Y., ROMINE, M.F., GAO, P.Q., GOTTLIEB, K. AND DESISSEROTH, A.B. (1993)
A novel interferon-inducible domain: structural and functional analysis of the human interferon regulatory factor 1 gene promoter.
Molecular and Cellular Biology **13** 690-702

SINA, B.J., WRIGHT, C., ATKINSON, C.T., BALLOU, R., AIKAWA, M., HOLLINGDALE, M. (1995)
Characterisation of a sporozoite antigen common to *Plasmodium falciparum* and *Plasmodium berghei*.
Molecular and Biochemical Parasitology **69** 239-246

SINDEN, R.E. AND SMITH, J.E. (1982)
The role of the Kupffer cell in the infection of rodents by sporozoites of *Plasmodium*: uptake of sporozoites by perfused liver and establishment of infection *in vivo*.
Acta Tropica **39** 11-27

SINGH, P.P., GERVAIS, F., SKAMENE, E. AND MORTENSEN, R.F. (1986)
Serum amyloid P-component-induced enhancement of macrophage listericidal activity.
Infection and Immunity **52** 688

SINIGAGLIA, F., MATILE, H. AND PINK, J.R.L. (1987)
Plasmodium falciparum specific human T cell clones: Evidence for helper and cytotoxic activities.
European Journal of Immunology **17** 187-192

SINIGAGLIA, F., AND PINK, J.R.L. (1985)
Human T lymphocyte clones specific for malaria (*Plasmodium falciparum*) antigens.
EMBO **13B** 3819-3822

SINTON, J.A., HARBHAGWAN, M. AND SINGH, J. (1931)
The numerical prevalence of parasites in relation to fever in chronic benign tertian malaria.
Indian Journal of Medical Research **18** 871-874

SIRIPONT, J., TEBO, J.M. AND MORTENSEN, R.F. (1988)
Receptor-mediated binding of the acute-phase reactant mouse serum amyloid P-component (SAP) to macrophages.
Cellular Immunology **117** 239-252

SLADE, S.J. AND LANGHORNE, J. (1989)
Production of interferon-gamma during infection of mice with *Plasmodium chabaudi chabaudi*.
Immunobiology **179** 353-365

SNAPPER, C.M. AND PAUL, W.E. (1987a)
Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production.
Science **236** 944-947

SNAPPER, C.M. AND PAUL, W.E. (1987b)
B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial lipopolysaccharide.
Journal of Immunology **139** 10-17

SNAPPER, C.M., FINKELMAN, F.D. AND PAUL, W.E. (1988)
Differential regulation of IgG1 and IgE synthesis by interleukin 4.
Journal of Experimental Medicine **167** 183-196

SNOW, R.W., BASTOS DE AZEVEDO, I., LOWE, B.S., KABIRU, E.W., NEVILL, C.G., MWANKUSYE, S., KASSIGA, G., MARSH, K. AND TEUSCHER, T. (1994)
Severe childhood malaria in two areas of markedly different falciparum transmission in East Africa.
Acta Tropica **57** 289-300

SNYDER, S.H. (1992)
NO release for good measure.
Nature **358** 623

SOHAL, A.K. AND ARNOT, D.E. (1993)
Plasmodium chabaudi: A short-term *in vitro* culture method and its application to chloroquine resistance testing.
Experimental Parasitology **76** 314-317

STECHSCHULTE, D.J. (1969)
Cell-mediated immunity in rats infected with *Plasmodium berghei.*
Military Medicine **134** 1147

STEEL, D.M. AND WHITEHEAD, A.S. (1994)
The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein.
Immunology Today **15** 81-88

STERNBERG, J., MABBOT, N., SUTHERLAND, I. AND LIEW, F.Y. (1994)
Inhibition of nitric oxide synthesis leads to reduced parasitaemia in murine *Trypanosoma brucei* infection.
Infection and Immunity **62** 2135-2137

STEVENSON, M.M., FONG TAM, M., BELOSEVIC, M., VAN DER MEIDE, P.H. AND PODOBA, J.E. (1990)
Role of endogenous gamma interferon in host response to infection with blood-stage *Plasmodium chabaudi* AS.
Infection and Immunity **60** 1193-1201

STEVENSON, M.M., FONG TAM, M., WOLF, S.F. AND SHER, A. (1995)
IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFNγ and TNFα and occurs via a nitric oxide-dependent mechanism.
Journal of Immunology **155** 2545-2556

STEVENSON, M.M. AND GHADIRIAN, E. (1989)
Human recombinant tumour necrosis factor alpha protects susceptible A/J mice against lethal *Plasmodium chabaudi* AS infection.
Infection and Immunity **57** 3936-3939

STEVENSON, M.M., NOWOTARSKI, M. AND YAP, G. (1990)
Review: cytokines and malaria.
Clinical and Investigative Medicine **13** 353-359

STEVENSON, M.M. AND TAM, M.F. (1993)
Differential induction of helper T cell subsets during blood-stage *Plasmodium chabaudi* AS infection in resistant and susceptible mice.
Clinical and Experimental Immunology **92** 77-83

STEVENSON, M.M., TAM, M.F. AND RAE, D. (1990)
Dependence on cell mediated mechanisms for the appearance of crisis forms during *Plasmodium chabaudi* AS infection in C57BL/6 mice.
Microbial Pathogenesis **9** 303-314

STEWART, M.J. AND VANDERBERG, J.P. (1988)
Malaria sporozoites leave behind trails of circumsporozoite protein during gliding motility.
Journal of Protozoology **38** 411-421

STEWART, M.J. AND VANDERBERG, J.P. (1991)
Malaria sporozoite release circumsporozoite protein from their apical end and translocate it along their surface.
Journal of Protozoology **38** 411-421

STOUTE, J.A., SLAOUI, M., HEPPNER, G., MOMIN, P., KESTER, K.E., DESMONS, P., WELLDE, B.T., GARCON, N., KRZYCH, U., MARCHAND, M., BALLOU, W.R. AND COHEN, J.D. (1997)
A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria.
New England Journal of Medicine **336** 86-91

STUART, P.M., ZLOTNIK, A. AND WOODWARD, J.G. (1988)
Induction of class I and class II MHC antigen expression on murine bone marrow-derived macrophages by IL-4 (B cell stimulatory factor 1).
Journal of Immunology **140** 1542-1547

STUBBE, J.A. (1990)
Ribonucleotide reductases: Amazing and confusing.
Journal of Biological Chemistry **265** 5329-5332

STUEHR, D.J. AND NATHAN, C.F. (1989)
Nitric oxide: A macrophage product responsible for cytostasis and respiratory inhibition in tumour cells.
Journal of Experimental Medicine **169** 1543-1555

SUBCHAREON, A., BURNOUF, T., OUATTARA, D., ATTANATH, P., BOUHAROUN-TAYOUN, H., CHANTAVANICH, P., FOUCAULT, C., CHONGSUPHAJAISIDDHI, T. AND DRUILHE, P. (1991)
Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria.
American Journal of Tropical Medicine and Hygiene **45** 297-308

SUHRBIER, A. (1991)
Immunity to the liver stage of malaria.
Parasitology Today **7** 160-163

SUHRBIER, A., WINGER, L.A., CASTELLANO, E. AND SINDEN, R.E. (1990)
Survival and antigenic profile of irradiated malarial sporozoites in infected liver cells.
Infection and Immunity **58** 2834-2839

SUSS, G., EICHMANN, K., KURY, E., LINKE, A. AND LANGHORNE, J. (1988)
Roles of CD4- and CD8-bearing lymphocytes in the immune response to the erythrocytic stages of *Plasmodium chabaudi* infection.
Infection and Immunity **56** 3081-3088

SUZUKI,Y., RANI, S., LIESENFELD, O., KOJIMA, T., LIM, S., NGUYEN, T.A., DALRYMPLE, S.A., MURRAY, R. AND REMINGTON, J.S. (1997)
Impaired resistance to the development of toxoplasmic enchephalitis in interleukin-6-deficient mice.
Infection and Immunity **65** 2339-2345

SZALAI, A.J., BRILES, D.E. AND VOLANAKIS, J.E. (1995)
Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in transgenic mice.
Journal of Immunology **155** 2557-2563

TAKAI, Y., WONG, G.G., CLARK, S.C. BURAKOFF, S.J., AND HERMANN, S.H. (1988)
B cell stimulatory factor-2 is involved in the differentiation of cytotoxic T lymphocytes.
Journal of Immunology **140** 508-512

TAKATSUKI, F., OKANO, A., SUZUKI, C., MIYASAKA, Y., HIRANO, T., KISHIMOTO, T., EJIMA, D. AND AKIYAMA, Y. (1990)
Interleukin 6 perfusion stimulates reconstitution of the immune and hematopoietic systems after 5-fluorouracil treatment.
Cancer Research **50** 2885-2890

TARLETON, R.L., KOLLER, B.H., LATORU, A. AND POSTAN, M. (1992)
Susceptibility of β_2-microglobulin-deficient mice to *Trypanosoma cruzi* infection.
Nature **356** 338-340

TAVERNE, J., BATE, C.A.W., KWIATKOWSKI, D., JAKOBSEN, P.H. AND PLAYFAIR, J.H.L. (1990a)
Two soluble antigens of *Plasmodium falciparum* induce tumour necrosis factor release from macrophages.
Infection and Immunity **58** 2923-2928

TAVERNE, J., BATE, C.A.W., SARKAR, D.A., MEAGER, A., ROOK, G.A.W. AND PLAYFAIR, J.H.L. (1990b)
Human and murine macrophages produce TNF in response to soluble antigens of *Plasmodium falciparum.*
Parasite Immunology **12** 33-43

TAVERNE, J., TAVERNIER, J., FIERS, W. AND PLAYFAIR, J.H.L. (1987)
Recombinant tumour necrosis factor inhibits malaria parasites *in vivo* but not *in vitro.*
Clinical and Experimental Immunology **67** 1-4

TAYLOR, D.N., WASI, C. AND BERNARD, K. (1984)
Chloroquine prophylaxis associated with a poor antibody response to human diploid cell rabies vaccine.
Lancet **i** 1405-1406

TAYLOR, D.W., LEVANDER, O.A., KRISHNA, V.R., EVANS, C.B., MORRIS, V.C. AND BARTA, J.R. (1997)
Vitamin E-deficient diets enriched with fish oil suppress lethal *Plasmodium yoelii* infections in athymic and scid/bg mice.
Infection and Immunity **65** 197-202

TAYLOR-ROBINSON, A.W. (1995)
Regulation of Immunity to Malaria: Valuble lessons learned from murine models.
Parasitology Today **11** 334-342

TAYLOR-ROBINSON, A.W. AND PHILLIPS, R.S. (1992)
Functional characterisation of protective $CD4^+$ T-cell clones reactive to the murine malaria parasite *Plasmodium chabaudi.*
Immunology **77** 99-105

TAYLOR-ROBINSON, A.W. AND PHILLIPS, R.S. (1993)
Protective $CD4^+$ T-cell lines raised against *Plasmodium chabaudi* show characteristics of either T_H1 and T_H2 cells.
Parasite Immunology **15** 301-310

TAYLOR-ROBINSON, A.W., PHILLIPS, R.S., SEVERN, A., MONCADA, S. AND LIEW, F.Y. (1993)
The role of Th1 and Th2 cells in a rodent malaria system.
Science **260** 1931-1934

TAYLOR-ROBINSON, A.W. AND PHILLIPS, R.S. (1994a)
B cells are required for the switch from Th1-to Th2-regulated immune responses to *Plasmodium chabaudi* infection.
Infection and Immunity **62** 2490-2499

TAYLOR-ROBINSON, A.W., PHILLIPS, R.S. (1994b)
Th1 and Th2 $CD4^+$ T cell clones specific for *Plasmodium chabaudi* but not for an unrelated antigen protect against blood stage *P. chabaudi* infection.
European Journal of Immunology **24** 158-164

TAYLOR-ROBINSON, A.W., LIEW, F.Y., SEVERN, A., XU, D. M^{C}SORLEY, S.J., GARSIDE, P., PADRON, J. AND PHILLIPS, R.S. (1994a)
Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells.
European Journal of Immunology **24** 980-984

TAYLOR-ROBINSON, A.W., PHILLIPS, R.S., LIEW, F.Y., CLARK, A. AND OWEN, M. (1994b)
$\alpha\beta$ but not $\gamma\delta$ T cells contribute to protective immunity to blood-stage *Plasmodium chabaudi* infection.
Transactions of the Royal Society of Tropical Medicine and Hygiene **88** 505-506

TEPPER, R.I., LEVINSON, D.A., STANGER, B.Z., CAMPOS-TORRES, J., ABBAS, A.K. AND LEDER, P. (1990)
IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice.
Cell **62** 457-467

TERANISHI, T., HIRANO, T., NAOMICHI, A. AND ONOOUE, K. (1982)
Human helper T cell factor(s) (ThF). II. Induction of IgG production in B lymphoblastoid cell lines and identification of T cell-replacing factor-(TRF) like factor(s).
Journal of Immunology **128** 193-198

THEANDER, T.G., BYGBJERG, I.C., ANDERSON, B.J., JEPSEN, S., KHARSAZMI, A. AND ODUM, N. (1986)
Suppression of parasite-specific responses in *Plasmodium falciparum* malaria. A longitudinal study of blood mononuclear cell proliferation and subset composition.
Scandinavian Journal of Immunology **24** 73-81

THOMPSON, G.R. AND BARTHOLOMEW, L. (1964)
The effect of chloroquine on antibody production.
University of Michigan Medical Centre Journal **30** 227-230

TOWNSEND, A. AND BODMER, H. (1989)
Antigen recognition by class I-restricted T lymphocytes.
Annual Review of Immunology **7** 601-624

TRAGER, W. AND JENSON, J.B. (1976)
Human malaria parasites in continuous culture.
Science **193** 673-675

TRENN, G., TAKAYAMA, H., HU-LI, J., PAUL, W.E. AND SITKOVSKY, M.V.
B cell stimulatory factor 1 (IL-4) enhances the development of cytotoxic T cells from Lyt-2+ resting murine T lymphocytes.(1988)
Journal of Immunology **140** 1101-1106

TRINCHIERI, G. (1995)
Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity.
Annual Review of Immunology **13** 251-276

TRIST, D.G. AND WEATHERALL, M. (1981)
Inhibition of lymphocyte transformation by mepacrine and chloroquine.
Journal of Pharmacy and Pharmacology **33** 434-438

TROYE-BLOMBERG, M., ANDERSSON, G., STOCZKOWSKA, M., SHABO, R., ROMERO, P., PATARROYO, M.E., WIGZELL, H. AND PERLMANN, P. (1985)
Production of IL-2 and IFN-γ by T cells from malaria patients in response to *Plasmodium* or erythrocyte antigens *in vitro*.
Journal of Immunology **135** 3498-3504

TROYE-BLOMBERG, M., BERZINS, K. AND PERLMANN, P. (1994)
T-cell control of immunity to the asexual blood stages of the malaria parasite.
Critical Reviews in Immunology **14** 131-155

TROYE-BLOMBERG, M. AND PERLMANN, P. (1988)
T cell functions in *Plasmodium falciparum* and other malarias.
Progress in Allergy **41** 253-287

TROYE-BLOMBERG, M., RILEY, E.M., KABILAN, L., HOLMBERG, M., PERLMANN, H., ANDERSSON, U., HEUSSER, C.H. AND PERLMANN, P. (1990a)
Production by activated human T cells of interleukin 4 but not interferon-γ is associated with elevated levels of serum antibodies to activating malaria antigens.
Proceedings of the National Academy of Sciences USA **87** 5484-5488

TROYE-BLOMBERG, M., SJOBERG, K., OLERUP, O., RILEY, E.M., KABILAN, L., PERLMANN, H., MARBIAH, N.T. AND PERLMANN, P. (1990b)
Characterisation of regulatory T cell responses to defined immunodominant T cell epitopes of the *Plasmodium falciparum* antigen Pf155/RESA.
Immunology Letters **25** 129-134

TROYE-BLOMBERG, M., ROMERO, P., PATARROYO, M.E., BJORKMAN, A. AND PERLMANN, P. (1984)
Regulation of the immune response in *Plasmodium falciparum* malaria. III. Proliferative response to antigen *in vitro* and subset composition of T cells from patients with acute infection or from immune donors.
Clinical and Experimental Immunology **58** 380-387

TRUYENS, C., ANGELO-BARRIOS, A., TORRICO, F., VAN DAMME, J., HEREMANS, H. AND CARLIER, Y. (1994)
Interleukin-6 (IL-6) production in mice infected with Trypanosoma cruzi: Effect of its paradoxical increase by anti-Il-6 monoclonal antibody treatment on infection and acute-phase and humoral immune responses.
Infection and Immunity **62** 692-696

TSUJI, M., MIYAHIRA, Y., NUSSENZWEIG, R.S., AGUET, M., REICHEL, M. AND ZAVALA, F. (1995)
Development of antimalaria immunity in mice lacking IFN-gamma receptor.
Journal of Immunology **154** 5338-5344

TSUJII, M., ROMERO, P., NUSSENZWIEG, R.S. AND ZAVALA, F. (1990)
$CD4^+$ cytolytic T cell clone confers protection against murine malaria.
Journal of Experimental Medicine **172** 1353-1357

ULICH, T.R., GUO, K., REMICK, D., DEL CSTILLO, J. AND YIN, S. (1991)
Endotoxin-induced cytokine gene expression *in vivo*. III. IL-6 mRNA and serum protein expression and the *in vivo* hematologic effects of IL-6.
Journal of Immunology **146** 2316-2323

ULLMAN, K.S., NORTHROP, J.P., VERWEIJ, C.L. AND CRABTREE, G.R. (1990)
Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function. The missing link.
Annual Review of Immunology **8** 421-452

URBAN, J.J., KATONA, I.M., PAUL, W.E. AND FINKELMAN, F.D. (1991)
Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice.
Proceedings of the National Academy of Sciences USA **88** 5513-5517

URBAN, J.J., MADDEN, K.B., SVETIC, A., CHEEVER, A., TROTTA, P.P., GAUSE, W.C., KATONA, I.M. AND FINKLEMAN, F.O. (1992)
The importance of Th2 cytokines in protective immunity to nematodes.
Immunological Reviews **127** 205-220

URBAN, Jr, J.F., MALISZEWSKI, C.R., MADDEN, K.B., KATONA, I.M. AND FINKELMAN, F.D. (1995)
IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice.
Journal of Immunology **154** 4675-4684

VALENTE, G., OZMEN, L., NOVELLI, F., GEUNA, M., PALESTRO, G., FORNI, G. AND GAROTTA, G. (1992)
Distribution of interferon-gamma receptor in human tissue.
European Journal of Immunology **22** 2403-2412

VALERO, M.V., AMADOR, L.R., GALINDO, C., FIGEROA, J., BELLO, M.S., MURILLO, L.A., MORA, A.L., PATARROYO, G., ROCHA, C.L., ROJAS, M., APONTE, J.J., SARMIENTO, L.E., LOZADO, D.M., CORONELL, C.G., ORTEGA, N.M., ROSAS, J.E., ALONSO, P.L. AND PATARROYO, M.E. (1993)
Vaccination with Spf66, a chemically synthesised vaccine, against *Plasmodium falciparum* malaria in Colombia.
The Lancet **341** 705-710

VAN DAMME, J., CAYPHAS, S., OPDENAKER, G., BILLIOU, A. AND VAN SNICK, J. (1987)
Interleukin-1 and poly(rI)poly(rC) induce production of a hybridoma growth factor by human fibroblasts.
European Journal of Immunology **17** 1-7

VANDERBERG, J.P. (1975)
Development of infectivity by the *Plasmodium berghei* sporozoite.
Journal of Parasitology **61** 43-50

VAN DER HEYDE, H.C., MANNING, D.D., ROOPENIAN, D.C. AND WEIDANZ, W.P. (1993a)
Resolution of blood-stage malarial infections in $CD8^+$ cell deficient $\beta 2\text{-}m^{o/o}$ mice.
Journal of Immunology **151** 3187-3191

VAN DER HEYDE, H.C., MANNING, D.D., ROOPENIAN, D.C. AND WEIDANZ, W.P. (1993b)
Role of $CD4^+$ T cells in the expansion of $CD4^-CD8^-\gamma\delta$ T cell subset in the spleens of mice during blood-stage malaria.
Journal of Immunology **151** 6311-6317

VAN DER HEYDE, H.C., PEPPER, B., BATCHELDER, J., CIGEL, F. AND WEIDANZ, W.P. (1997)
The time course of selected malarial infections in cytokine-deficient mice.
Experimental Parasitology **85** 206-213

VANDER JAGT, D.L., HUNSAKER, L.A. AND CAMPOS, N.M. (1986)
Characterisation of hemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum.*
Molecular and Biochemical Parasitology **18** 389-400

VAN MEIRVENNE, N., JANSSENS, P. G. AND MAGNUS, E. (1975)
Antigenic variation in syringe passaged populations of *Trypanosoma (Trypanozoon) brucei.* I Rationalization of the experimental approach.
Annals of the Belgian Society of Tropical Medicine **55** 1

VERGARA, U., FERREIRA, A., SCHELLEKENS, H. AND NUSSENZWEIG, V. (1987)
Mechanism of escape of exoerythrocytic forms (EEF) of malaria parasites from the inhibitory effects of interferon-gamma.
Journal of Immunology **138** 4447-4449

VESPA, G.N.R., CUNHA, F.Q. AND SILVA, J.S. (1994)
Nitric oxide is involved in control of *Trypansoma cruzi*-induced parasitaemia and directly kills the parasite *in vitro*.
Infection and Immunity **62** 5177-5182

VICARI, A.P. AND ZLOTNIK, A. (1996)
Mouse NK1.1$^+$ T cells. A new family of T cells.
Immunology Today **17** 71-76

VINETZ, J.M., KUMAR, S., GOOD, M.F., FOWLKES, B.J., BERZOFSKY, J.A. AND MILLER, L.H. (1990)
Adoptive transfer of CD8$^+$ T cells from immune animals does not transfer immunity to blood stage *Plasmodium yoelii* malaria.
Journal of Immunology **144** 1069-1074

VITETTA, E.S., O'HARA, J., MYERS, C.D., LAYTON, J.E., KRAMMER, P.H. AND PAUL, W.E. (1985)
Serological, biochemical and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1.
Journal of Experimental Medicine **162** 1726-1731

VODOVOTZ, Y. AND BOGDAN, C. (1994)
Control of nitric oxide synthase expression by transforming growth factor-β: implications for hemeostasis.
Progress in Growth Factor Research **5** 341-351

VOLANAKIS, J.E. AND KAPLAN, M.D. (1971)
Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide.
Proceedings of the Society of Experimental Medicine and Biology **136** 612-614

VOLLER, A. (1964)
Fluorescent antibody methods and their use in malaria research.
Bulletin of the World Health Organisation **30** 343-354

VON DER WEID, T., KITAMURA, D., RAJEWSKY, K. AND LANGHORNE, J. (1994a)
A dual role for B cells in *Plasmodium chabaudi chabaudi* AS infection.
Research in Immunology **145** 412-419

VON DER WEID, T., KOPF, M., KOHLER, G. AND LANGHORNE, J. (1994b)
The immune response to *Plasmodium chabaudi* malaria in interleukin-4-deficient mice.
European Journal of Immunology **24** 2285-2293

VREDEN, S.G.G, VAN DEN BROEK, M.F., OETTINGER, M.C., BOERS, W., VAN ROOJIEN, N., MEUWISSEN, J.H.E.T. AND SAUERWEIN, R.W. (1995)
Susceptibility to *Plasmodium berghei* infection in rats is modulated by the acute phase response.
Parasite Immunology **17** 445-450

VREDEN, S.G.S., VAN DEN BROCK, M.F., OETTINGER, M.C., VERHAVE, J.P., MEUWISSEN, J.H.E.T. AND SAUERWEIN, R.W. (1992)
Cytokines inhibit the development of liver schizonts of the malaria parasite *Plasmodium berghei in vivo*.
European Journal of Immunology **22** 2271-2275

WAKI, S., UEHARA, S., KARIBE, K., NARLUCH, H. AND SUZUKI, M. (1995)
Interferon-gamma and the induction of protective IgG2a antibodies in non-lethal *Plasmodium berghei* infections of mice.
Parasite Immunology **17** 503-508

WAKI, S., UEHARA, S., KANBE, K., ONO, K., SUZUKI, M. AND NARIUCHI, H. (1992)
The role of T cells in pathogenesis and protective immunity to murine malaria.
Immunology **75** 646-651

WALLIKER, D., CARTER, R. AND MORGAN, S. (1971)
Genetic recombination in malaria parasites.
Nature **232** 561-562

WARRELL, D.A. (1993)
Leishmaniasis, malaria and schistosomiasis in Saudi Arabia.
Saudi Medical Journal **14** 203-208

WATANABE,H., MIYAJI, C., KAWACHI, Y., IIAI, T., OHTSUKA, K., IWANAGE, T., TAKAHASHI-IWANAGA, H. AND ABO, T. (1995)
Relationships between intermediate TCR cells and NK1.1 T cells in various immune organs.
Journal of Immunology **155** 2972-2983

WEIDANZ, W.P. (1982)
Malaria and alterations in immune reactivity.
In *Malaria, British Medical Bulletin* 167-172

WEIDANZ, W.P., MELANCON-KAPLAN, J. AND CAVACINI, L.A. (1990)
Cell-mediated immunity to the asexual stages of malarial parasite: animal models.
Immunology Letters **25** 87-96

WEINBAUM, F.I., EVANS, C.B. AND TIGELAAR, R.E. (1976)
Immunity to *Plasmodium berghei yoelii* in mice. I The course of infection in T and B cell deficient mice.
Journal of Immunology **117** 1999-2004

WEINBERG, J.B., CHAPMAN, H.A. Jr. AND HIBBS, J.B. Jr. (1978)
Characterisation of the effects of endotoxin on macrophage tumour cell killing.
Journal of Immunology **178** 72-80

WEISS, G., WERNER-FELMAYER, G., WERNER, E.R., GRUNEWALD, K., WACHTER, H. AND HENTZE, M.W. (1994)
Iron regulates nitric oxide synthase activity by controlling nuclear transcription.
Journal of Experimental Medicine **180** 969-976

WEISS, L. (1990)
The spleen in malaria: the role of barrier cells.
Immunology Letters **25** 165-172

WEISS, W.R., SEDEGAH, M., BEAUDON, R.L., MILLER, L.H. AND GOOD, M.F. (1988)
$CD8^+$ T Cells (Cytotoxic/Suppressors) are required for protection in mice immunised with malaria sporozoites.
Proceedings of the National Academy of Sciences USA **85** 573-576

WENNER, C.A., GULER, M.L., MACATONIA, S.E., O'GARRA, A. AND MURPHY, K.M. (1996)
Roles of IFN-γ and IFN-α in IL-12-induced T helper cell-1 development.
Journal of Immunology **156** 1350-1358

WHEELOCK, E.F. (1965)
Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin.
Science **149** 310-311

WHITE, K.L., JARBOE, D.L. AND KRZYCH, U. (1994)
Immunization with irradiated *Plasmodium berghei* sporozoites induces IL-2 and IFNγ but not IL-4.
Parasite Immunology **16** 479-491

WHITE, K.L., SNYDER, H.L. AND KRZYCH, U. (1996)
MHC Class I-dependent presentation of exoerythrocytic antigens to $CD8^+$ T lymphocytes is required for protective immunity against *Plasmodium berghei*.
Journal of Immunology **156** 3374-3381

WHITE, N.J. AND HO, M. (1992)
The pathophysiology of malaria.
Advances in Parasitology **31** 83-127

WHO (1993)
World malaria situation in 1991.
Weekly Epidemiological Record **68** 245

WIDMER, M.B. AND GRABSTEIN, K.H. (1987)
Regulation of cytolytic T-lymphocyte generation by B-cell stimulatory factor.
Nature **326** 795-798

WILLIAMSON, W.A. AND GREENWOOD, B.M. (1978)
Impairment of the immune response to vaccination after acute malaria.
TheLancet **i** 1328-1329

WILLIAMS, T.N., MAITLAND, K., BENNETT, S., GANCZAKOWSKI, M., PETO, T.E.A., NEWBOLD, C.I., BOWDEN, D.K., WEATHERALL, D.J. AND CLEGG, J.B. (1996)
High incidence of malaria in α-thalassaemic children.
Nature 383 522-525

WIZEL, B., HOUGHTEN, R., CHURCH, P., TINE, J.A., LANAR, D.E., GORDON, D.M., BALLOU, W.R., SETTE, A. AND HOFFMAN, S.L. (1995)
HLA-A2-restricted cytotoxic T lymphocyte responses to multiple *Plasmodium falciparum* sporozoite surface protein 2 epitopes in sporozoite-immunised volunteers.
Journal of Immunology **155** 766-775

WORKU, S., BJORKMAN, A., TROYE-BLOMBERG, M., JEMANEH, L., FARNERT, A. AND CHRISTENSSON, B. (1997)
Lymphocyte activation and subset redistribution in the peripheral blood in acute malaria illness: γδ T cell patterns in *Plasmodium falciparum* and *P. vivax* infections.
Clinical and Experimental Immunology **108** 34-41

WRIGHT, D.H. (1968)
The effect of neonatal thymectomy on the survival of golden hamsters infected with *Plasmodium berghei.*
British Journal of Experimental Pathology 49 379-384

XIE, Q-W., CHO, H., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D., DING, A., TROSO, T. AND NATHAN, C. (1992)
Cloning and characterisation of inducible nitric oxide synthase from mouse macrophages.
Science **256** 225-228

XIE, Q-W., KASHIWABARA, Y. AND NATHAN, C. (1994)
Role of transcription factor NF-κB/Rel in the induction of nitric oxide synthase.
Journal of Biological Chemistry **269** 4705-4708

XIE, Q., WHISNANT, R. AND NATHAN, C.F. (1993)
Promoter of the mouse gene encoding calcium-independent NOS confers inducibility by IFN-γ and bacterial LPS.
Journal of Experimental Medicine **177** 1779-1784

YAP. G.S., JACOBS, P. AND STEVENSON, M.M. (1994)
Th cell regulation of host resistance to blood-stage *Plasmodium chabaudi* AS.
Research in Immunology **145** 419

YING, S.C., GEWURZ, A.T., JIANG, H. AND GEWURZ, H. (1993)
Human serum amyloid P component oligomers bind and activate the classical complement pathway via residues 14-26 and 76-92 of the A chain collagen-like region of C1q.
Journal of Immunology **150** 169-176

ZAHEDI, K. AND WHITEHEAD, A.S. (1989)
Acute phase induction of mouse serum amyloid P component. Correlation with other parameters of inflammation.
Journal of Immunology **143** 2880-2886

ZHANG, Y., LIN, J.X. AND VILCEK, J. (1988)
Synthesis of interleukin 6 (interferon-β2/B cell stimulatory factor 2) in human fibroblasts is triggered by an increase in intracellular cyclic AMP.
Journal of Biological Chemistry **263** 6177-6182

ZIDOVETZKI, R., SHERMAN, I.W. AND O'BRIEN, L. (1993)
Inhibition of *Plasmodium falciparum* phospholipase A2 by chloroquine, quinine and artemether.
Journal of Parasitology **79** 565-570

ZLOTNIK, A., FISCHER, M., ROEHM, N. AND ZIPORI, D. (1987)
Evidence for effects of interleukin 4 (B cell stimulatory factor 1) on macrophages: Enhancement of antigen presenting ability of bone marrow-derived macrophages.
Journal of Immunology **138** 4275-4279

ZVERING, Y., AMANTE, F., SMILLIE, A., CURRIER, J., SMITH, G., HOUGHTEN, R.A. AND GOOD, M.F. (1992)
High frequency of malaria-specific T cells in non-exposed humans.
European Journal of Immunology 22 689-696