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**Investigating the effects of *Plasmodium chabaudi chabaudi* AS-infected  
erythrocytes on Dendritic Cell function *in vitro***

**Volume I**

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A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

Division of Immunology, Infection and Inflammation  
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Caterina Di Lorenzo,  
University of Glasgow,  
April 2006

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## Abbreviations

Ab	Antibody
ACAD	Activated cell autonomous death
AICD	Activation induced cell death
ANOVA	Analysis Of Variance
APC	Antigen Presenting Cell
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of Differentiation
cDNA	Complementary DNA
CFSE	5-Carboxyfluorescein diacetate succinimidyl ester
cGMP	Guanosine 3', 5'-Cyclic Monophosphate
COX	Cyclooxygenase
eNOS	Constitutive nitric oxide synthase
CpG	A site where cytosine (C) lies next to guanine (G) in the DNA sequence. P indicates that C and G are connected by a phosphodiester bond
CR	Complement receptor
CSP	Circumsporozoite Protein
CT	Cholera Toxin
CTL	Cytotoxic T Lymphocytes
CTLA	Cytotoxic T lymphocyte associated antigen
DC	Dendritic Cell
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing nonintegrin
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot technique
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorter

FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
<sup>3</sup> H	Tritium
HLA	Human leukocyte antigen
HO	Haeme-oxygenase
i.p.	Intraperitoneal
i.v.	Intravenous
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
iGA	Iterative group analysis
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 Receptor Associated Kinase
IRF	Interferon regulatory factor
JNK	Ras-c-jun N-terminal kinase
kDa	Kilo Dalton
KLH	Keyhole limpet haemocyanin
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LSA	Liver Stage Antigen
MA	Malarial antigen
mAb	Monoclonal antibody
ManLAM	Mannosylated lipoarabinomannans
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIP	Macrophage inflammatory protein
MM	Mismatch probe
mRNA	Messenger RNA
MSP	Merozoite surface protein
MT	Methyl tryptophan
MyD	Myeloid differentiation factor
NFAT	Nuclear factor of activated T cells
NF- $\kappa$ B	Nuclear factor kappa B
NIK	Nuclear factor kappaB-inducible kinase
NO	Nitric oxide
OD	optical density
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PfEMP-1	<i>P. falciparum</i> erythrocyte membrane protein-1
PG	Prostaglandin
PI	Propidium iodide
PM	Perfect match probe
PPAR	Peroxisome proliferation-activated receptor
pRBC	Parasitised Red Blood Cell
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RBC	Red Blood Cell
rIL	Recombinant interleukin
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
RP	Rank Product

rRNA	Ribosomal RNA
rTNF	Recombinant tumour necrosis factor
RTQ-PCR	Real-time quantitative polymerase chain reaction
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SLS	Sodium lauryl sulphate
SSC	Side scatter
STAT	Signal transducers and activators of transcription
STRAP	Sporozoite threonine/asparagine-rich protein
TAP	Transporter for antigen presentation
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th	T helper cell
TIR	Toll-IL-1 receptor
TIRAP	Toll-IL-1 receptor adapter protein
TLR	Toll like receptor
TMB	3,3',5,5'-tetramethylbenzidine peroxidase
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor-associated factor
TRAIL	TNF-related apoptosis inducing ligand
TRAM	TNF receptor apoptosis-mediating protein
TRANCE	TNF-related activation induced cytokine
TRAP	Thrombospondin-related anonymous protein
TRIF	TIR-domain containing adaptor inducing interferon-beta
TSP	Thrombospondin
UV	Ultra-violet
VCA	Viral capsid antigen
Ci	Curie(s)

cpm	Count per minute
<	Less than
>	More than
%	Percentage point(s)
°C	Degree centigrade
g	gram
mg	milligram
µg	microgram
ng	nanogram
M	molar
mM	millimolar
µM	micromolar
ml	millilitres
l	Litres
g	times gravity
ρ	density

## Abstract

During malaria infection there is significant depression of the host's immune response to concurrent infections and heterologous vaccine antigens as well as the response to the parasite itself. Using the murine *Plasmodium chabaudi chabaudi* AS model, I examined whether the malaria parasite can suppress immune responses *via* effects on dendritic cells (DC), which are important for initiating primary immune responses. As the morbidity and mortality associated with malaria are derived exclusively from the asexual erythrocytic stages, I investigated the effects that this stage has on DC function.

I found that malaria infected erythrocytes (pRBC) do not directly activate bone marrow-derived DCs *in vitro*; rather, they inhibit the LPS induced upregulation of Class II MHC and costimulatory molecules (CD40 and CD86) on DCs and significantly reduce the ability of these cells to induce proliferation and effector function of naïve T cells. Nevertheless, pRBC-treated DCs induce equivalent levels of the very early activation marker CD69 on antigen-specific naïve T cells compared with RBC-treated DCs, indicating that T cells in both conditions are antigen experienced. Furthermore, the proliferation of T cells cultured with pRBC-treated DCs could be recovered by the addition of exogenous IL-2, suggesting that they are functionally anergic.

In search of a mechanistic explanation for this parasite-mediated inhibition of DC function, I investigated the possible role of selected parasite components. Haemozoin, the end-product of the haemoglobin catabolism by intracrythrocytic malaria parasite, was found to contribute significantly to the immunosuppressive effects induced in DCs. A soluble factor(s) secreted by pRBCs was also found to affect the ability of DCs to mature in response to LPS treatment *in vitro*. Furthermore, following gene array analysis, I have identified a number of candidate molecules in pRBC-treated DC that may be involved in the observed alterations in functional activity.

The present study provides a better insight of how the malaria parasite affects DC function and identifies a number of parasite-induced changes in these cells related to their

decreased ability to induce effective primary immune responses. These observations may therefore facilitate the development of more effective vaccines and immunisation protocols.

# 1. Introduction

## 1.1 *Plasmodium* and malaria

### 1.1.1 Life cycle

Malaria is an arthropod-borne disease caused by protozoan parasites of the genus *Plasmodium* (Wakelin, 1996). Four species infect humans: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. The life cycle of all species of human malaria parasites is essentially the same (Phillips, 2001)(Figure 1). It comprises an exogenous sexual phase (sporogony) with multiplication in certain female *Anopheles* mosquitoes and an endogenous asexual phase (schizogony) with multiplication in the vertebrate host.

Infection is initiated by inoculation of sporozoites through mosquito bites. The sporozoites circulate in the blood for 30-45 minutes before invading liver cells (hepatocytes) (Fairley, 1947; Sinden and Smith, 1982). Here they undergo a process of development and multiplication known as 'pre-erythrocytic schizogony' leading to the development of merozoites (Garnham, 1966).

In *P. vivax* and *P. ovale* infections, the sporozoites differentiate into 'hypnozoites' as well as merozoites (Krotoski *et al.*, 1982). The hypnozoites remain dormant in hepatocytes for considerable periods of time. The mechanism of activating the growth of these parasites is not known. When "reactivated" they grow and undergo 'exo-erythrocytic schizogony' forming a wave of merozoites that invade the blood and produce a clinical relapse.

In *P. falciparum* and *P. malariae*, hypnozoites are not formed and the parasite develops directly into pre-erythrocytic schizonts. Upon reaching maturity, the schizonts rupture releasing merozoites into the blood stream, where they invade erythrocytes and initiate a schizogonic cycle in the blood. Within the circulating erythrocytes, the merozoite begins to feed on haemoglobin leaving, as the product of digestion, a pigment called haemozoin, a combination of haematin with protein (Arese and Schwarzer, 1997). This iron-containing pigment is seen in the body of the parasite in the form of dark granules, which

are more obvious in the later stages of development.

During their development within the red blood cells, the parasites transform into a uninucleated feeding stage called a 'trophozoite', shortly after which the nucleus begins to divide into a variable number of small nuclei. This is soon followed by the division of the cytoplasm, leading to schizont formation. In *P. falciparum* infections, mature trophozoite stages disappear from the peripheral circulation and cytoadhere to endothelial cells lining post-capillary venules within different organs, a process known as 'sequestration' (David *et al.*, 1983). When sequestration of infected erythrocytes occurs in the vessels of the brain, it is believed to cause the severe disease syndrome known as cerebral malaria, which is associated with high mortality (Garnham, 1966).

Mature schizonts are fully developed forms in which, as a result of segmentation of the nucleus and the cytoplasm, a number of small rounded merozoites are produced. When the process of schizogony is completed, the red cell lyses and the merozoites are released into the blood stream (Dvorak *et al.*, 1975). The merozoites then invade fresh red cells in which another generation of parasites is produced by the same process. This erythrocytic cycle of schizogony can be repeated many times in the course of infection, leading to a progressive increase of parasitaemia until the process is slowed down by the immune response of the host (Phillips, 1983).

After several rounds of blood schizogony, a proportion of the merozoites develop into the sexual stages of the parasites - the gametocytes. Microgametocytes (male) and macrogametocytes (female) circulate in the peripheral blood where they remain infective for a limited period of time (Garnham, 1966). When these infective stages are taken up by a mosquito, they develop into gametes and fertilization occurs. The resulting zygote matures within 24h to the motile ookinete, which burrows through the stomach wall leading to the formation of an oocyst (Sinden and Strong, 1978). Within the developing oocyst many mitotic divisions occur, resulting in oocysts full of sporozoites. Rupture of oocysts releases the sporozoites, which migrate to the mosquito's salivary glands (Vanderberg, 1975) to complete the cycle approximately 7 to 18 days after gametocyte

ingestion.

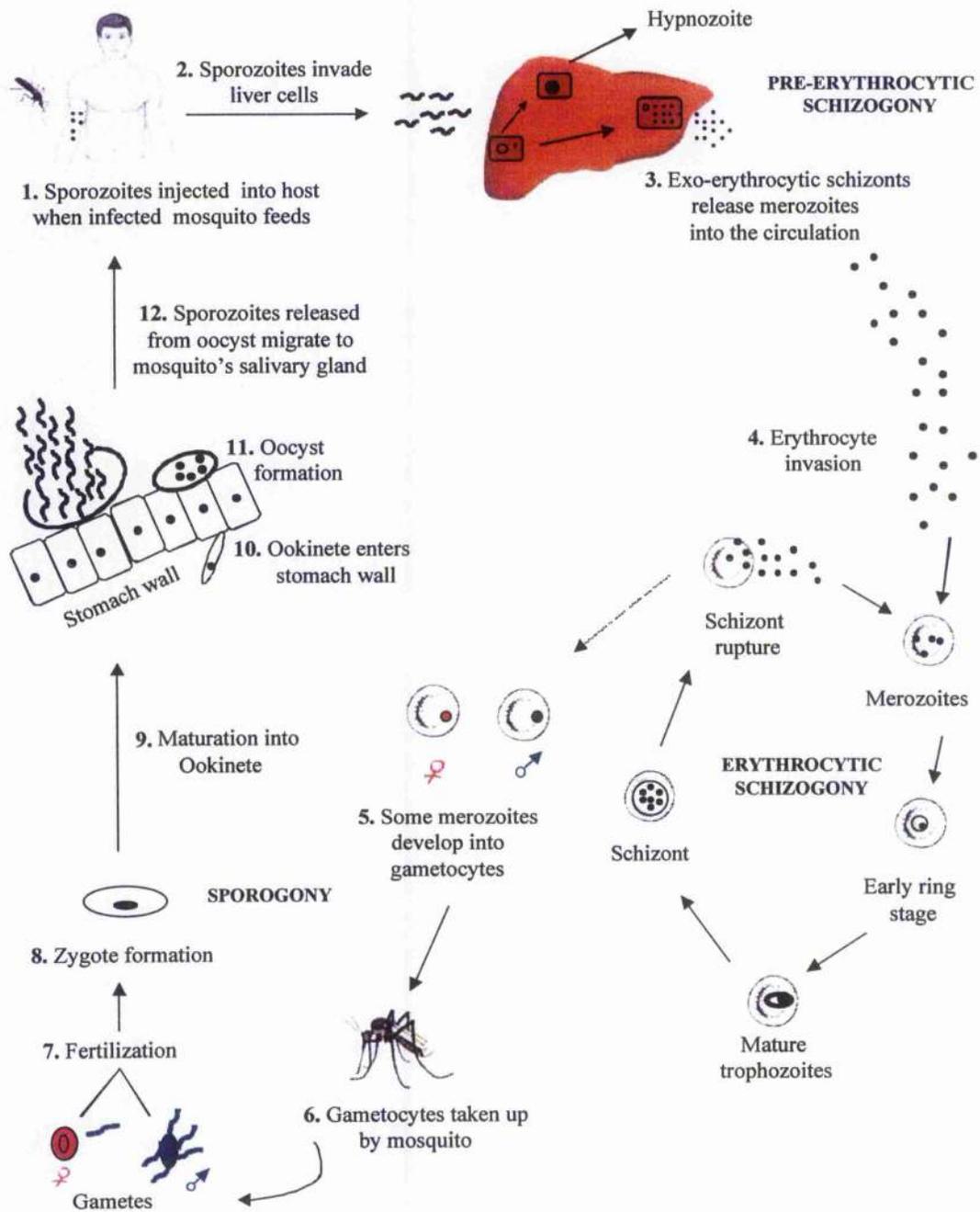


Figure 1.1 *Plasmodium* life cycle

## 1.1.2 Human malaria parasites

### 1.1.2.1 *Plasmodium vivax*

*P. vivax* is found mostly in Asia, Latin America, and in some parts of Africa (Sina, 2002). Because of the population densities, especially in Asia, it is probably the most prevalent human malaria parasite (Sina, 2002). *P. vivax* (as well as *P. ovale*) has dormant liver stages ("hypnozoites") that can activate and invade the blood ("relapse") several months or years after the infecting mosquito bite (Krotoski *et al.*, 1982). It is a selective parasite as it only invades young, immature erythrocytes (Kitchen, 1939). After an incubation period of usually 10-17 days, the patient experiences influenza-like symptoms with headache, muscle pain, nausea and vomiting (Garnham, 1966). As the infection progresses, increased numbers of rupturing erythrocytes liberate merozoites as well as toxic debris into the circulation. Together, these produce the typical pattern of chills and fever. These symptoms usually re-appear periodically, every 48 hours, which is why *P. vivax* is said to cause tertian malaria or benign malaria as most patients can tolerate the attacks and can survive for years without treatment (Garnham, 1966). However, there are occasional reports of severe and fatal *P. vivax* infections. These are associated with lung injury and respiratory distress (Carlini *et al.*, 1999; Torres *et al.*, 1997), splenic rupture and associated pathology (Leclerc *et al.*, 1989), and even instances of cerebral malaria associated with apparently pure *P. vivax* infections (Sachdev and Mohan, 1985). Thus, under rare and yet poorly understood circumstances, *P. vivax* infections can present in dangerous forms.

### 1.1.2.2 *Plasmodium ovale*

*P. ovale* is found mostly in Africa (especially West Africa) and the islands of the western Pacific (Collins and Jeffery, 2005). It is biologically and morphological very similar to *P. vivax* including its selectivity for young erythrocytes (Garnham, 1966). However, differently from *P. vivax*, it can infect individuals who are negative for the Duffy blood

group, which is the case for many residents of sub-Saharan Africa (Collins and Jeffery, 2005). This explains the greater prevalence of *P. ovale* (rather than *P. vivax*) in most of Africa. Following parasite invasion of erythrocytes, the host cell becomes enlarged and distorted, usually assuming an oval form (Garnham, 1966). It causes benign tertian malaria and infection is similar to *P. vivax*. Untreated infections however last only about one year (Collins and Jeffery, 2005).

#### 1.1.2.3 *Plasmodium malariae*

*P. malariae*, in contrast to *P. ovale* and *P. vivax*, only infects mature erythrocytes (Chwatt, 1948). The incubation period is the longest of the *Plasmodia*, usually 18 to 40 days, but possibly several months or years. The early symptoms are influenza-like, with fever occurring every 72 hours and hence said to cause quartan malaria (Phillips, 1983). Attacks are moderate to severe and last several hours. It causes a long-lasting chronic infection that in some cases can last a lifetime (Garnham, 1966).

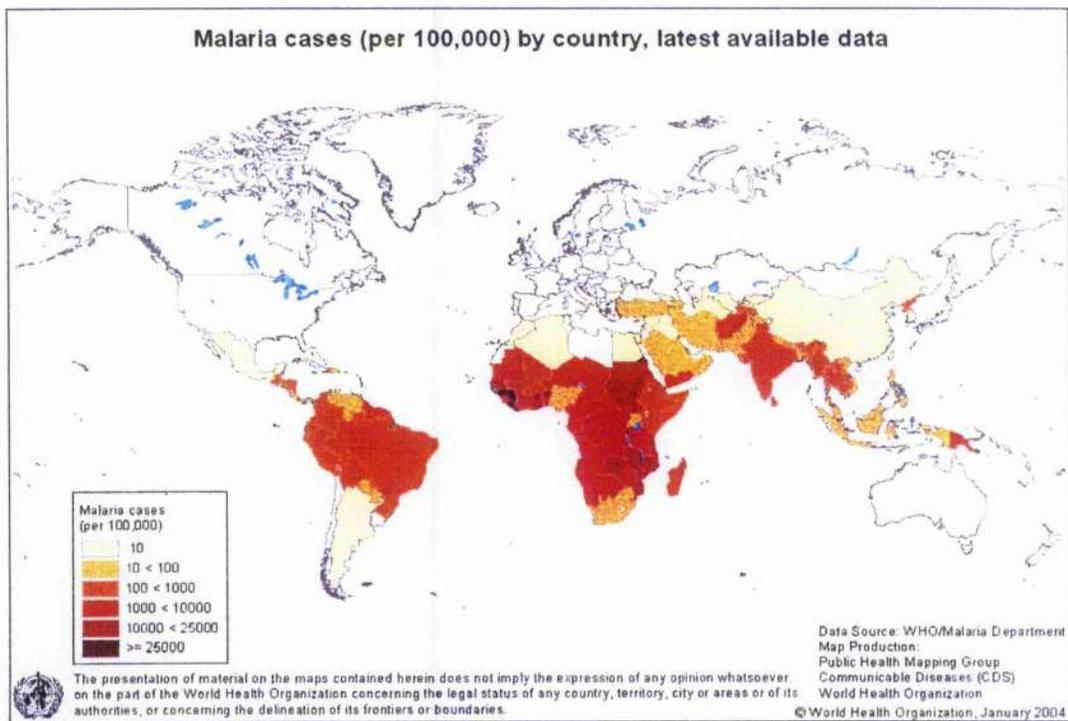
#### 1.1.2.4 *Plasmodium falciparum*

*P. falciparum* is found in tropical and subtropical areas (Phillips, 1983). It demonstrates no selectivity in host erythrocytes and invades any red blood cell at any stage in its existence (Chwatt, 1948). The incubation period is the shortest of all the *plasmodia* ranging from 7 to 10 days. After the early influenza-like symptoms, *P. falciparum* rapidly produces daily chills and fever as well as severe nausea and vomiting. The periodicity of the attacks then becomes tertian (Garnham, 1966). Although any malaria infection may be fatal, *P. falciparum* is the most likely to result in death if left untreated. It is estimated that every year 700,000 to 2.7 million people are killed by *P. falciparum*, especially in Africa where this species predominates (Snow *et al.*, 2005). *P. falciparum* can cause severe malaria because it multiplies rapidly in the blood, and can thus cause severe loss of red blood cells (anaemia). In addition, the infected red cells can sequester in capillaries of internal organs, attaching through 'knobs' on the red cell (Crabb *et al.*, 1997). Thus,

parasitized erythrocytes can obstruct small blood vessels and when this occurs in the brain, cerebral malaria results, a complication that can be fatal.

### 1.1.3 Geographic distribution of malaria

Despite advances in understanding malaria ecology and development of interventions, more than 50% of the world's population is exposed to malaria. In many parts of the world, changes in the environment, population migration and financial problems have led to serious increases in malaria. For example, deforestation can increase human-vector contact and create improved breeding conditions for several vector species (Collins and Paskewitz, 1995). Water management projects such as irrigation, impoundments and dams can also create additional breeding grounds (Keiser *et al.*, 2005). Furthermore, economic and political stress often results in population migration, which increases movement of non-immune individuals into malarious areas and of carriers into non-endemic areas.



**Figure 1.2.** Malaria world distribution (Source: World Health Organization, 2004)

Intensive malaria is confined to the tropical and subtropical areas (Figure 1.2). The African continent is the most affected, accounting for more than 90% of the burden (Breman *et al.*, 2004). Recently, malaria outbreaks have been reported in some locations of Africa that had been previously thought to be at elevations too high for malaria transmission, such as the highlands of Kenya. This might be due to climatic change or to human migration. Also, malaria has resurged in certain locations of Africa that previously had effective control programmes, such as Madagascar, South Africa, and Zanzibar (Kiszewski and Teklehaimanot, 2004).

Malaria epidemics generally afflict immunologically vulnerable populations. The very instability of malaria epidemics exacerbates their clinical threat (Kiszewski and Teklehaimanot, 2004). Long inter-epidemic periods of low transmission allow immunity to wane in populations. Thus in areas where malaria is transmitted less stably, the risk of severe disease in children is greater (Snow *et al.*, 1998), and older children and adults are more likely to have cerebral manifestations that are often fatal (Imbert *et al.*, 1997).

In Africa, malaria is most prevalent in the highlands of the eastern and southern parts of the continent. In the tropics, the upper limits of transmission are often set by an altitude of approximately 2,000 metres, but anomalous weather conditions may occasionally render higher altitudes permissive for vector and parasite development (Fisher, 1985). In east Africa, unstable highland malaria affects Ethiopia, Eritrea, western Kenya, southwestern Uganda, the highlands of Tanzania and much of Rwanda and Burundi (Kiszewski and Teklehaimanot, 2004). Ethiopia has historically had the most intensive experience with recurrent malaria epidemics (Kiszewski and Teklehaimanot, 2004). Localised malaria epidemics occur every year somewhere in Ethiopia with occasional massive outbreaks affecting most of the country (Kiszewski and Teklehaimanot, 2004). The highlands of Kenya are sometimes affected concurrently with the Ethiopian malaria outbreaks, perhaps triggered by the same climatic determinants. Some reports have suggested that Kenyan epidemics simply represent unusually high peaks in seasonal transmission (Hay *et al.*, 2002a; Hay *et al.*, 2002b). Others report a growing risk of

widespread malaria epidemics in Kenya due to environmental changes associated with agricultural development that favour the breeding of the vector mosquitoes (Malakooti *et al.*, 1998). Malaria eradication efforts apparently succeeded in temporarily interrupting transmission in the highlands, which remained almost malaria free from the late 1950s to the early 1970s (Roberts, 1964a, b, c). A trend of increasing transmission has developed since then with an upsurge of epidemic outbreaks since 1990 (Brown *et al.*, 1998).

In Tanzania, epidemic malaria is concentrated in the highlands along the Rift Valley, an area that includes approximately 8.4 million people, or approximately 25% of the country's population (Kiszewski and Teklehaimanot, 2004). Epidemic-prone districts in Uganda are clustered in the southwestern corners of the country near the Tanzanian and Rwandan borders. Generally malaria in Uganda is transmitted stably below 1,500 meters in elevation, with less stable transmission occurring between 1,500 and 2,300 meters (Kiszewski and Teklehaimanot, 2004).

In Southern Africa, unstable transmission affects parts of Mozambique, Malawi, Zimbabwe, Swaziland and the northern provinces of South Africa. (Kiszewski and Teklehaimanot, 2004). In Swaziland, epidemics may have strong links with human factors, particular nutritional status (Packard, 1984, 1986).

Malaria epidemics have been regularly reported in the arid and semi-arid regions of Mali, Senegal, Chad, Niger and Sudan (Kiszewski and Teklehaimanot, 2004). Many other parts of Africa have localised malaria epidemics including Angola, Botswana, Cape Verde, Namibia, Somalia and Zambia (Kiszewski and Teklehaimanot, 2004).

In Asia, a diverse array of mosquito vectors imposes different dynamics on malaria transmission. *P. vivax* occurs more frequently in Asia than in Africa where *P. falciparum* dominates (Sina, 2002; Snow *et al.*, 2005). Malaria epidemics have long afflicted parts of India, Sri Lanka and Pakistan (Kiszewski and Teklehaimanot, 2004). Along the borders of Thailand and Cambodia, increased malaria transmission has been associated with gem mining and other human activities (Luxemburger *et al.*, 1997). Population movements

from these sites to less endemic regions have also triggered localised outbreaks through the return of infected people into vulnerable communities (Singhanetra-Renard, 1993). Epidemic malaria transmission in Irian Jaya and Papua New Guinea is due to the *Anopheles punctulatus* mosquitoes, occurring in extensive areas of populated highlands (Bangs *et al.*, 1996). Thus, epidemic malaria is usually diverse across Asia due to the wide variety of mosquito vectors.

In South America, malaria transmission is less frequent than in Africa, but can break out in occasional localised epidemics. *P. vivax* is the predominant cause of disease. In Brazil, gold mining has regularly brought immunologically vulnerable people into seasonally intense malaria transmission areas (Camargo *et al.*, 1994). Other nations that are repeatedly afflicted with outbreaks of *P. vivax* and/or *P. falciparum* malaria include Colombia, Venezuela, Peru and Ecuador (Kiszewski and Teklehaimanot, 2004). Outbreaks in Colombia and Venezuela often severely affect isolated Native American communities. Malaria in Venezuela has been concentrated in Roraima State where it occasionally flares into epidemics mainly affecting the indigenous populations. Rain-fall associated outbreaks of *P. falciparum* malaria occasionally occur along the Pacific coasts of Peru and Ecuador, where malaria is generally much less stable than in the interior Amazonian lowlands (Kiszewski and Teklehaimanot, 2004).

Malaria occurs in low-altitude areas of the countries of Central America including Honduras, Nicaragua, and Guatemala. Limited numbers of cases occur in Panama, Costa Rica, and southern Mexico. Active control programmes are in place in several of these countries. *P. vivax* is the dominant species, and fortunately remains susceptible to chloroquine (Kiszewski and Teklehaimanot, 2004).

Malaria has been eliminated from several countries of the Caribbean. However, *P. falciparum* occurs in Haiti and there are a limited numbers of cases in the Dominican Republic (Kiszewski and Teklehaimanot, 2004).

Malaria eradication programmes during the 1940s and 1950s, along with widespread use of screening, eliminated malaria from North America, and there is no year-to-year local transmission. However, isolated, rare cases of local transmission have occurred. North America, the Caribbean region, Europe, Australia, Mexico and Central America are the major success stories of malaria vector control efforts (Collins and Paskewitz, 1995).

#### **1.1.4 Animal models of malaria**

Various animal model systems have been developed to examine the interaction of the malaria parasite and its host. The most common host-pathogen combinations are simian models, either with their natural parasites or adapted human parasites, and rodent models with parasites isolated from African wild rodents (Langhorne, 1994). Rodent models, particularly those using mouse and rat, offer an advantage over simian malaria models in that they are easy and cheap to maintain and above all a great deal is known about their immune system. Therefore dissective studies of mechanisms can be carried out (Langhorne, 1994). However, all laboratory rodents are artificial hosts of malaria parasites to which they have been adapted and care must be taken in extrapolating results from these models to human malaria.

##### **1.1.4.1 Murine models**

Studies using murine models of malaria have greatly contributed to our understanding of the immunobiology of host responses to the malaria parasite and elucidation of the mechanism of anti-malaria immunity. Analogous to four human *Plasmodium* species, there are four rodent species, *P. chabaudi*, *P. vinkei*, *P. yoelii* and *P. berghei*, which cause malaria infection in mice with varying degrees of morbidity and mortality (Stevenson, 1989). The outcome of infections with various strains of *P. berghei*, *P. yoelii* and *P. chabaudi* has been described to depend on the genetic background of the host (Stevenson, 1989). Moreover the sex of the host also influences the outcome of malaria infection in mice as well as in humans, and testosterone has been found to be immunosuppressive (Benten *et al.*, 1993).

Murine malaria parasites can be divided into two groups based on the immune response required for control of acute infection and elimination of the parasite (Sherman, 1998). Infection with *P. yoelii* and *P. berghei* cannot be controlled or eliminated in the absence of B cells. Immunity to these parasites is therefore antibody-dependent. Acute infections with *P. chabaudi adami* or *P. chabaudi* AS and secondary infections with *P. vinckei* can be controlled in the absence of B cells, implicating antibody-independent mechanisms. In the absence of B cells however, parasitaemia in the peripheral blood of *P. chabaudi* AS infected animals is reduced to low levels but the parasites are not completely eliminated. Collectively these observations stress the importance of both antibody-independent and antibody-dependent mechanisms of acquired immunity against blood-stage malaria parasite as it has been extensively discussed in section 1.1.6.2.

The most characterised murine model is *P. chabaudi* AS, which can result in non-lethal infections in mouse strains such as BALB/c, C57/BL6, C57/BL10 and NIH mice and lethal infections in A/J and DBA/2 mice (Langhorne *et al.*, 2002). *P. chabaudi* AS has been used in many studies as a model for examining acquired immunity to the asexual erythrocytic stages of malaria parasites as it is considered to be the most similar to *P. falciparum* infection in humans. Like *P. falciparum*, *P. chabaudi* AS has been shown to sequester in the post-capillary venules (Cox *et al.*, 1987) although with a different site of sequestration. In the case of *P. falciparum*, the asexual stage-infected erythrocytes tend to sequester in the heart, brain, lungs, kidneys, small intestine and liver (Aikawa *et al.*, 1990; Pongponratn *et al.*, 1991) while *P. chabaudi* AS-infected erythrocytes preferentially sequester in the liver. Both *P. falciparum* and *P. chabaudi* AS exhibit antigenic variation during the asexual erythrocytic stage (McLean *et al.*, 1982) and in resistant strains of laboratory mice recovery from the acute primary parasitaemia of a *P. chabaudi* AS infection is followed by one or more patent recrudescences (Cox *et al.*, 1987; Gilks *et al.*, 1990). Furthermore, *P. chabaudi*-infected erythrocytes have been shown to be able to adhere to specific cell types and sequester by interacting with molecules such as CD36 (Mota *et al.*, 2000), which has been described to mediate suppression of DC function by *P. falciparum*-infected erythrocytes (Urban *et al.*, 1999).

## 1.1.5 Protective immunity to malaria

The complex life cycle of malaria poses a number of challenges to the immune response. Since stages in the cycle express varying antigen profiles and have different locations, they require different effector mechanisms. Even with repeated infection, protective immunity is non-sterile and it is species-, stage-, strain- and variant-specific (Andrysiak *et al.*, 1986; Fandeur and Chalvet, 1998; Rotman *et al.*, 1999). Acquired protective immunity induced by malaria parasites involves both antibody-mediated and cell-mediated immunity.

### 1.1.5.1 Immunity to pre-erythrocytic stages

#### 1.1.5.1.1 Antibody-mediated immunity

It has been known for many years that immunisation with irradiated sporozoites induces protective immunity against malaria in both humans and animals (Clyde *et al.*, 1973; Nussenzweig *et al.*, 1967). Early studies provided clear evidence that this protective immunity is mediated by antibodies (Abs) that recognise sporozoite surface proteins, thereby inhibiting the ability of the parasite to invade hepatocytes (Weiss, 1990). The strongest evidence of an important role of anti-sporozoite antibodies in protective immunity has been obtained in experiments showing that protection against sporozoites occurred after passive transfer of monoclonal antibodies (mAbs) against the repeat domain of the circumsporozoite protein (CSP) of *P. yoelii* (Charoenvit *et al.*, 1987) and by immunisation of mice with synthetic subunit vaccines designed to induce high levels of anti-CSP antibodies (Zavala *et al.*, 1987). In humans, more than 90% of the adult population in West Africa was shown to have Abs to *P. falciparum* sporozoites in the blood (Nardin *et al.*, 1979). Less than half the children had this Ab indicating that repeated exposure to sporozoites over many years is necessary to induce its formation (Nardin *et al.*, 1979). Nevertheless, the observation that B cell-deficient mice could also

mount a protective immune response after sporozoite immunisation strongly suggested that T cells also played a protective role (Chen *et al.*, 1977).

#### 1.1.5.1.2 Cell-mediated immunity

##### 1.1.5.1.2.1 Role of CD8<sup>+</sup> T cells

A number of studies using the rodent parasites *P. yoelii* and *P. berghei* have demonstrated that CD8<sup>+</sup> T cells are critical for protective immunity against *Plasmodium* liver stages. Early studies established that the *in vivo* depletion of CD8<sup>+</sup> T cells reduces the immunity induced by immunisation with irradiated sporozoites (Schofield *et al.*, 1987; Weiss *et al.*, 1988). Furthermore, it was demonstrated that immunisation with irradiated sporozoites induced CD8<sup>+</sup> T cells specific for defined epitopes located in the CSP of *P. berghei* and *P. yoelii*. The identification of these epitopes allowed the generation of epitope-specific CD8<sup>+</sup> T cell-clones that were used to study the *in vivo* anti-parasite activity of these T cells (Rodrigues *et al.*, 1991; Romero *et al.*, 1989; Weiss *et al.*, 1992). Transfer of these CD8<sup>+</sup> T cell clones into mice subsequently challenged with viable sporozoites, inhibited the development of liver stages and thus prevented infection of red blood cells (Rodrigues *et al.*, 1991). This protective activity was shown to be stage-specific as transfer of the T cells did not protect mice from a challenge with infected erythrocytes.

The molecular mechanisms by which CD8<sup>+</sup> T cells inhibit the development of *Plasmodium* liver stages of malaria remain to be elucidated. It is known that mice lacking perforin or Fas molecules can still develop protective immunity against liver stages (Doolan and Hoffman, 2000; Renggli *et al.*, 1997). Furthermore, it has been demonstrated that memory CD8<sup>+</sup> T cells lacking perforin and Fas, can still efficiently inhibit the development of *P. yoelii* liver stages (Morrot and Zavala, 2004). These findings raised the possibility that CD8<sup>+</sup> T cell-derived cytokines such as IFN- $\gamma$  may act as effector molecules thus destroying intracellular parasites. In fact, early studies have shown that injection of IFN- $\gamma$  in mice and rats infected with *P. berghei* sporozoites a few

hours before sporozoite challenge, inhibited the development of the exoerythrocytic forms in the liver cells of the hosts (Ferreira *et al.*, 1986). Instead, administration of neutralising mAbs against IFN- $\gamma$  to immune hosts reversed sterile immunity to sporozoite challenge, by allowing the growth of exoerythrocytic forms and thus the development of parasitaemia (Schofield *et al.*, 1987). Furthermore, a potential role for nitric oxide (NO) as part of the downstream effector mechanisms associated with protection conferred by attenuated sporozoites has also been proposed. Irradiated *P. berghei* sporozoite immunised mice subsequently challenged with viable sporozoites developed parasitaemia when treated orally with substrate inhibitors of nitric oxide synthase (NOS), suggesting that the production of NO prevents the development of exoerythrocytic stages of malaria in liver (Seguin *et al.*, 1994).

Studies in humans living in malaria-endemic areas indicate that CD8<sup>+</sup> T cell responses to liver stages are rather restricted because they are generally found at very low levels, even in individuals living in regions with intense malaria transmission (Lalvani *et al.*, 1996; Plebanski *et al.*, 1997). Limiting dilution analysis of CTL precursor frequencies was performed on peripheral blood of previously identified West African responders to a wide range of epitopes derived from different liver stage antigens (LSA-1, CSP, TRAP and STRAP) (Plebanski *et al.*, 1997). The results indicated that in this population there was normally a low precursor frequency of specific memory CTL against any single malarial epitope. By comparison, a secondary response to an influenza epitope in the same population was 3-fold higher (Plebanski *et al.*, 1997). Interestingly, low CTL responses were found even in areas where malaria transmission is approximately 300-fold higher (Lalvani *et al.*, 1996).

The situation found in human populations is not entirely different from that observed in experimental rodent models. Immunisation of mice with irradiated *P. yoelii* sporozoites induces rather low CD8<sup>+</sup> T cell responses, barely detectable by highly sensitive *ex vivo* assays (Carvalho *et al.*, 2001). The generation of transgenic (Tg) mice expressing a T cell receptor (TCR) specific for the CD8<sup>+</sup> T-cell epitope located in the *P. yoelii* CSP has proven to be a valuable tool to study basic features of this T-cell response such as the

kinetics of differentiation and proliferation as it occurs *in vivo*.

It was shown that in contrast to memory cells, which can eliminate infected cells immediately after antigen recognition naïve CD8<sup>+</sup> T cells need to differentiate before achieving effector status (Sano *et al.*, 2001). Nevertheless the time required for this process was shown to be very short. In fact sporozoites induced a rapid activation of naïve CD8<sup>+</sup> T cells, which in only 24h displayed clear signs of differentiation and produced IFN- $\gamma$  and perforin (Sano *et al.*, 2001). The maximum proliferation and cytotoxic activity of specific CD8<sup>+</sup> T cells was instead observed 48h after immunisation (Sano *et al.*, 2001). Furthermore, the CD8<sup>+</sup> T cell anti-parasitic activity was shown to depend on the number of naïve cell precursors transferred into mice before immunisation (Sano *et al.*, 2001). Therefore the limited efficacy of primary CD8<sup>+</sup> T cell responses observed in humans and normal mice did not appear to be due to the time it takes for naïve cells to achieve an effector status as this was attained after just 24h post immunisation. Instead it was the low frequency of antigen-specific precursors present before immunisation that seemed to limit the protective activity of primary CD8<sup>+</sup> T cell responses. Interestingly it was demonstrated that the proper development of this CD8<sup>+</sup> T cell response was strictly dependent on CD4<sup>+</sup> T cells (Carvalho *et al.*, 2002). BALB/c mice were depleted of CD4<sup>+</sup> T cells by using a mAb specific for CD4 and were immunised two days later with irradiated *P. yoelii* sporozoites. Epitope-specific CD8<sup>+</sup> T cell responses were measured at different time points using an ELISPOT assay (Carvalho *et al.*, 2001) to detect antigen-specific IFN- $\gamma$  secretion in the spleen. Parasite-induced CD8<sup>+</sup> T cell responses were detectable in CD4-depleted and control mice with no major differences in the responses in both groups. As this T cell response evolved, however, it became evident that the absence of CD4<sup>+</sup> T cells greatly reduced the magnitude of the CD8<sup>+</sup> T cell response (Carvalho *et al.*, 2002). The CD8<sup>+</sup> T cell response was in fact reduced by 96% in CD4-depleted mice as compared to that in normal mice. Thus, CD4<sup>+</sup> T cells are important in maintaining or enhancing arising CD8<sup>+</sup> T cell responses. It was found that IL-4 secreted by CD4<sup>+</sup> T cells was essential for the full development of the CD8<sup>+</sup> T cell response although it is still not clear whether this cytokine acts directly on CD8<sup>+</sup> T cells or through other cell types (Carvalho *et al.*, 2002).

The findings described above are similar to those obtained by Bourgeois *et al.* who re-assessed the role of CD4<sup>+</sup> T cells on CD8<sup>+</sup> T cell responses and demonstrated that CD4<sup>+</sup> T cells are absolutely essential for the generation of CD8 memory cells (Bourgeois and Tanchot, 2003). Bourgeois and colleagues investigated the role of CD4<sup>+</sup> T cells in the response of CD8<sup>+</sup> Tg T cells to the male antigen HY (Bourgeois and Tanchot, 2003). It was shown that the absence of CD4<sup>+</sup> T cell help had no impact on the early events of the primary immune response. Naïve CD8<sup>+</sup> Tg T cells stimulated *in vivo* by HY were able to proliferate and eliminate the antigen in the absence of CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells, however, played a role in the expansion of CD8<sup>+</sup> T cells, since the recovery of CD8<sup>+</sup> Tg T cells at the end of the primary response was 3-4-fold less in the absence of CD4<sup>+</sup> T cells. Moreover, CD8<sup>+</sup> T cells primed in the absence of CD4<sup>+</sup> cells did not acquire the properties of memory cells. They proliferated poorly both *in vitro* and *in vivo* in secondary immune responses, and their cytokine secretion upon *in vitro* re-stimulation with the male antigen was very low and similar to that of naïve cells (Bourgeois and Tanchot, 2003).

Although it is clear that the magnitude of the primary CD8<sup>+</sup> T cell response closely correlates with the number of parasites used for immunisation (Sano *et al.*, 2001), increasing the time of antigen presentation by daily immunisations did not enhance the magnitude of this response (Hafalla *et al.*, 2002). These results suggest that naïve CD8<sup>+</sup> T cells become refractory to further antigenic stimulation. Thus while recently activated T cells continue their differentiation and proliferation process, naïve T cells are somehow prevented from being activated despite additional antigen supply.

The inability of CD8<sup>+</sup> T cells to expand further despite an increased antigen exposure during the development of the primary response is reminiscent of what is observed in endemic areas where very low frequencies of T cells against liver-stage antigens are observed despite continued sporozoite inoculations through the bites of malaria-infected mosquitoes (Lalvani *et al.*, 1996; Plebanski *et al.*, 1997). Indeed it was also demonstrated in mice that repeated bites of infected mosquitoes failed to expand the number of activated cells once a primary CD8<sup>+</sup> T cell response was established (Hafalla *et al.*,

2002). Transfer of primed spleen cells from infected mice into normal mice that subsequently received naïve specific-CD8<sup>+</sup> T cells and were then immunised with parasites strongly inhibited the activation of the naïve T cells (Hafalla *et al.*, 2003). These results indicate that primed/activated antigen specific T cells themselves contributed to or were directly responsible for preventing activation of naïve CD8<sup>+</sup> T cells in immunised hosts (Hafalla *et al.*, 2003).

To identify the inhibitory T cell population, spleen cells from parasite-immunised animals were systematically depleted of different T cell populations before transfer into normal mice. Depletion of NK cells,  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells did not affect the inhibitory capacity of the primed spleen cells (Hafalla *et al.*, 2003). However, depletion of CD8<sup>+</sup> T cells allowed the naïve CD8<sup>+</sup> specific T cells to proliferate normally. To confirm this observation CD8<sup>+</sup> T cells purified from spleens of parasite-immunised mice were shown to inhibit the activation of naïve CD8<sup>+</sup> T cells as efficiently as the whole primed spleen (Hafalla *et al.*, 2003). Furthermore the expansion of naïve CD8<sup>+</sup> T cells was shown to decrease drastically as the number of transferred activated CD8<sup>+</sup> T cells increased (Hafalla *et al.*, 2003).

Interestingly, the transfer of large numbers of CD8<sup>+</sup> T cell-specific peptide-loaded DCs into the animals overcame the inhibitory effect exerted by activated cells on naïve CD8<sup>+</sup> T cells (Hafalla *et al.*, 2003). These studies indicated that DCs play a key role in mediating the self-regulatory mechanism among activated and naïve CD8<sup>+</sup> T cells. They also strongly suggested that the limited activation and proliferation of naïve cells within an immunogenic environment were not due to a functional disability that was induced upon these T cells, i.e. anergy; rather, they appeared to be caused by limited access of naïve CD8<sup>+</sup> T cells to antigen presenting DCs (Hafalla *et al.*, 2003).

Collectively these results suggest that immediately after the initial clonal burst, and together with the rapid development of CD8<sup>+</sup> T cell populations expressing effector functions, inducible regulatory mechanisms are switched on to prevent the activation of naïve CD8<sup>+</sup> T cells after exposure to antigen. While these regulatory mechanisms may

limit the magnitude of the anti-malaria primary response, they may also play a key physiological role by preventing an overwhelming T cell activation that may be deleterious to the tissues and organs infected by the parasite. However, while these studies establish that competition between naïve and activated CD8<sup>+</sup> T cells at the level of APCs is an early regulatory mechanism, it is likely that additional regulatory mechanisms are subsequently developed as the immune response evolves. In fact as mentioned earlier, CD4<sup>+</sup> T cells have been shown to be important in sustaining the development of CD8<sup>+</sup> T cell responses after clonal expansion (Carvalho *et al.*, 2002).

#### 1.1.5.1.2.2 Role of CD4<sup>+</sup> T cells

CD4<sup>+</sup> T cells are known to play an important role in the induction of protective anti-sporozoite antibodies. In fact, early studies using *P. yoelii* infection in mice have demonstrated the importance of CD4<sup>+</sup> T cells in the induction of humoral responses (Weiss *et al.*, 1993). Mice treated with anti-CD4 mAbs prior to sporozoite immunisation produced very little anti-sporozoite Abs (Weiss *et al.*, 1993). This observation suggested that CD4<sup>+</sup> T cells played a crucial role in protection against sporozoites by helping B cells to induce anti-malaria humoral responses. However passive transfer of hyperimmune serum containing high titres of anti-Abs revealed that CD4-depleted mice were still not protected against small numbers of sporozoites (Weiss *et al.*, 1993). These observations indicated that the defect in immunity observed in anti-CD4 treated animals was not solely due to the lack of anti-sporozoite Abs and suggested that CD4<sup>+</sup> T cells can exert an inhibitory effect upon the development of intrahepatocytic parasites. One of the first studies demonstrating a direct inhibitory effect of CD4<sup>+</sup> T cells against liver stages of malaria parasites described a CD4<sup>+</sup> T cell clone obtained from a mouse immunised with irradiated *P. berghei* sporozoite that recognised an unidentified antigen shared by sporozoites and blood-stages and differed from the CSP (Tsuji *et al.*, 1990). Passive transfer of these T cell clones into naïve mice protected most of these animals against sporozoite challenge. Furthermore, these clones were shown to display cytotoxic activity upon stimulation with antigen, as they lysed antigen-pulsed B cells (A20.2J cell line) *in vitro*. In addition, they produced IFN- $\gamma$  and IL-2 *in vitro*, and recognised the plasmodial

antigen in the context of class II MHC (Tsuji *et al.*, 1990). Other studies using CD4<sup>+</sup> T cell clones specific for defined epitopes of the *P. yoelii* CSP also confirmed the ability of CD4<sup>+</sup> T cells to inhibit the development of malaria liver stages directly both *in vitro* and *in vivo* (Renia *et al.*, 1993; Takita-Sonoda *et al.*, 1996). It was shown that some of these clones eliminated liver stage parasites from cultured hepatocytes in an MHC restricted manner (Renia *et al.*, 1993). Although MHC class II molecules are not normally expressed at the surface of hepatocytes, the expression of these molecules was shown to be induced by IFN- $\gamma$  during viral infections (Franco *et al.*, 1988). Therefore, it has been proposed that activated, parasite antigen-specific T cells could produce cytokines that induce class II expression in neighbouring infected cells (Renia *et al.*, 1993). Therefore, CD4<sup>+</sup> T cells, as well as assisting and sustaining the proper development of CD8<sup>+</sup> T cell responses, they help B cells to induce a high level of anti-malaria humoral response and directly inhibit the development of liver stage parasites.

#### 1.1.5.1.2.3 Role of $\gamma\delta$ T cells

The role of  $\gamma\delta$  T cells in protective immunity against the liver stages was studied in mice deficient of  $\alpha\beta$  T cells (Tsuji *et al.*, 1994).  $\alpha\beta$  T cell-deficient mice were subjected daily to the bites of irradiated malaria-infected mosquitoes for a period of two weeks. Mice exposed to the bites of irradiated non-infected mosquitoes were used as controls. Five days after the last exposure to mosquitoes,  $\alpha\beta$  T cell-deficient mice and their respective heterozygous counterparts, which develop normal  $\alpha\beta$  T cells, were challenged intravenously with viable sporozoites. As expected, parasite development in the liver of sporozoite-immunised heterozygous mice was strongly inhibited. Most importantly,  $\alpha\beta$  T cell-deficient mice also mounted an immune response that significantly inhibited the development of intrahepatic parasites (assessed by measuring plasmodial rRNA), suggesting that  $\gamma\delta$  T cells might be responsible for the observed decrease in liver-stage parasites (Tsuji *et al.*, 1994). To define further the possible involvement of  $\gamma\delta$  T cells in protective immunity against liver stages, sporozoite-immunised  $\alpha\beta$  T cell-deficient mice were transiently depleted of  $\gamma\delta$  T cells by injection of a mAb specific for all TCR  $\gamma$  chains (Tsuji *et al.*, 1994). The injection of this anti-TCR  $\gamma$  antibody into sporozoite-immunised

$\alpha\beta$  T cell-deficient mice abrogated the inhibition of liver stages induced by their immunisation (Tsuji *et al.*, 1994). These findings suggest that  $\gamma\delta$  T cells play an important role in the anti-parasitic activity observed in sporozoite-immunised  $\alpha\beta$  T cell-deficient animals. Furthermore, when  $\gamma\delta$ T cell clones, derived from spleen cells of sporozoite-immunised  $\alpha\beta$  T cell-deficient mice, were transferred into normal mice, that were later inoculated with viable sporozoites, parasite development was shown to be significantly inhibited, confirming the protective role of  $\gamma\delta$  T cells against the liver stages of malaria parasites (Tsuji *et al.*, 1994). Further studies in  $\gamma\delta$  T cell-deficient mice immunised with irradiated sporozoites, revealed that these animals had increased liver parasite burden when challenged with *P. yoelii* sporozoites compared to similarly challenged immunocompetent mice, suggesting that  $\gamma\delta$  T cells are a component of early immunity directed against malaria pre-erythrocytic parasites (McKenna *et al.*, 2000).

### 1.1.6.2 Immunity to erythrocytic stages

#### 1.1.6.2.1 Antibody-mediated Immunity

Early studies showed that passive immunisation with purified immunoglobulin obtained from adults living in malaria endemic areas transferred clinical protection against *P. falciparum* (Cohen *et al.*, 1961; Sabchareon *et al.*, 1991). Similarly, passive transfer of mAbs against parasite antigens was shown to confer protection in naive mice (Narum *et al.*, 2000; Spencer Valero *et al.*, 1998). Various studies using animal models further confirmed the involvement of B cells in parasite clearance. Mice rendered deficient of B cells due to a targeted mutation of the transmembrane exon of the IgM m-chain (m-MT (Kitamura *et al.*, 1991)), were unable to resolve a primary infection with the erythrocytic stages of *P. chabaudi* AS (von der Weid *et al.*, 1996). m-MT mice could reduce the initial acute infection to relatively low levels of parasitaemia with kinetics similar to wild type mice. Mutated animals however developed chronic unresolved infections characterised by relapsing peaks of parasitaemia (von der Weid *et al.*, 1996). This indicated that B cell-independent mechanisms of immunity were sufficient to reduce the acute parasitaemia of a primary *P. chabaudi* AS infection, but were not able to resolve the infection (von der

Weid *et al.*, 1996). B cell transfers into these chronically infected mice demonstrated that B cells were potent effectors of parasite clearance (von der Weid *et al.*, 1996). Studies in B cell deficient mice also indicated that B cells played an essential role in switching from Th1 to Th2 responses, which is critical for the complete resolution of *P. chabaudi* infection (Langhorne *et al.*, 1998; Taylor-Robinson and Phillips, 1994).

It has been demonstrated that naturally acquired immunity to malaria in individuals living in endemic areas depends on the acquisition of a repertoire of specific, protective Abs directed against the polymorphic target antigen, *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1)(Bull *et al.*, 1998). In 1986, Marsh and Howard (Marsh and Howard, 1986) showed that sera taken from children after an infection were able to agglutinate *in vitro* RBCs infected with the same strain of parasite that the child was exposed to but not strains to which other children were exposed. Sera taken from immune adults, by contrast, were able to agglutinate most strains circulating in the community. It was subsequently shown that each agglutinate formed with sera from immune adults contained parasite of the same strain, suggesting that the immune sera does not recognise a common epitope found on all infected RBCs, but epitopes that are strain specific (Newbold *et al.*, 1992). These strain-specific antigens were principally the 'variant' antigens that are termed PfEMP-1, which are expressed on the surface of trophozoite-infected RBCs (Leech *et al.*, 1984) and encoded by approximately 60 genes of the 'var' multigene family (Smith *et al.*, 1995; Su *et al.*, 1995). PfEMP-1 has been shown to mediate cytoadherence of infected RBCs to endothelial receptors such as CD36, and ICAM-1 (Baruch *et al.*, 1996), a process that is believed to be involved in the pathogenesis of cerebral malaria and placental malaria. Abs to PfEMP-1 were reported to prevent the cytoadherence property of infected cells (Smith *et al.*, 2000). This may therefore avoid the development of cerebral malaria (Smith *et al.*, 2000) and presumably allow infected cells to be removed by the spleen (David *et al.*, 1983). Similarly, it was reported that Abs disrupt spontaneous binding of uninfected RBCs to *Plasmodium*-infected RBCs (rosetting) (Carlson *et al.*, 1990). This *in vitro* phenomenon is thought to be associated with cerebral malaria although its occurrence *in vivo* has not been demonstrated yet.

Typically, only a single PfEMP-1 antigen is expressed at any one time and the ability of the parasite to switch expression from one variant to another is believed to be primarily responsible for the ability of the parasite to evade immune responses, a strategy now known as antigenic variation (Biggs *et al.*, 1991). Thus immunity to the parasite develops once an individual acquires Abs against multiple PfEMP-1 variants, which might explain why natural immunity takes several years to develop. This idea is substantiated by the fact that the levels of variant-specific Abs increase in an age dependent manner and correlate with a decline in both the prevalence and density of parasitaemia (Piper *et al.*, 1999).

Therefore the degree of protective immunity in humans correlates with the level of Abs against asexual blood-stage antigens, and has been shown to depend on Ab isotypes (Astagneau *et al.*, 1995; Chizzolini *et al.*, 1988; Piper *et al.*, 1999). Various studies have reported that Abs, which were protective *in vivo* by passive transfer of immune immunoglobulin in humans had no effect *in vitro* on the growth of *P. falciparum* asexual blood-stages, unless they were allowed to cooperate with blood monocytes by binding to Fc receptors (Bouharoun-Tayoun *et al.*, 1990; Khusmith and Druilhe, 1983). It was also shown that Abs, which were not protective *in vivo* were unable to exert an anti-malaria effect *in vitro* by cooperation with monocytes (Bouharoun-Tayoun *et al.*, 1990; Khusmith and Druilhe, 1983). For these reasons, the subclasses of Abs produced in response to infection are of particular importance, since certain isotypes, being non cytophilic (i.e. they do not have an affinity to cells), are unable to cooperate with cells in parasite-killing effector responses such as opsonization and antibody-dependent cellular inhibition (Bouharoun-Tayoun *et al.*, 1990).

Resistance to *P. falciparum* infection in humans was reported to be mainly associated with the ability to produce IgG1 and IgG3, two cytophilic Abs, and to reduce the proportion of the non-cytophilic isotypes IgG2, IgM and IgG4 of the same specificity (Bouharoun-Tayoun and Druilhe, 1992). The non-cytophilic Abs were suggested to block the effector mechanism induced by cytophilic isotypes because of their higher

concentration in serum and their greater affinity to the target antigen, thus providing a means of escape induced by the parasite (Bouharoun-Tayoun and Druilhe, 1992). Furthermore, the IgG2 isotype appeared to be the main blocking Ab in some adult patients, whereas, in young children, who are unable to produce high levels of IgG2, the parasite has been shown to induce the synthesis of IgM preferentially (Bouharoun-Tayoun and Druilhe, 1992). Interestingly, IgG2 has also been reported to be associated with low risk of infection and protection against *P. falciparum* (Aucan *et al.*, 2000). A polymorphism of the gene encoding FcγRIIa has been found that explains such conflicting results.

FcγRIIa (CD32) is expressed on the surface of lymphocytes and monocyte/macrophages, and provides an important link between humoral and cellular immune systems (Nimmerjahn and Ravetch, 2006). It is a low affinity IgG receptor capable of binding immunoglobulin subtypes IgG<sub>1-4</sub>. However the recognition of IgG by CD32 is influenced by a polymorphism within the gene (Cooke *et al.*, 2003). A single nucleotide change within the CD32 molecule alters a histidine (H) to an arginine (R) residue at position 131 and changes its function *in vitro*. The presence of the H131 allele is essential for the efficient binding of IgG2 subtypes (Warmerdam *et al.*, 1991). In its absence, those with the R131 genotype have preferential affinity for IgG3 and IgG1 subtypes. Therefore the functional activity of IgG subtypes is likely to depend, at least in part, on the CD32 genotype of the host.

In mice, the isotype IgG2a is associated with protection against *Plasmodium* infection (Matsumoto *et al.*, 2000; Smith *et al.*, 1997; Su and Stevenson, 2000). IgG2a is predominant during the primary ascending parasitaemia in mice infected with *P. chabaudi* AS followed by an IgG1 response during the chronic stage of infection, as a consequence of Th1 to Th2 switching (Taylor-Robinson and Phillips, 1994). It has been shown that IgG1 and IgG2b can confer protection against lethal challenge infection with *P. yoelii* in mice immunised with MSP-1 (Hirunpetcharat *et al.*, 1997). IgG3 may also be important as passive transfer of anti-MSP-1 IgG3 into naïve recipient resolves *P. yoelii* infection (Majarian *et al.*, 1984).

Ab responses directed against surface proteins of the merozoite may function either by blocking RBC invasion or by making the merozoite susceptible to phagocytosis. MSP-1 is the best studied of the merozoite antigens (Holder *et al.*, 1999). Following release from infected RBCs, the membrane-bound component of MSP-1 is subjected to a single proteolytic cleavage known as 'secondary processing' (Blackman *et al.*, 1991a). The cleavage product is shed prior to merozoite invasion (Blackman *et al.*, 1991b) and only the small carboxyl-terminal 19 KDa domain MSP-1<sub>19</sub>, is carried into the newly infected RBC (Blackman *et al.*, 1990). It was shown that Abs inhibited merozoite invasion by both binding to MSP-1<sub>19</sub> and blocking secondary processing of the MSP-1 protein (Blackman *et al.*, 1990; Blackman *et al.*, 1994). Similarly, Abs to the Duffy binding protein, which is necessary for *P. vivax* merozoite invasion of RBC (Michon *et al.*, 2000), may inhibit the establishment of *P. vivax* infections. Furthermore, it has been reported that Abs use other mechanism to block merozoite invasion of RBCs, such as causing complement-mediated damage and preventing merozoites release by causing agglutination of merozoites in rupturing schizonts (Ramasamy *et al.*, 2001).

Despite the importance of Ab responses in malaria infection, it seems that not all Abs are protective. Polyclonal Abs specific for MSP-2 but not mAbs specific to the same antigen have been shown to enhance invasion of multiple merozoites into RBC (Ramasamy *et al.*, 2001; Ramasamy *et al.*, 1999). Furthermore, high-titre Abs against MSP-2 fails to induce complement-mediated damage (Ramasamy *et al.*, 1999). In this regard, it has been suggested that merozoites might have evolved a mechanism to limit complement-mediated damage by adopting strategies such as rapid shedding of the surface proteins (Ramasamy *et al.*, 2001). To explain the increase in multiple invasion instead, it has been proposed that merozoites crosslinked by anti-MSA-2 Abs can recognize RBC ligands, reorientate one or more of their apical surfaces to contact the RBC membrane and successfully complete invasion together (Ramasamy *et al.*, 1999). Alternatively, Ab-cross-linked merozoites attracted to the same RBC, might dissociate and invade the RBC independently (Ramasamy *et al.*, 1999). The formation of ring-stages lying within discrete parasitophorous vacuoles favours the latter model (Ramasamy *et al.*, 2001).

Moreover it has been reported that Abs, which inhibit MSP-1 processing and erythrocyte invasion can be blocked by naturally acquired antibodies (Guevara Patino *et al.*, 1997). All of the above *in vitro* observations illustrate the importance of identifying epitopes that induce protective Abs when designing a vaccine against malaria.

#### 1.1.6.2.2 Cell-mediated Immunity

The role of cell-mediated immune responses directed against the erythrocytic stages of malaria has been extensively studied in many experimental animal models (Taylor-Robinson, 1995).

##### 1.1.6.2.2.1 Role of CD4<sup>+</sup> T cells

Both Th1 and Th2 cells have been shown to be involved in protective immunity against blood-stage malaria. *P. berghei* and *P. yoelii* were reported to induce a rapid and strong Th2 immune response whereas *P. chabaudi adami* a strong Th1 response (Taylor-Robinson, 1995). These findings were substantiated by the fact that severe combined immunodeficient (SCID) mice reconstituted with enriched immune T cells suppressed acute *P. chabaudi adami* parasitaemia, suggesting that T, but not B, cells are required to clear this strain of malaria (van der Heyde *et al.*, 1994). In addition, B cell deficient mice were also shown to resolve *P. chabaudi adami*, *P. vinckei petteri* and *P. chabaudi chabaudi* malaria at the same rate as normal mice (van der Heyde *et al.*, 1994). Collectively, these observations establish that T cells alone can mediate the clearance of acute malaria caused by these subspecies of *Plasmodium*. Moreover, the protective immune response was shown to depend upon CD4<sup>+</sup> T cells, as B cell deficient mice treated with anti-CD4 mAb did not resolve their infections (van der Heyde *et al.*, 1994).

Nevertheless, several studies in *P. chabaudi* AS have shown that protective immunity to blood-stage malaria requires both CD4<sup>+</sup> T cells and Abs, and sequential activation of Th1 and Th2 cells is critical for protection (Langhorne *et al.*, 1998; Taylor-Robinson and Phillips, 1994). The importance of Th1 and Th2 cells in protection against *P. chabaudi*

AS infection has been investigated in resistant and susceptible mice (Stevenson and Tam, 1993). It is now well established that in resistant mice, the acute phase of *P. chabaudi* AS infection is predominantly characterised by the production of IFN- $\gamma$  during the first week of infection, which declines as the parasitaemia decreases, and is replaced by IL-4 and IL-10 production during the later stages of infection (Taylor-Robinson and Phillips, 1994). In contrast, mouse strains for which *P. chabaudi* infection is fatal, produced high levels of IL-5 within the first week of infection (Stevenson and Tam, 1993). These observations indicate that resolution of primary blood-stage *P. chabaudi* infection occurs by sequential activation of Th1 CD4<sup>+</sup> T cells followed by activation of the Th2 subset, and that induction of a strong Th2 response early in infection may lead to a severe and lethal course of malaria. Furthermore, the size of the infective dose, thus the level of antigen presented to the immune system, was reported to affect significantly the nature of the prevailing immune response to *P. chabaudi* infection (Taylor-Robinson and Phillips, 1998). In resistant mice, production of IFN- $\gamma$  was directly proportional to the size of the inoculum whereas IL-4 production correlated indirectly with the size of the infective dose (Taylor-Robinson and Phillips, 1998). In contrast, increasing the infective dose in a susceptible mouse strain led to a prominent and accelerated up-regulation of IL-4 production (Taylor-Robinson and Phillips, 1998).

It has been speculated that the initial Th1 type response observed during *P. chabaudi* infection could be due to the high concentration of antigens that the host is exposed to following schizont rupture due to the synchronous nature of the asexual erythrocytic cycle (Taylor-Robinson and Phillips, 1998). Conversely, Th2 type responses were suggested to be due to the continuous and increasing stimulation with antigen which would occur in asynchronous infections such as in *P. berghei* and *P. yoelii* infections. Indeed there is strong evidence indicating that antigen dose affects Th1/Th2 responses (Constant and Bottomly, 1997). However, there are no clear-cut conclusions regarding whether 'high' or 'low' doses of antigen are best suited to induce each type of immune response. Interestingly, most of the studies in which low doses of antigen induced Th1-like responses used parasites as immunogens, whereas low doses of soluble proteins tended to skew toward Th2-type cells (Constant and Bottomly, 1997). Therefore, it may

be that the antigen itself can influence the type of response initiated. However, whether the type of the immune response elicited during malaria and the synchronicity of bloodstream infection are linked requires further studies. Nevertheless, collectively these results show that CD4<sup>+</sup> T cells are crucial for resistance to malaria. Both Th1 and Th2 cells contribute to protective immunity at different times of infection and the balance between these two subsets determines the outcome of the disease.

The role of CD4<sup>+</sup> T cells in immunity to malaria in humans is less well understood. Individuals lacking previous exposure to *P. falciparum*, as well as malaria-exposed individuals, have T cells that proliferate and secrete IFN- $\gamma$  in response to parasite antigen and inhibit parasite growth *in vitro* (Fell *et al.*, 1994). The degree of proliferation of peripheral blood mononuclear cells (PBMCs) isolated from malaria-exposed individuals and stimulated *in vitro* with parasite antigens, correlated with the number of previous malaria episodes (Carvalho *et al.*, 1999). In children living in highly endemic areas, protection against *P. falciparum* correlates with the level of antigen-specific proliferative responses (Migot-Nabias *et al.*, 1999). However, acute *P. falciparum* malaria is associated with loss of *in vitro* T cell responsiveness to antigenic stimulation as well as reduction in the number of T cells in the peripheral circulation (Elhassan *et al.*, 1994; Hviid *et al.*, 1991). This cellular hyporesponsiveness has been associated with the sequestration of T cells expressing the adhesion molecule leukocyte function-associated molecule-1 (LFA-1) on their surface (Elhassan *et al.*, 1994; Hviid *et al.*, 1991). Particularly, depressed frequencies of CD4<sup>+</sup> T cells were observed in patients with cerebral malaria (Hviid *et al.*, 1997). After the clearance of parasitaemia, previously sequestered cells re-emerge into the periphery, with subsequent restoration of immune responsiveness, thus explaining why acute *P. falciparum* malaria is associated with a transient inability of peripheral blood cells to respond to antigenic stimulation *in vitro* (Elhassan *et al.*, 1994). A shift from a Th2 response to a more pronounced Th1 response has been suggested to resolve *P. falciparum* infection (Winkler *et al.*, 1998). Nevertheless, the precise role of effector CD4<sup>+</sup> T cells in protection of humans against malaria needs further investigation.

#### 1.1.6.2.2.2 Role of $\gamma\delta$ T cells

$\gamma\delta$  T cells are found in increased numbers in the spleens of humans and mice infected with malaria (Nakazawa *et al.*, 1994), but their role in immunity to blood-stage infection remains unclear. It has been shown that depletion of  $\gamma\delta$  T cells does not alter parasitaemia, anaemia or survival rates of mice infected with the avirulent *P.c. adami* or virulent *P. c. chabaudi* CB, whereas mice depleted of  $\alpha\beta$  T cells had higher levels of parasitaemia, lower RBC counts, and decreased survival rates (Sayles and Rakhmievich, 1996). These results indicate that  $\alpha\beta$  but not  $\gamma\delta$  T cells play an essential role in host defence against *P. chabaudi* infection in mice (Sayles and Rakhmievich, 1996).

In contrast, other studies emphasise the importance of this population of T cells in the resolution of malaria. Mice lacking  $\gamma\delta$  T cells were shown to develop chronic parasitaemia following *P. c. chabaudi* AS infection (Langhorne *et al.*, 1995; Seixas and Langhorne, 1999). Proliferation of  $\gamma\delta$  T cells was also associated with parasite clearance from the blood in *P. c. adami* infected mice (Nakazawa *et al.*, 1994). As the parasitaemia decreased, the number of  $\gamma\delta$  T cells was shown to decline towards basal levels as observed in immunohistochemical preparations of spleen sections (Nakazawa *et al.*, 1994). The expansion of  $\gamma\delta$  T cells was suggested to require the presence of  $\alpha\beta$  T cells and was supported by the observation that the expansion of  $\gamma\delta$  T cells did not occur in anti-CD4 treated mice and by the finding of large numbers of  $\gamma\delta$  T cells in the spleens of infected SCID mice which received CD4-enriched fractions containing few  $\gamma\delta$  T cells (van der Heyde *et al.*, 1993b). Furthermore, the fact that the  $\alpha\beta$  T cell population was found to be maximally activated to become blast cells during the period of ascending parasitaemia, whereas the maximal  $\gamma\delta$  T cell blast response was found during the period of descending parasitaemia, suggests that these cells may function in the resolution of blood-stage malaria (van der Heyde *et al.*, 1993b). The detection of high levels of  $\gamma\delta$  T cells only in models that resolve their infection further reinforces this possibility (van der Heyde *et al.*, 1993b).

Human  $\gamma\delta$  T cells isolated from peripheral blood of malaria non-immune individuals can inhibit growth of the late or extracellular stages of *P. falciparum in vitro* (Troye-Blomberg *et al.*, 1999). The inhibitory activity correlates with the number of  $\gamma\delta$  T cells present in the culture, suggesting that  $\gamma\delta$  T cells contribute to the control of infection (Troye-Blomberg *et al.*, 1999). Thus a role of  $\gamma\delta$  T cells in providing protective immunity against malaria cannot be excluded and requires further investigation.

#### 1.1.6.2.2.3 Role of CD8<sup>+</sup> T cells

Regarding CD8<sup>+</sup> T cells, as described earlier, there is clear evidence for their contribution to immune effector mechanisms aimed at pre-erythrocytic stages of the malaria life cycle. However, in contrast to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells have been shown not to be essential for control of asexual erythrocytic malaria parasites.  $\beta$ 2-microglobulin ( $\beta$ 2m) deficient mice effectively lack major histocompatibility complex (MHC) class I molecules, thus they virtually lack CD8  $\alpha\beta$  T cells. When infected with *P. c. adami*, *P. c. chabaudi* or *P. yoelii* 17xNL,  $\beta$ 2m-deficient mice were as resistant to blood-stage infection as control animals (van der Heyde *et al.*, 1993a). Together with selective lymphocyte depletion experiments performed with *P. c. chabaudi* (Suss *et al.*, 1988), these data demonstrate that CD8<sup>+</sup> T cells are not required to suppress blood-stage malaria and that the suppression mechanism is not MHC class I-restricted.

#### 1.1.6.2.2.4 Cytokines involved in protection against blood-stage malaria

T cells play a central role in the elimination of blood-stage malaria parasites through the release of cytokines that activate other effector cells. Cytokines involved in immunity to blood-stage malaria include IL-12, IFN- $\gamma$  and TNF- $\alpha$ .

##### (a) IL-12

The major immunological role of IL-12, that is of relevance to protective immunity to

blood-stage malaria, is its ability to induce IFN- $\gamma$  production by both natural killer (NK) and CD4<sup>+</sup> T cells (Trinchieri, 2003). Systemic administration of IL-12 was shown to protect susceptible A/J mice against lethal *P. chabaudi* AS infection (Stevenson *et al.*, 2001). Moreover, this IL-12-induced protection occurred through IFN- $\gamma$ , TNF- $\alpha$ , and NO-dependent mechanisms, associated with Th1 cell-mediated immune responses (Sam and Stevenson, 1999). Administration of anti-IL-12 to mice infected with *P. berghei* led to increased parasitaemia and fatal outcome (Yoshimoto *et al.*, 1998b). Anti-IL-12 treatment significantly reduced the secretion and mRNA expression of IFN- $\gamma$ , showing that IL-12 is a potent inducer of IFN- $\gamma$  during malaria infection (Yoshimoto *et al.*, 1998b). In humans, the levels of IL-12 were found to be significantly elevated in patients who suffered less severe disease whereas they were found to be lower in patients who suffered more severely from the disease (Luty *et al.*, 2000; Malaguarnera *et al.*, 2002).

While many studies provide evidence for a protective role of IL-12 in immunity to blood-stage malaria, others have shown that IL-12 is also implicated in mediating pathology during malaria. Yoshimoto *et al.* demonstrated that IL-12p40 mRNA expression is upregulated in the spleen and liver during early infection in mice inoculated with lethal blood-stage *P. berghei* parasites (Yoshimoto *et al.*, 1998a). Infection with this parasite was also found to upregulate mRNA expression of IFN- $\gamma$ , IL-4, IL-10, TNF- $\alpha$  and iNOS in these organs. Infected mice were found to experience severe liver damage, reduced body weight and severe anaemia. Treatment of infected mice with mAb against IL-12 significantly decreased liver injury and prolonged survival, although all the animals eventually succumbed. Anti-IL-12 treatment also significantly reduced mRNA expression and secretion of IFN- $\gamma$  in the liver as well as the spleen (Yoshimoto *et al.*, 1998a). These results provide evidence that IL-12 is involved in the pathogenesis of liver injury *via* production of IFN- $\gamma$  during lethal *P. berghei* infection. Therefore, while IL-12 has protective effects against blood-stage malaria, this molecule could also be involved in malaria pathology due to its ability to induce excessive production of inflammatory mediators.

## **(b) IFN- $\gamma$**

IFN- $\gamma$  plays an important role in resistance to blood-stage malaria infection. *P. falciparum* erythrocytic stages were lethally susceptible to oxygen-dependent and oxygen-independent mediators released by IFN- $\gamma$ -activated, monocyte-derived human macrophages (Ockenhouse *et al.*, 1984). PBMCs from children with mild *P. falciparum* infection produce high levels of IFN- $\gamma$  when stimulated *in vitro* with merozoite antigens, and these children have a lower risk of re-infection (Luty *et al.*, 1999). In contrast, children with severe malaria show lower levels of IFN- $\gamma$  production by PBMCs and are more susceptible to re-infection (Luty *et al.*, 1999). In the mouse model, infection of IFN- $\gamma$  deficient mice with *P. chabaudi* AS results in increased morbidity and mortality indicating a role for this cytokine in protection (Su and Stevenson, 2000). Furthermore, mice defective in IFN- $\gamma$  and its receptor show a predominantly Th2 response, which is associated with susceptibility to *P. chabaudi* infection (Balmer *et al.*, 2000; Su and Stevenson, 2000). These results therefore provide evidence for a protective role for IFN- $\gamma$  in blood-stage malaria.

### (c) TNF- $\alpha$

TNF- $\alpha$  production is greatly augmented during malaria infection, as shown by elevated levels of TNF- $\alpha$  in plasma of patients with malaria (Luty *et al.*, 2000; Perkins *et al.*, 2000) and infected mice (Choudhury *et al.*, 2000; Stevenson *et al.*, 1995). Malaria parasites and their products, such as malaria pigment (haemozoin) (Pichyangkul *et al.*, 1994) and glycosylphosphatidyl-inositols (Schofield and Hackett, 1993) can directly induce the release of TNF- $\alpha$  by macrophages. Early studies reported that parasites incubated overnight with recombinant TNF- $\alpha$  (rTNF- $\alpha$ ) showed no loss of viability, but repeated injection of rTNF- $\alpha$  into infected mice reduced parasitaemia and significantly prolonged survival of mice infected with a lethal variant of *P. yoelii* parasite (Taverne *et al.*, 1987). These observations suggested that TNF- $\alpha$  acted on blood-stage malaria *in vivo* via host cells. This hypothesis was confirmed in later studies where rTNF- $\alpha$  was shown to cause an increase in the phagocytosis of *P. falciparum*-infected erythrocytes by human

monocytes (Muniz-Junqueira *et al.*, 2001). The phagocytosis-enhancing effect of TNF- $\alpha$  was observed exclusively when anti-*P.falciparum* antibody was present in the culture (Muniz-Junqueira *et al.*, 2001). Other studies using neutrophils as phagocytic cells, also observed an increase in phagocytosis induced by this cytokine (Kumaratilake *et al.*, 1991; Kumaratilake *et al.*, 1990), suggesting that TNF- $\alpha$  is an important component of the phagocytic effector mechanisms, which are involved in the destruction of the malarial parasite.

In addition to its protective role, TNF- $\alpha$  is also associated with pathology of malaria. The balance between the protective and pathologic roles of TNF- $\alpha$  is dependent on the amount, timing and location of TNF- $\alpha$  expression. In fact, resistant C57BL/6 mice had higher levels of TNF- $\alpha$  mRNA in the spleen and liver early during infection than did susceptible A/J mice, which succumb to the disease 10 days after initiation of infection. Administration of anti-TNF- $\alpha$  Ab to these resistant mice abrogated the immunity, indicating a protective role for TNF- $\alpha$ . (Jacobs *et al.*, 1996). Furthermore, resistant C57BL/6 mice also expressed high levels of IFN- $\gamma$  mRNA and low levels of IL-4 mRNA in the spleen, whereas susceptible A/J mice had low levels of IFN- $\gamma$  mRNA but high levels of TNF- $\alpha$  mRNA in the liver as well as having high levels of TNF- $\alpha$  protein in serum, later during infection just before death occurred (Jacobs *et al.*, 1996). These results demonstrate that a Th1-associated increase in TNF- $\alpha$  mRNA expression in the spleen early during infection correlates with resistance to *P. chabaudi* AS, whereas increased TNF- $\alpha$  mRNA levels in the liver and excessive levels of the TNF- $\alpha$  protein in serum later during infection correlate with susceptibility.

Injections of neutralizing mAb against recombinant murine IFN- $\gamma$  in *P. berghei* infected mice, markedly reduced serum levels of TNF- $\alpha$ , which led to reduced incidence of experimental cerebral malaria, suggesting that these cytokines contribute to pathology in *P. berghei* infection (Grau *et al.*, 1989a). Furthermore, experiments with blocking Abs revealed that production of TNF- $\alpha$  during *P. berghei* blood-stage malaria infection was responsible for weight loss and anaemia (Hirunpetcharat *et al.*, 1999). These findings

concurrent with *P. falciparum* infection in humans where analysis of post-mortem human tissues showed expression of TNF- $\alpha$  in brains of patients with cerebral malaria (Brown *et al.*, 1999). In addition, high levels of TNF- $\alpha$  in children with *P. falciparum* malaria correlated with hypoglycaemia and high mortality rates (Grau *et al.*, 1989b).

Collectively, the data suggest that infection control is cell mediated but that immune pathology from cell mediated mechanisms contributes to disease. To achieve desirable outcomes, the balance of mediators that are involved in the immune response to malaria must be tightly controlled. An understanding of the mechanisms by which cytokines induce protection and/or pathology in malaria will prove to be fundamental for designing vaccines and developing new therapies for malaria.

### **1.1.7 Malaria-induced immunosuppression**

Asymptomatic and symptomatic malaria patients often show reduced immune responses not only to malaria parasites but also to unrelated antigens, including vaccines (Williamson and Greenwood, 1978), suggesting that an active immune suppression mechanism may operate during the course of malaria. In humans, the depressed responsiveness to heterologous antigens was originally reported in 1962 by measuring the antibody responses of infected and uninfected children to vaccination with tetanus toxoid (McGregor, 1962). Standard doses of the same batch of tetanus toxoid failed to induce an anti-toxin response in significantly more Gambian children with malarial parasitaemia than in counterparts who had been kept free from malaria by continuous chemoprophylaxis (McGregor, 1962). Later studies confirmed this diminished antibody response to tetanus toxoid and also reported that children with acute malaria showed a significantly reduced antibody response to the O antigen of *Salmonella typhimurium* (Greenwood *et al.*, 1972). However, their antibody response to the H antigen of *S. typhimurium* and their cellular immune responses were normal (Greenwood *et al.*, 1972).

In an attempt to establish the duration of this immune defect, the immune response of children with acute malaria to vaccination with *S. typhimurium* and meningococcal

vaccines were studied at varying times after the onset of their illness (Williamson and Greenwood, 1978). Children with acute malaria were randomly allocated to one of three groups. Children in group I were immunised at the time of their presentation at hospital. Group II received the immunisations 7 days after presentation with acute malaria and group III were vaccinated 28 days after presentation. All children were treated immediately with chloroquine and weekly pyrimethamine was given to patients and controls to prevent any further attacks of malaria during the study period. A significant correlation was found between the level of parasitaemia and the degree of immunosuppression, children with the highest parasitaemia making almost no antibody response to vaccination. After treatment immune responsiveness to *S. typhimurium* vaccine rapidly returned. However, recovery of immune responsiveness to meningococcal vaccine was delayed. These results suggested that an attack of acute malaria, even when treated promptly, could have a prolonged suppressive effect on the humoral immune system (Williamson and Greenwood, 1978).

In addition to studies documenting impaired responses to heterologous antigens in children with acute *falciparum* malaria (Greenwood *et al.*, 1972; McGregor, 1962; Williamson and Greenwood, 1978), there is also evidence of suppression of immune responses to malaria-specific antigens (Brasseur *et al.*, 1983; Ho *et al.*, 1986; Troye-Blomberg *et al.*, 1984). Malarial antigens (MA) were prepared from a natural isolate of *P. falciparum* collected in eastern Thailand. PBMCs were collected from patients with acute malaria or from healthy controls and stimulated either with a mitogen or with MA or a control antigen prepared from cultured uninfected erythrocytes obtained from the same donor who was used for parasite cultures. Patients with acute *P. falciparum* malaria were found to have an antigen-specific T cell proliferative defect that persisted for more than four weeks following treatment (Ho *et al.*, 1986). The duration of this specific immunosuppression correlated with the initial parasite count and the severity of clinical illness and was more prolonged in patients with more serious disease (Ho *et al.*, 1986). The antigen-specific immunosuppression may explain the difficulty associated with the development of protective immunity to *P. falciparum* malaria. These results indicate that blood-stage malaria infections may suppress responses important for immunity to malaria

thereby allowing the parasite to survive. Furthermore, they suggest that patients infected with *P. falciparum* may not respond as well to a malaria vaccine as would uninfected individuals.

While malaria-induced immunosuppression may have evolved as a mechanism by which the parasite can avoid immune-mediated clearance, it leaves malaria-infected individuals more susceptible to secondary infections. *Salmonella* septicaemia was found to be more common and more severe during outbreaks of malaria (Mabey *et al.*, 1987) and it has been suggested that malaria-induced immunosuppression was one of the reasons for the persistence of tuberculosis in malaria-endemic regions (Enwere *et al.*, 1999). In addition, malaria infections have been implicated in the development of Burkitt's lymphoma, a tumour in which B lymphocytes, infected with Epstein-Barr virus (EBV), proliferate abnormally in immunosuppressed patients (Lam *et al.*, 1991; Moormann *et al.*, 2005; Whittle *et al.*, 1984). An early study reported that during an attack of *P. falciparum* malaria, T cell subpopulations are altered so that, *in vitro*, B lymphocytes infected with EBV proliferate abnormally to secrete large amounts of Abs (Whittle *et al.*, 1984). PBMCs were obtained from patients during and three weeks after an acute attack of *P. falciparum* malaria and were used in *in vitro* regression assays, which provide a measure of the strength of T cell control of EBV-infected B cells. All patients had antibodies to the viral capsid antigen (VCA) of EBV. It was found that regression indices were high during the attack, denoting loss of T cell control, but they fell on recovery, signifying a return to normal control (Whittle *et al.*, 1984). These observations concurred with a later study by Lam *et al.* that demonstrated that the numbers of EBV-infected B cells were higher in acute malaria patients than in convalescent malaria patients and in healthy control adults (Lam *et al.*, 1991). More recently, it was reported that elevated EBV loads were found in children 1-4 years of age living in a region in Kenya where malaria is endemic (Moormann *et al.*, 2005).

The incidence of herpes zoster was also shown to be markedly increased in malaria-infected individuals (Cook, 1985). Papua New Guinean children under 9 years of age were reported to have developed herpes zoster following an episode of malaria, due to *P.*

*falciparum* or *P. vivax* (Cook, 1985). The reactivation of the varicella-zoster virus in these patients may reflect transient depression of cell-mediated immunity by these malaria parasites. Furthermore, a high rate of parvovirus B19 and *P. falciparum* co-infection has been reported in children from Nigeria (Jones *et al.*, 1990) and patients with malaria showed severe anaemia as a result of concomitant parvovirus B19 infection (Scarлата *et al.*, 2002). These results confirm the possibility that the depression of cell-mediated immunity during *P. falciparum* malaria may favour the appearance of opportunistic viral infections.

Hepatitis B virus carriage was found to be significantly increased among cases of severe malaria (Thursz *et al.*, 1995). It was suggested that in individuals with persistent hepatitis B virus infection the protective role of cytotoxic T cells in lysing parasite-infected hepatocytes could be impaired due to the reduced levels of HLA class I on hepatocytes infected by hepatitis B virus, leading to increased susceptibility to severe malaria (Thursz *et al.*, 1995).

The pattern of immunosuppression observed in children with acute malaria is similar to that observed in experimental animal models. At the time of maximal parasitaemia, *P. berghei* infection in mice caused profound suppression of antibody response to some antigens including human- $\gamma$ -globulin, but not to others, such as to keyhole limpet haemocyanin (KLH)(Greenwood *et al.*, 1971). However, no suppressive effect of cell-mediated immunity was demonstrated, as skin graft rejection and contact hypersensitivity were not impaired (Greenwood *et al.*, 1971).

Back in 1965, Kaye *et al.* demonstrated lowered resistance to *S. typhimurium* in mice infected with *P. berghei* (Kaye *et al.*, 1965). Depression of antibody responses to tetanus toxoid was demonstrated in malaria-infected mice (Voller *et al.*, 1972). Similarly, a reduced immune response to sheep erythrocytes was reported during malaria infection in mice (Salaman *et al.*, 1969). Acute *P. yoelii* and chronic *P. berghei* infections in mice were accompanied by a reduced capacity to mount an antibody response to type III pneumococcal polysaccharide (McBride *et al.*, 1977). *P. yoelii* infection in mice was

shown to suppress the response to killed *Bordetella pertussis* (whooping cough) vaccine (Viens *et al.*, 1974), depress the primary antibody response to alum-absorbed bovine serum albumin (McBride and Micklem, 1977) and to have depressed immune responses to Moloney leukemia virus (MLV), the latter resulting in the induction of lymphomas, accompanied by reduced levels of circulating neutralising Ab to MLV and in particular by the absence of IgG antibody (Bomford and Wedderburn, 1973).

The observations described above suggest that repeated episodes of malaria-induced immunosuppression may contribute to a high incidence of bacterial infections and recrudescence of viral infections, may interfere with vaccination programmes and may have other long-term effects such as predisposing to the development of Burkitt's lymphoma. Furthermore, the demonstration that *P. falciparum* infection in humans is associated with a similar type of immunosuppression observed in experimental animal models support the view that animal experiments have relevance to human disease and they may aid in determining and overcoming the mechanisms of malaria immunosuppression as well as the development of a much needed effective vaccine against malaria.

Dendritic cells (DCs) play a pivotal role in the activation of T cells and consequently the induction of adaptive immune responses and immunity (Adams *et al.*, 2005). Therefore, it is reasonable to assume that DCs also play a critical role in initiating immune responses to malaria. There is evidence that many pathogens have evolved mechanisms to subvert the function of these important cells thereby modulating the host's immune response to their advantage (Rescigno, 2002; Rescigno and Borrow, 2001). Thus, it is likely that malaria parasites may modulate key functions of DCs that could potentially lead to or contribute to more generalised immunosuppression observed in infected individuals.

## 1.2 Dendritic Cell biology

Dendritic cells (DCs) are bone marrow-derived mononuclear cells that are found in tissues throughout the body and are specialised for the uptake, transport, processing and presentation of antigens to T cells (Adams *et al.*, 2005). It is now clear that DCs are a heterogeneous group of cells that vary in cell-surface marker expression and function. Originally, they have been divided into two subpopulations, myeloid and lymphoid, as they can efficiently derive from myeloid and lymphoid precursors respectively (Ardavin, 1997; Inaba *et al.*, 1993). The concept that DC subtypes are the product of entirely separate developmental lineages however, has been challenged by studies showing that both subsets can arise from common myeloid progenitors (Traver *et al.*, 2000). Therefore, a second model for the generation of functionally distinct DC subtypes has been proposed whereby all DCs are thought to belong to a single haematopoietic lineage, the different subtypes of DCs being generated by local environmental signals (Shortman and Liu, 2002). The reality is probably a mixture of these two models, and a large degree of functional plasticity seems to be a general feature of both DCs and their precursors (Shortman and Liu, 2002).

Despite the tremendous interest in DCs, there are great difficulties in studying the development and lineage of a cell, which is present in such low numbers within multiple tissues. The *in vitro* culture of DC using cocktails of cytokines and growth factors has provided a reliable source of these rare cells for functional studies. The majority of studies to date have identified subsets of cells on the basis of cell surface phenotype or function (Shortman and Liu, 2002).

### 1.2.1 Murine DCs

Murine DCs are defined by their expression of a range of cell surface markers including CD11c (Metlay *et al.*, 1990), CD11b (Crowley *et al.*, 1990), CD205 (Inaba *et al.*, 1995) and MHC class II (Pierre *et al.*, 1997). Furthermore, the T cell markers CD4 and CD8 are

also expressed on mouse DCs and are useful for segregating subtypes (Vremec *et al.*, 2000). Using these surface markers, five DC subtypes are consistently found in the lymphoid tissues of uninfected laboratory mice. Three common subtypes of CD11c<sup>+</sup> MHCII<sup>+</sup> DCs can be found in spleens and lymph nodes: CD4<sup>+</sup>CD8 $\alpha$ <sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup> DC, CD4<sup>-</sup>CD8 $\alpha$ <sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup> DC and CD4<sup>-</sup>CD8 $\alpha$ <sup>+</sup>CD205<sup>+</sup>CD11b<sup>-</sup> DC (Shortman and Liu, 2002). These are all thought to be blood-derived although it is unclear whether they undergo maturation within the spleen or arrive in a mature state (Wilson and O'Neill, 2003). Both CD4<sup>-</sup>CD8 $\alpha$ <sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup> DC and CD4<sup>+</sup>CD8 $\alpha$ <sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup> DC resemble myeloid lineage cells since they express myeloid markers such as CD11b and F4/80 and because they lack CD8 $\alpha$  expression (Vremec *et al.*, 2000). Therefore, CD8 $\alpha$ <sup>-</sup> DCs are generally referred to as 'myeloid DCs'. The CD4<sup>+</sup>CD8 $\alpha$ <sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup> subset differs from the CD4<sup>-</sup>CD8 $\alpha$ <sup>-</sup>CD205<sup>-</sup>CD11b<sup>+</sup> DCs by their greater adhesion capacity, higher levels of F4/80 expression (Vremec *et al.*, 2000) and lower cytokine production following stimulation (Hochrein *et al.*, 2001). However both CD8 $\alpha$ <sup>-</sup> subsets are efficient stimulators of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and show efficient MHC Class II presentation to antigen-specific CD4<sup>+</sup> T cells (Kronin *et al.*, 1996; Pooley *et al.*, 2001). Other reports have shown that CD8 $\alpha$ <sup>-</sup> DCs can induce Th2 type immune responses since they stimulate cells that produce IL-4 but little IFN- $\gamma$  (Maldonado-Lopez *et al.*, 1999). Both subsets of CD8 $\alpha$ <sup>-</sup> DCs are located in the marginal zone of the spleen between the white and red pulp (Vremec and Shortman, 1997) but they migrate into the T cell zone on stimulation with microbial products (Reis e Sousa *et al.*, 1997).

CD4<sup>-</sup>CD8 $\alpha$ <sup>+</sup>CD205<sup>+</sup>CD11b<sup>-</sup> DCs are generally referred to as 'lymphoid' DCs and are concentrated in the T cell areas of the spleen (Vremec and Shortman, 1997). They exhibit different functional capacities compared with CD8 $\alpha$ <sup>-</sup> DCs. Freshly isolated CD8 $\alpha$ <sup>+</sup> DCs can have regulatory effects on T cells. They activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, but can induce apoptosis in CD4<sup>+</sup> T cells (Suss and Shortman, 1996) and limit CD8<sup>+</sup> T cell proliferation by reducing IL-2 production (Kronin *et al.*, 1996). They have also been identified as the DCs responsible for maintaining T cell tolerance in lymphoid organs in the absence of infection (Belz *et al.*, 2002). In contrast, under conditions consistent with activation, such as the presence of IL-2 (Pooley *et al.*, 2001), CD8 $\alpha$ <sup>+</sup> DCs can efficiently

present antigen to CD8<sup>+</sup> T cells in the context of MHC class I (den Haan *et al.*, 2000; Pooley *et al.*, 2001). They can cross-present exogenous soluble antigens for stimulation of cytotoxic T cells (den Haan *et al.*, 2000) and it has been reported that they can induce Th1 type immune responses *in vivo*, consistent with activated CD8 $\alpha$ <sup>+</sup> DCs being the major producers of IL-12 (Hochrein *et al.*, 2001; Maldonado-Lopez *et al.*, 1999).

In addition to the three subsets described in the spleen, lymph nodes contain two extra DC subtypes: CD4<sup>+</sup>CD8 $\alpha$ <sup>-</sup>CD11b<sup>+</sup> DCs, which express moderate levels of CD205 and are believed to be the mature form of tissue interstitial DCs and CD4<sup>+</sup>CD8 $\alpha$ <sup>lo</sup>CD205<sup>+</sup>CD11b<sup>+</sup> DCs, only found in skin-draining lymph nodes and express high levels of langerin, a characteristic marker of epidermal Langerhans cells. This latter subset is believed to be the mature form of Langerhans cells (Henri *et al.*, 2001). Langerhans DCs in lymph nodes are also distinguished by their high surface expression of MHC class II, CD40, CD80 and CD86, typical characteristic of activated DCs (Henri *et al.*, 2001).

Murine plasmacytoid DC represent a distinct class of DC recently identified in bone marrow, thymus, spleen and lymph nodes. This population had been missed in mouse cell suspensions because it expresses B220 (CD45) (so might be eliminated along with B cells during selection procedures), Gr-1 (so might be eliminated along with granulocytes) together with only low levels of CD11c and MHC class II (Martin *et al.*, 2002; Nakano *et al.*, 2001; Nikolic *et al.*, 2002). They have a typical morphology of large, round cells with a diffuse nucleus and rare dendrites, with some cells expressing CD62L (Nakano *et al.*, 2001; Nikolic *et al.*, 2002). Murine plasmacytoid DCs, like their human counterparts, have been shown to produce IFN- $\alpha$  following virus infection and thus may play important roles in antiviral responses once activated (Nakano *et al.*, 2001). A summary of mouse DC subsets is shown in Table 1.1.

### 1.2.2 Human DCs

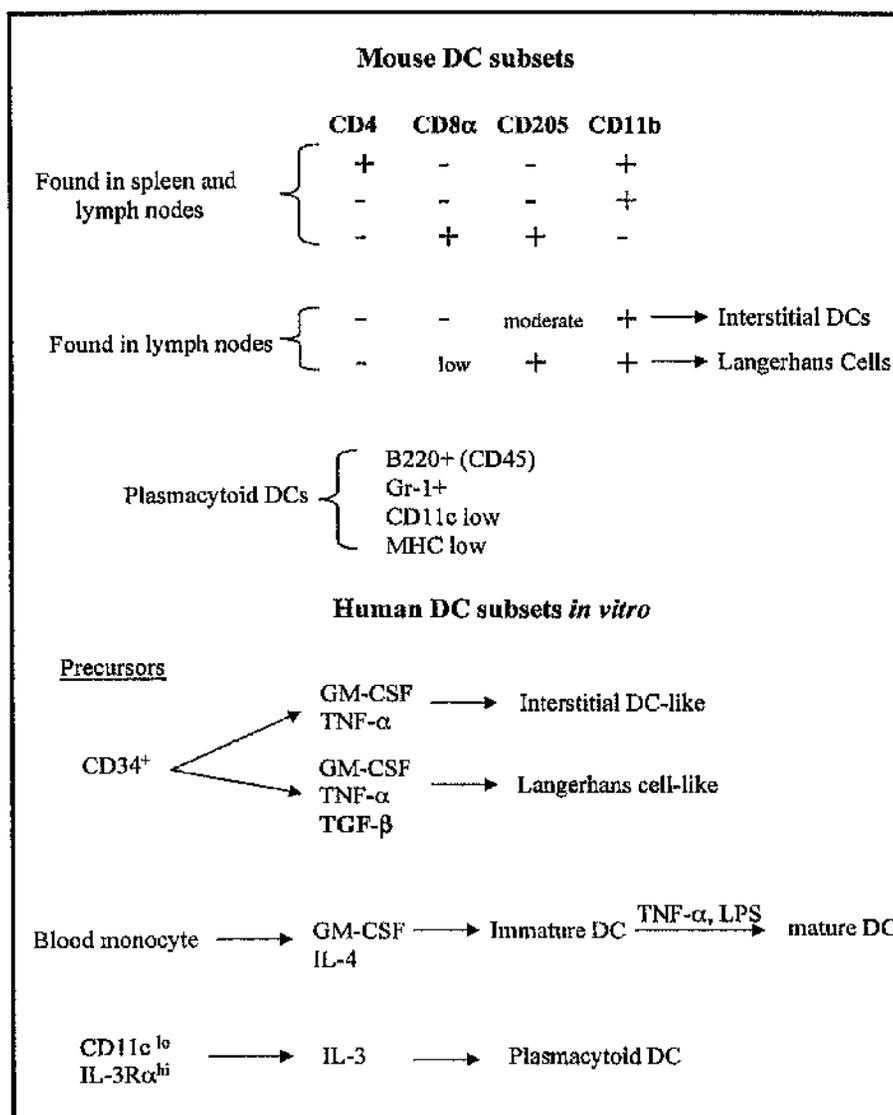
A direct comparison of human DCs with mouse DCs has been challenging at a number of levels. First of all, there are relatively few studies on human DCs freshly isolated from

tissues. Moreover, *ex vivo* DCs are likely to differ from their *in vivo* counterparts since they are easily activated upon handling, leading to altered phenotypes. This problem further emphasises the difficulties associated with subset analysis for both human and murine DCs. Blood is the only readily available source of human DCs although it is more a source of precursor DCs and even when some blood DCs are sufficiently mature, their surface expression of different markers may represent differences in activation states rather than separate DC subsets (Shortman and Liu, 2002). Another barrier to comparing human DCs directly with mouse DCs is the lack of expression of CD8 on human DCs, so the human DC equivalent of mouse CD8 $\alpha^+$  DC remains elusive.

In a few cases, human DCs have been isolated from lymphoid tissues and directly analysed without any incubation steps that may promote their differentiation *ex vivo*. Only in these cases it is possible to compare these DCs with murine DC subtypes directly and there are now studies on human thymic DCs supporting the idea of subset segregation, similar to that observed in the mouse (Bendriss-Vermare *et al.*, 2001; Vandenabeele *et al.*, 2001). Most human thymic DCs are CD11c $^+$ CD11b $^-$ CD45RO $^lo$ , lack myeloid markers and therefore resemble mouse thymic CD8 $\alpha^+$  DCs. A minority of human DCs are CD11c $^{hi}$ CD11b $^+$ CD45RO $^{hi}$  and express many myeloid markers, so resemble mouse CD8 $\alpha^-$  DCs (Shortman and Liu, 2002). However, most of the insights into human DC subsets have not come from direct isolation of mature DCs from tissues but from studies on their development in culture from immature DCs or precursors DCs.

Three different precursor-cells have been used to generate human DC in culture: CD34 $^+$  blood monocytes and CD11c $^-$ IL-3R $^{hi}$  precursors (Shortman and Liu, 2002). CD34 $^+$  precursors yield different DC subsets depending on the combination of cytokines used. Culture with GM-CSF and TNF- $\alpha$  leads to the production of DCs resembling Langerhans cells and interstitial DCs, the former strongly depending on TGF- $\beta$  for development (Shortman and Liu, 2002). Blood monocytes are the most common precursors used for the generation of DCs in culture. They yield immature DCs following culture with GM-CSF and IL-4 and final maturation is achieved by stimulation with pro-inflammatory cytokines such as TNF- $\alpha$  or microbial products such as LPS (Shortman and Liu, 2002).

The final pathway for human DC development involves culture of CD11c<sup>lo</sup>IL-3R $\alpha$ <sup>hi</sup> precursors with IL-3, which leads to the formation of IFN- $\alpha$  producing plasmacytoid DCs (Shortman and Liu, 2002). Human DC subsets are summarised below in Table 1.1.



**Table 1.1** Murine and human Dendritic cell subtypes

Although a number of different DC subtypes has now been identified, myeloid DCs are the focus of the present study. Thus from this point onwards any reference to DCs should be taken to mean myeloid DCs, unless otherwise stated.

### 1.2.3 Antigen uptake, processing and presentation

DC precursors from the bone marrow pass through the blood to peripheral tissues where they reside as immature DCs, continuously sampling the antigenic environment. Immature DCs have been shown to recognise invading pathogens *via* conserved pattern-recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) within microbial carbohydrates, lipids and nucleic acids (Janeway and Medzhitov, 2002). Toll-like receptors (TLRs) have emerged as a major family of PRRs, recognising a wide range of molecules including prokaryote-derived lipoproteins, glycolipids, flagellin, CpG DNA, and lipopolysaccharides (LPS)(Akira *et al.*, 2001). There is now evidence that TLR expression is not uniform among DCs, raising the possibility that different DC subtypes may be preferentially involved in recognition of distinct classes of pathogens (Edwards *et al.*, 2003; Kadowaki *et al.*, 2001). For instance, plasmacytoid DCs uniquely express TLR9 and TLR7, which allow them to respond to viral CpG DNA and viral single-stranded RNA respectively (Coccia *et al.*, 2004; Ito *et al.*, 2005). However, plasmacytoid DCs do not express TLR4 and therefore respond to LPS relatively weakly. In fact, myeloid DCs, which express TLR4, primarily mediate the responses to bacterial infections (Pitha, 2004). A summary of the many TLR ligands and the distribution of these receptors among the different DC subsets are shown in Tables 1.2 and 1.3 respectively.

TLR	Ligands
TLR-1	Tri-acyl lipopeptides (bacteria, mycobacteria) Soluble factors ( <i>Neisseria meningitidis</i> )
TLR-2	Lipoprotein/lipopeptides (a variety of pathogens) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) A phenol-soluble modulin ( <i>Staphylococcus epidermidis</i> ) Glycoinositolphospholipids ( <i>Trypanosoma cruzi</i> ) Glycolipids ( <i>Treponema maltophilum</i> ) Porins ( <i>Neisseria</i> ) Zymosan (fungi) Atypical LPS ( <i>Leptospira interrogans</i> ) Atypical LPS ( <i>Porphyromonas gingivalis</i> ) HSP70 (host)
TLR-3	Double-stranded RNA (virus)
TLR-4	LPS (Gram-negative bacteria) Taxol (plant) Fusion protein (RSV) Envelope proteins HSP60 ( <i>Chlamydia pneumoniae</i> ) HSP60 (host) HSP70 (host) Type III repeat extra domain A of fibronectin (host) Oligosaccharides of hyaluronic acid (host) Polysaccharide fragments of heparan sulfate (host) Fibrinogen (host)
TLR-5	Flagellin (bacteria)
TLR-6	Di-acyl lipopeptides (mycoplasma)
TLR-7	Imidazoquinoline (synthetic compounds) Loxoribine (synthetic compounds) Bropirimine (synthetic compounds)
TLR-8	?
TLR-9	CpG DNA (bacteria)
TLR-10	?

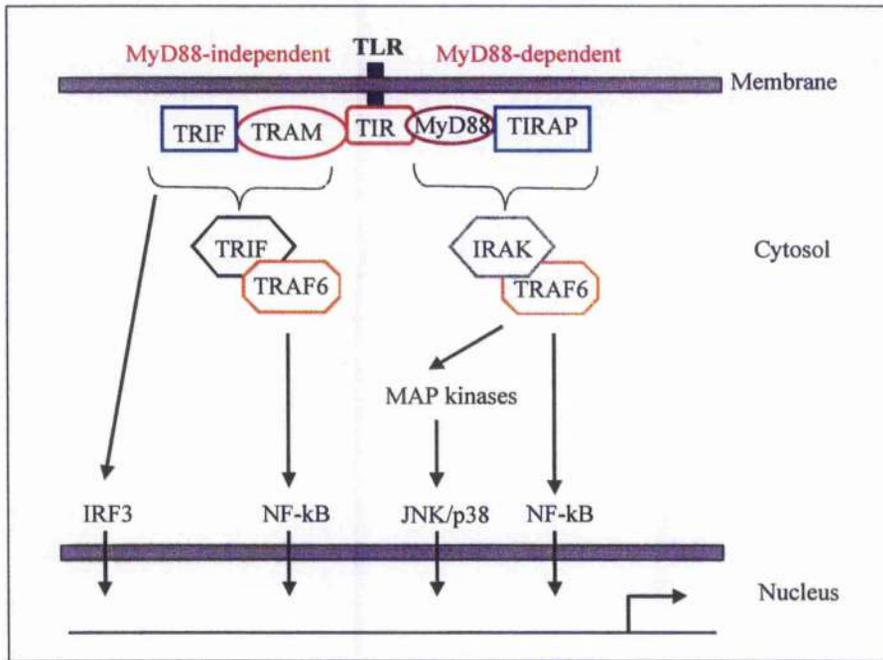
**Table 1.2** TLRs and their ligands as reported in reference (Takeda *et al.*, 2003).

	Human blood		Mouse spleen			
	CD11c <sup>+</sup>	PDC	CD4 <sup>+</sup>	CD8 $\alpha$ <sup>+</sup>	CD4 <sup>+</sup> CD8 $\alpha$ <sup>-</sup>	PDC
TLR-1	high	low	moderate $\longrightarrow$			
TLR-2	high	-	moderate $\longrightarrow$			
TLR-3	high	-	low	high	moderate	low
TLR-4	moderate	-	low	low	low	low
TLR-5	low	-	high	low	moderate $\longrightarrow$	
TLR-6	low	low	high	moderate $\longrightarrow$		
TLR-7	-	high	low	-	low	high
TLR-8	low	-	high	low	low	high
TLR-9	-	high	moderate $\longrightarrow$			high
TLR-10	low	low	N/A	N/A	N/A	N/A

**Table 1.3** TLR expression on DC subsets isolated from human blood and murine spleen as reported in references (Edwards *et al.*, 2003; Kadowaki *et al.*, 2001). PDC, plasmacytoid DCs; N/A, not applicable.

TLRs are members of the TLR-IL-1 receptor superfamily, all of which share an intracytoplasmic Toll-IL-1 receptor (TIR) domain that mediates the recruitment of TIR-containing adaptor molecules such as MyD88, TIRAP, TRIF and TRAM (Akira *et al.*, 2001). These adaptor molecules function to recruit other signalling molecules, notably the IL-1 receptor-associated kinase complex (IRAK), which activates the TRAF6 protein that is required for DC maturation in response to a number of different stimuli (Kobayashi *et al.*, 2003). In mice, all TLRs can initiate signalling through the MyD88-IRAK-TRAF6 pathway, which results in the activation of the transcription factor NF- $\kappa$ B and mitogen-activated protein (MAP) kinases, inducing the transcription of genes such as TNF- $\alpha$ , IL-1 and IL-6 (Akira *et al.*, 2001). MyD88-independent signalling pathways have also been described. For example TRIF controls a MyD88-independent pathway that is

unique to TLR3 and TLR4 signalling and is important for the secretion of IFN- $\beta$  (Hoebe *et al.*, 2003; Yamamoto *et al.*, 2003) (Figure 1.3).



**Figure 1.3** Toll-like receptor-signalling pathways. Interaction of microbial products with TLRs initiates two major signalling pathways. The MyD88-dependent pathway leads to the phosphorylation of IRAK, recruitment of TRAF6, activation of NF- $\kappa$ B and JNK/p38 and release of pro-inflammatory cytokines. Activation of NF- $\kappa$ B can also occur *via* MyD88-independent pathway, which also leads to the activation of IRF3 and the induction of IFN- $\beta$  and RANTES.

Besides TLRs, immature DCs also express several C-type lectin receptors, which recognise carbohydrate structures on pathogens (Figdor *et al.*, 2002). Examples include mannose receptor, DC-SIGN, DEC-205 and langerin (Geijtenbeek *et al.*, 2000; Sallusto *et al.*, 1995; Valladeau *et al.*, 2000). Once in contact with antigen, immature DCs use several pathways to facilitate uptake. These include receptor-mediated endocytosis through C-type lectins, Fc receptors (Fc $\gamma$ RI, Fc $\gamma$ RII, Fc $\gamma$ RIII) and complement receptors (CR3) (Banchereau *et al.*, 2000; Peiser *et al.*, 2002; Sallusto *et al.*, 1995). They also have

high capacity to non-specifically endocytose particulates and solutes through phagocytosis and macropinocytosis (Sallusto *et al.*, 1995). Although many of these pathways are used for uptake of pathogen-related molecules, they may also be utilised for uptake of self-antigens (Steinman *et al.*, 2000). Indeed, immature DCs express  $\alpha\text{v}\beta 3$ - and  $\alpha\text{v}\beta 5$ -integrins and CD36, which help to facilitate continuous uptake of apoptotic material in the immune steady state (Albert *et al.*, 1998). These may be important in DC-mediated maintenance of peripheral self-tolerance (Steinman *et al.*, 2000).

Following antigen recognition and uptake, DCs process protein antigens into peptides, which are loaded onto MHC class I and II molecules and transported to the cell surface for recognition by antigen-specific T cells (Quah and O'Neill, 2005). Endogenous protein antigens, which are processed onto MHC class I, are degraded into peptides by the proteasome in the cytosol. These are then transported *via* transporters for antigen presentation (TAP) molecules into the endoplasmic reticulum (ER), where they are loaded on MHC class I. The peptide-MHC I complexes are then transported from the ER *via* the trans-Golgi network to the cell surface for presentation to  $\text{CD}8^+$  T cells (Quah and O'Neill, 2005).

Exogenously acquired protein antigens are instead engulfed and processed in endosomes, which then fuse with lysosomes, where proteases degrade the proteins into peptides that are loaded onto MHC II molecules (Quah and O'Neill, 2005). This requires proteolytic degradation of the MHC II-associated invariant chain that normally blocks access to the peptide-binding pocket of MHC II (Guermontprez *et al.*, 2002). Peptide-MHC II complexes are then transported to the cell surface for presentation to  $\text{CD}4^+$  T cells (Chow *et al.*, 2002). Exogenous antigens may also be processed by DCs onto MHC I (Albert *et al.*, 1998). This phenomenon called 'cross-presentation' or 'cross-priming' allows DCs to elicit  $\text{CD}8^+$  as well as  $\text{CD}4^+$  T cell responses to exogenously acquired antigens (Ackerman *et al.*, 2003; Fonteneau *et al.*, 2002; Guermontprez *et al.*, 2003). Furthermore, lipid antigens expressed on pathogens (such as mycobacterial mycolates) or self-tissues (such as sphingolipids) are presented on DCs by CD1 molecules, which heterodimerise

with  $\beta$ 2-microglobulin and are structurally similar to MHC I (Joyce and Van Kaer, 2003; Moody and Porcelli, 2003).

#### 1.2.4 DC maturation

The antigen generally induces immature DCs to undergo phenotypic and functional changes that culminate in the activation of immature DCs. This maturation process is accompanied by cytoskeletal reorganization, reduced phagocytic activity, acquisition of cellular motility, migration to lymphoid tissues, enhanced T cell activation potential and the development of characteristic cytoplasmic extensions usually referred to as 'dendrites' (Quah and O'Neill, 2005). Mature DCs secrete cytokines that determine the type of ensuing immune response and upregulate the expression of adhesion and costimulatory molecules, which are involved in bidirectional signalling between DCs and T cells, modulating both T cell activation and DC function (Quah and O'Neill, 2005). Furthermore, maturation imparts on the peripheral DCs the ability to migrate from the tissues to T cell zones of lymph nodes. This is accomplished through downregulation of CCR1 and CCR5 and upregulation of CCR7, which helps localise DCs to lymphatic vessels and lymph nodes *via* chemokines CCL19 and CCL21. Maturation also induces DCs to secrete chemokines such as IP-10, which recruits various T cell subsets and RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$ , which recruit monocytes and DCs into the local environment (Adams *et al.*, 2005).

#### 1.2.5 Outcomes of DC-T cell interaction

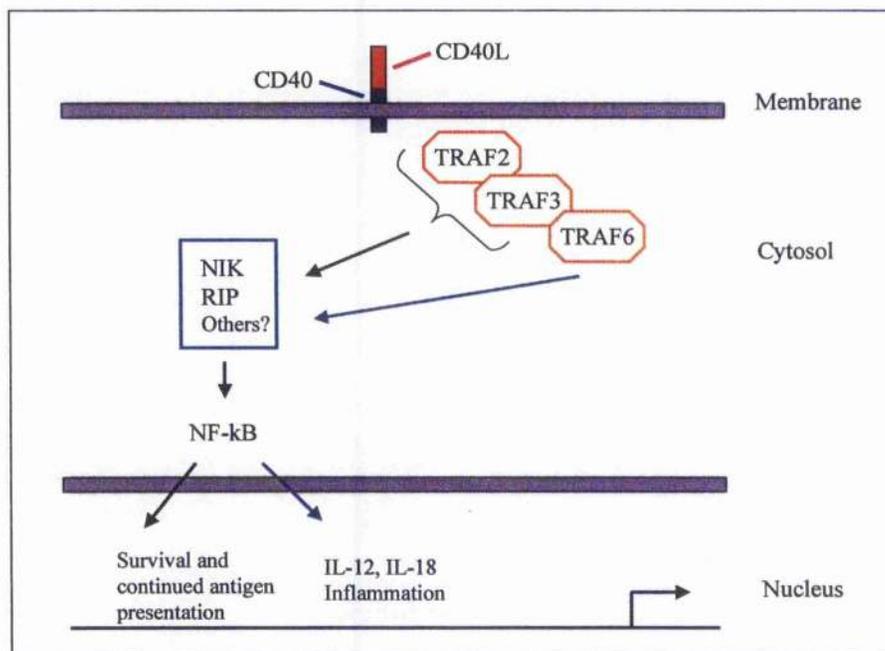
##### 1.2.5.1 Induction of Th1/Th2 responses

Effective priming of naïve T cells results in their clonal expansion and differentiation into cytokine secreting effector cells and memory cells. The ensuing T cell response is dependent on many factors, including the concentration of antigen on the DC, the affinity of the T cell receptor (TCR) for the peptide-MHC complex, the duration of the DC-T cell

interaction, the state of DC maturation and the type of DC maturation stimulus (Gell *et al.*, 2003). Following priming, CD4<sup>+</sup> T cells may differentiate towards T helper 1 (Th1) cells, which produce IFN- $\gamma$  and support CD8<sup>+</sup> cytotoxic T-lymphocyte (CTL) responses, or toward T helper 2 (Th2) cells, which produce IL-4, IL-5 and IL-13, support humoral immunity and downregulate Th1 responses (Adams *et al.*, 2005). The secreted cytokine profile of the stimulating DCs determines the direction of this Th polarization. IL-12, IL-18 and IL-27 polarize toward Th1, whereas CCL17, CCL-2, and the absence of IL-12 skew the response toward Th2 (Adams *et al.*, 2005).

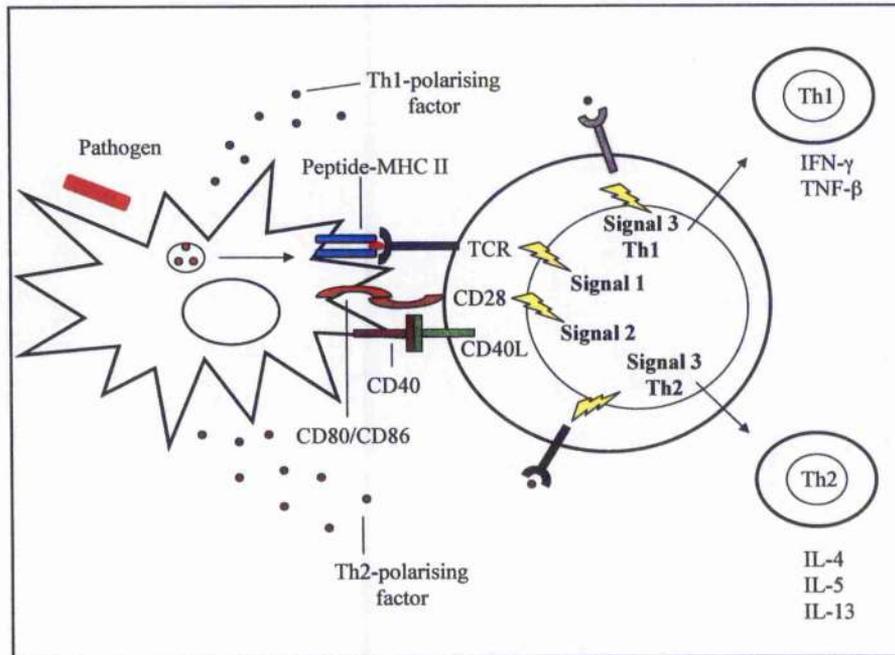
T cell stimulation and Th1/Th2 polarization requires three DC-derived signals: the stimulatory signal 1, which results from the ligation of TCRs by pathogen-derived peptides, presented by MHC class II molecules on the cell surface of DCs and determines the antigen-specificity of the response (Kapsenberg, 2003); the co-stimulatory signal 2, provided by molecules such as CD80/CD86 on the DCs interacting with CD28 on the T cells (Harding *et al.*, 1992); and signal 3, which as mentioned above, is the polarising signal that is mediated by various soluble or membrane-bound factors such as IL-12 or CCL2 that promote the development of Th1 or Th2 cells respectively (Kalinski *et al.*, 1999). Most T cell polarising factors are either weakly or transiently expressed after pathogen recognition. High expression of such factors require a further signal as it has been reported in the case of IL-12 (Snijders *et al.*, 1998). For optimal expression of Th-cell polarising molecules during DC-T cell interaction, the primed DC requires intimate cross-talk with the T cells involving ligation of CD40 by CD40L, which is rapidly expressed by the T cells after their activation by the DC-derived signals 1 and 2 (Grewal and Flavell, 1998). Stimulation through CD40 results in a complex series of events within the DC. Engagement of CD40 by its ligand, CD154 (CD40L), leads to trimeric clustering of CD40 and the recruitment of adaptor proteins known as TNF-receptor-associated factors (TRAFs) to the cytoplasmic tail (Pullen *et al.*, 1998). Binding of TRAFs results in the formation of a signalling complex that includes multiple kinases such as NF- $\kappa$ B inducing kinase (NIK), receptor interacting protein (RIP), members of the mitogen-activated protein kinase (MAPK) family and possibly others (Quezada *et al.*, 2004). Clustering of these kinases then initiates a downstream cascade of signalling events,

resulting in activation of the MAPK and NF- $\kappa$ B pathways and finally transcription of target genes, leading to physiological effects, such as production of inflammatory mediators, DC survival and prolonged MHC/antigen complex presentation (Quezada *et al.*, 2004) (Figure 1.4).



**Figure 1.4** CD40 activation of NF- $\kappa$ B in DCs.

A variety of surface molecules other than CD40-CD40L and CD80/CD86-CD28 on DCs and T cells have been demonstrated to promote T cell and/or DC activation; these molecules include several members of the TNF receptor family such as TRANCE (Bachmann *et al.*, 1999), OX40 (Chen *et al.*, 1999), 4-1BB (Cannons *et al.*, 2001) and CD27 (Hendriks *et al.*, 2000) and their corresponding ligands. A diagram of the events involved in the induction of effector T cell responses is shown in Figure 1.5.



**Figure 1.5** Dendritic cell-derived signals involved in T cell stimulation and Th1/Th2-cell polarisation modified from (Kapsenberg, 2003).

It has to be noted that Figure 1.5 represents a very simplistic and basic diagram of the events involved in the induction of Th1/Th2 polarisation. In fact, it is now known that the type of costimulation may also control T cell polarisation. For instance, ICOS may play an important role in the development of Th2 cells (McAdam *et al.*, 2000), although a recent report showed that ICOS was upregulated on antigen-specific T and B cells following Th1 induction (Smith *et al.*, 2005). CD40-CD40L interaction may instead sustain Th1 responses (Grewal and Flavell, 1998).

#### 1.2.5.2 Induction of regulatory T cells

During the last few years it has become clear that DCs not only play a role in the promotion of effector Th1 and Th2 cell responses but under some circumstances, the T cell may be inactivated leading to immunological tolerance. Tolerance is a state in which

the immune system fails to make an active response to antigen and it is therefore critical for preventing immune responses to tissue antigens by self-reactive T cells as well as preventing inappropriate responses to harmless foreign antigens, particularly when such responses could lead to tissue damage (Fazekas de St Groth, 2001). The induction of regulatory T cells plays an important role in the maintenance of tolerance (Sakaguchi, 2005; Schwartz, 2005). Two subsets of regulatory T cells can be distinguished: naturally occurring and adaptive regulatory T cells (Bluestone and Abbas, 2003). Naturally occurring T regulatory cells are CD4<sup>+</sup>CD25<sup>+</sup> cells that obtain their regulatory function in the thymus and are primarily involved in tolerance to autoantigens (Bach, 2003). Adaptive regulatory T cells include Tr1 cells, which secrete high levels of IL-10, and Th3 cells, which secrete TGF- $\beta$  (Jonuleit and Schmitt, 2003). In contrast to natural regulatory T cells, adaptive regulatory cells originate, like Th1 and Th2 cells, from uncommitted peripheral naïve or memory Th cells upon activation by antigen-presenting DCs in a certain immunological context (Jonuleit and Schmitt, 2003). Adaptive regulatory T cells may normally respond to innocuous foreign antigens and are therefore primarily associated with tolerance to environmental antigens (Jonuleit and Schmitt, 2003).

The current belief is that mainly immature or semi-mature DCs play a role in T regulatory cell development. Several studies have suggested that immature DCs might acquire chemokine receptors (e.g. CCR7) under homeostatic conditions, enabling their migration towards lymph nodes (Dhodapkar and Steinman, 2002; Dhodapkar *et al.*, 2001; Geissmann *et al.*, 2002). It has been hypothesised that because of their total lack of expression of co-stimulatory and Th1/Th2 polarising molecules, immature DCs can drive the development of adaptive T regulatory cells from naïve T cells (Jonuleit *et al.*, 2000). Semi-mature DCs were also reported to induce T regulatory cell development. Immature DCs exposed to tissue-derived TNF- $\alpha$  in the absence of pathogen acquire part of the characteristics of fully mature DCs, including the expression of co-stimulatory molecules and the ability to migrate to draining lymph nodes (Lutz and Schuler, 2002). However, they lack the ability to produce polarising signals and, as a consequence, predominantly drive the development of adaptive T regulatory cells.

### 1.2.5.3 T cell anergy

Besides inducing functional Th1/Th2 cell responses and T regulatory cells, interaction of DCs with T cells may result in failure of T cell activation leading to T cell anergy, providing another important mechanism by which peripheral tolerance can be maintained. T cell anergy is defined as a state in which the cell is viable but fails to display certain functional responses (proliferation and IL-2 production) upon exposure to its specific antigen and results from partial T cell activation due to a lack of adequate co-stimulation (lack of signal 2) (Alegre *et al.*, 2001; Lechler *et al.*, 2001). This type of anergy may be reversed by the stimulation of T cells with IL-2 (DeSilva *et al.*, 1991). Another form of anergy has also been described and is associated with the failure to proliferate after activation, despite the presence of co-stimulatory signals (Wells *et al.*, 2001). This division-arrest-associated form of anergy cannot be reversed by IL-2 and is dependent on CTLA-4 (Wells *et al.*, 2001).

### 1.2.5.4 Deletion/apoptosis of antigen-specific T cells

Activation through the TCR can result in apoptosis of T cells and in this regard it is becoming clear that two pathways control the fate of antigen-specific T cells: activation-induced cell death (AICD) and activated T cell-autonomous death (ACAD) (Hildeman *et al.*, 2003; Hildeman *et al.*, 2002). AICD is driven by death receptors such as Fas (CD95), which, once engaged, activate downstream pathways that lead to caspase activation and cell apoptosis (Hildeman *et al.*, 2002). Furthermore, AICD requires repetitive or high dose stimulation *via* the TCR (Hildeman *et al.*, 2002). Conversely, ACAD does not require death receptors. It is instead controlled by members of the Bcl-2 family of proteins, which induce apoptosis by releasing cytochrome *c* and other apoptogenic factors from mitochondria (Li *et al.*, 2004a). Moreover, contrary to AICD, a single antigen exposure is enough to cause cell death (Hildeman *et al.*, 2002). Finally, in functional terms, ACAD plays a major role in the removal of T cells at the end of immune responses, whereas both AICD and ACAD seem to have a role in auto-reactive T cell removal (Hildeman *et al.*, 2002).

### 1.2.6 Pathogen-driven T cell responses

There is accumulating evidence that microbes drive the development of protective Th1 or Th2 responses by directly affecting DCs at the time of pathogen encounter. Both Cholera toxin (Gagliardi *et al.*, 2000) and a phosphorylcholine-containing glycoprotein (ES-62) secreted by the filarial nematode *Ancanthocheilonema viteae* (Whelan *et al.*, 2000) have been shown to induce the maturation of DCs with the capacity to drive Th2 responses. Conversely, influenza virus was shown to activate DCs to drive polarised Th1 responses (Cella *et al.*, 1999). Furthermore DCs have been reported to interact with the fungus *Candida albicans* in its different forms and to elicit different effector T cell responses (d'Ostiani *et al.*, 2000). *C. albicans* can reversibly switch between unicellular yeast to filamentous (hyphal) forms. Yeasts activated DCs for IL-12 production and priming of Th1 cells, whereas ingestion of hyphae inhibited IL-12 and Th1 responses and induced IL-4 production (d'Ostiani *et al.*, 2000).

More recently it has been demonstrated that microbial compounds induce Th cell polarisation *via* the polarisation of DCs into committed Th1 cell-promoting or Th2 cell-promoting effector DCs (de Jong *et al.*, 2002). Soluble egg antigens (SEA) of the helminth *Schistosoma mansoni* and Cholera toxin, both associated with Th2 cell responses, promoted immature DCs to develop into functional effector cells with polarised type 2 phenotypes (de Jong *et al.*, 2002). SEA induced the development of type 2 DCs, which promoted Th2 cells *via* the enhanced expression of OX40L, whereas Cholera toxin induced Th2 responses *via* an OX40L-independent mechanism (de Jong *et al.*, 2002). Instead, double-stranded RNA (poly (I:C), a mimic of viral RNA) and the toxin of the intracellular bacterium *Bordetella pertussis*, both associated with Th1 cell responses, promoted immature DCs to develop into functional effector cells with polarised type 1 phenotypes (de Jong *et al.*, 2002). The toxin from *Bordetella pertussis* induced the development of type 1 DCs with enhanced IL-12 production, which promotes Th1 cell development, whereas poly (I:C) induced the development of extremely potent Th1-inducing DCs without an enhanced IL-12 production (de Jong *et al.*, 2002).

These observations suggest that analogous to the development of polarised Th cell subsets from a single precursor population, DCs are guided by environmental stimuli to acquire stable polarised functional effector phenotypes, supporting the concept that the type of the immune response is optimally adapted to the type of the pathogen.

### 1.2.7 'Alternatively activated' DCs induced by pathogens

Extending the concept that pathogens cause priming of immature DCs to become mature effector DCs that drive Th1 or Th2 responses, accumulating information suggest that particular pathogens and /or tissue factors could prime for an alternative subset of DCs that may not be able to induce appropriate effector T cell responses. Some of these 'alternatively activated DCs' may drive the generation of regulatory T cells, in which case they are generally referred to as 'regulatory DCs'. Pathogens were originally thought to evade immunity by inducing T regulatory cells through inhibition of DC maturation. However more recent studies suggest that certain pathogens and tissue factors can prime for T regulatory cell development through inducing DC maturation into an 'alternatively activated' state.

#### 1.2.7.1 Pathogen-driven immature regulatory DCs

Immature regulatory DCs express low levels of MHC and co-stimulatory molecules and therefore do not support substantial development of effector T cells from naïve T cells (Smits *et al.*, 2005). However, they are different from steady-state immature DCs because they are unresponsive to inducers of DC maturation, such as LPS (Smits *et al.*, 2005). Furthermore, on CD40 stimulation they show decreased IL-12 production but increased levels of IL-10 (Smits *et al.*, 2005). These immature regulatory DCs can develop in response to a variety of pathogens or pathogen-derived compounds. Examples include hepatitis C virus, *Plasmodium falciparum* and mannosylated lipoarabinomannans (ManLAM) derived from *Mycobacterium tuberculosis* (Auffermann-Gretzinger *et al.*, 2001; Dolganiuc *et al.*, 2003; Geijtenbeek *et al.*, 2003; Kanto *et al.*, 1999; Nigou *et al.*, 2001; Ocana-Morgner *et al.*, 2003; Urban *et al.*, 1999).

Patients chronically infected with hepatitis C virus harbour IL-10 secreting CD4<sup>+</sup> T cells (MacDonald *et al.*, 2002). *In vitro* studies with human monocyte-derived DCs show that DC functions, such as maturation, expression of co-stimulatory molecules, cytokine production and T cell stimulatory capacity, are all severely hampered on exposure to hepatitis C virus core protein and non-structural protein 3 (Dolganiuc *et al.*, 2003). Similar defects are observed in monocyte-derived DCs generated from hepatitis C virus-infected patients and these defects cannot be overcome by stimulation with LPS, suggesting a role of regulatory DCs in the induction of regulatory T cells in hepatitis C virus infection (Auffermann-Gretzinger *et al.*, 2001; Kanto *et al.*, 1999).

ManLAM derived from *M. tuberculosis* and also from *M. leprae* or *M. bovis* BSG (Geijtenbeek *et al.*, 2003; Nigou *et al.*, 2001), has been shown to bind to DC-SIGN expressed on DC membrane. In the case of human monocyte-derived DCs, this interaction inhibits the LPS-induced DC maturation and IL-12 production and enhances DC expression of IL-10. In contrast to ManLAM, whole mycobacteria do not inhibit DC maturation, but do have the same DC-SIGN-dependent effect on cytokine production (Geijtenbeek *et al.*, 2003; Giacomini *et al.*, 2001; Nigou *et al.*, 2001).

*Plasmodium falciparum*-infected erythrocytes have been shown to bind to CD36 on DCs and inhibit the LPS-induced DC maturation and, thus, the capacity of DCs to stimulate naïve T cells (Urban *et al.*, 1999). Instead, infected erythrocytes induce high levels of IL-10 production by DCs (Urban *et al.*, 1999). CD36 normally binds apoptotic cells, preventing detrimental inflammation by regulation of DC function (Voll *et al.*, 1997). Apparently this system has been hijacked by *Plasmodium* parasites in order to evade immune responses directed against infected erythrocytes (Urban *et al.*, 2001b). A detailed analysis of *Plasmodium*-DC interaction is described further.

#### 1.2.7.2 Pathogen-driven mature regulatory DCs

In contrast to pathogen-driven immature regulatory DCs, pathogen-driven mature regulatory DCs normally express high levels of MHC and co-stimulatory molecules,

although compared to fully mature immunogenic DCs, CD40 expression is somewhat reduced in some studies (McGuirk *et al.*, 2000). Studies with human monocyte-derived DCs revealed that *Schistosoma*-derived lysophosphatidylserine primes, in a TLR2-dependent fashion, for mature DCs that drive the generation of IL-10-producing regulatory T cells (van der Kleij *et al.*, 2002). These DCs are characterised by decreased IL-12 and increased IL-10 production.

In a recent study using *Bordetella pertussis*, the causative agent of whooping cough, pathogen-specific Tr1 clones have been isolated from the lungs of infected mice (McGuirk *et al.*, 2002). These Tr1 cells were found to be specific for filamentous haemagglutinin (FHA), a virulence factor. FHA enhances the maturation of mouse DCs, inhibits IL-12 and induces some IL-10 production. These FHA-mature DCs drive the development of regulatory T cells, which are capable of blocking Th1 responses to secondary, unrelated infections, such as influenza virus (McGuirk *et al.*, 2002). Similar effects have been described for adenylate cyclase toxin from *B. pertussis* (Ross *et al.*, 2004) and for cholera toxin from *Vibrio cholerae* (Lavelle *et al.*, 2003).

Measles virus is another example of pathogen that induces mature regulatory DCs. Ligation of CD46, a complement regulatory transmembrane protein, by envelope haemagglutinin of Measles virus strongly decreases IL-12 production in human monocyte-derived DCs and murine DCs (Karp *et al.*, 1996) and profoundly inhibits their T cell-stimulatory capacity, despite normal maturation (Marie *et al.*, 2001; Schnorr *et al.*, 1997; Servet-Delprat *et al.*, 2000).

### 1.2.8 Pathogen-induced host factors promoting the formation of regulatory DCs

Besides directly affecting the activation state of DCs, pathogens may indirectly modulate DC maturation and function by inducing host cells to secrete cytokines or express molecules, which may, in turn, be responsible for the failure of DCs to mount an

appropriate T cell response directed against the pathogen itself. Some examples of possible 'immunosuppressive factors' secreted /induced by DCs are explained below.

#### 1.2.8.1 Role of eicosanoids on DC function

Eicosanoids, which include prostaglandins (PGs) and leukotrienes (LTs), are potent modulators of immune responses in addition to playing a role in numerous basic host physiological processes (Funk, 2001). The biological functions of the E series prostaglandins (PGE) have been the most intensively studied, PGE<sub>2</sub> being one of the best-known eicosanoids to date. PGE<sub>2</sub> formation is initiated by the action of phospholipase A<sub>2</sub>, which releases arachidonic acid from the membrane phospholipid bilayer in response to inflammatory stimuli (Murakami *et al.*, 1997). Free arachidonic acid is rapidly metabolised to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) *via* the enzymatic activity of the cyclooxygenase (COX) enzymes, of which there are 2 distinct isoforms encoded by separate genes: COX-1 and COX-2 (Appleton *et al.*, 1996; Vane *et al.*, 1998). COX-1 is constitutively expressed in most tissues and generates PGs for physiological homeostasis, whereas COX-2 is an inducible gene that generates high levels of PGs during inflammation (Seibert and Masferrer, 1994). Following formation of PGH<sub>2</sub> by COX, PGE<sub>2</sub> synthase converts PGH<sub>2</sub> to PGE<sub>2</sub> (Smith *et al.*, 1991).

In the immune system, eicosanoids are predominantly produced by APCs and act as autocrine and paracrine lipid mediators. For many years macrophages have been the focus of studies on the generation of arachidonic acid-derived mediators and these cells were considered to be the major source of eicosanoids, which also drastically affect macrophage functions (Humes *et al.*, 1977; Nathan, 1987). In fact exogenous PGE<sub>2</sub> has been shown to inhibit macrophage proliferation (Pelus *et al.*, 1979), oxygen radical generation (Smith and Weidemann, 1980), the production of IL-1 (Kunkel *et al.*, 1986a) and the accumulation of LPS-induced TNF- $\alpha$  mRNA (Kunkel *et al.*, 1988; Kunkel *et al.*, 1986b).

There is now evidence demonstrating the ability of DCs to produce arachidonic acid metabolites. *In vitro* generated DCs have been reported to possess the whole enzymatic equipment necessary for the biosynthesis of eicosanoids starting with endogenous arachidonic acid and produce a full range of arachidonic acid products, especially PGE<sub>2</sub> (Harizi *et al.*, 2001). Furthermore, *in vitro* generated BM-DCs have been shown to express all the PGE receptors (EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub>) with EP<sub>2</sub> and EP<sub>4</sub> playing a central role in the modulation of DC functions by PGE<sub>2</sub> (Harizi *et al.*, 2003; Mcja *et al.*, 1997). Depending on the site of encounter and the maturation stage, PGE<sub>2</sub> has opposite effects on DC activation. In peripheral tissues, PGE<sub>2</sub> appears to have stimulatory effects on DCs. In fact PGE<sub>2</sub> cooperates with inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, to promote DC maturation (Gualde and Harizi, 2004). Furthermore, PGE<sub>2</sub> upregulates the DC expression of CCR7 chemokine receptor and promotes their migration to lymphoid organs (Luft *et al.*, 2002; Scandella *et al.*, 2002). Conversely, in lymphoid organs PGE<sub>2</sub> plays an inhibitory role, reducing the activation of DCs and their ability to present antigen to T cells (Harizi *et al.*, 2001). Another established action of PGE<sub>2</sub> on DC function is the regulation of cytokine production, depending on the context and the microenvironment. For instance, the presence of PGE<sub>2</sub> during the priming of DC with antigen completely inhibits their ability to produce the Th1-driving cytokine IL-12. Instead PGE<sub>2</sub>-primed DCs have been shown to produce high levels of IL-10 (Kalinski *et al.*, 1997). In addition to its potential to modulate the maturation, IL-12-producing capacity and Th-cell-polarising ability of DCs, there is also evidence that PGE<sub>2</sub> inhibits the production of inflammatory chemokines CCL3 and CCL4 in mouse bone marrow-derived DCs (Jing *et al.*, 2003).

Although PGE<sub>2</sub> is known as the major COX metabolite generated by APC, DCs can also produce other prostanoids including thromboxanes A<sub>2</sub> (TXA<sub>2</sub>) and PGD<sub>2</sub>. It has been demonstrated that DC-derived TXA<sub>2</sub> affects, in a paracrine manner, the activity of T cells and modulates acquired immunity by negatively regulating DC/T cell interactions (Kabashima *et al.*, 2003a). Other studies have explored the effects of PGD<sub>2</sub> as a potential regulator of the adaptive immune response through its action on DCs. PGD<sub>2</sub> has been shown to affect the migratory properties of Langerhans cells (Angeli *et al.*, 2001) and the

maturation of human DCs (Gosset *et al.*, 2003). Moreover, it has been reported that PGD<sub>2</sub> suppresses the CD40- and LPS-induced IL-12 secretion by murine splenic DCs (Faveeuw *et al.*, 2003).

The effects of PGE<sub>2</sub> on lymphocytes have also been extensively studied. PGE<sub>2</sub> has been shown to induce B cell unresponsiveness (Schad and Phipps, 1989) and inhibition of B cell proliferation and antibody production (Kurland *et al.*, 1977; Simkin *et al.*, 1987; Thompson *et al.*, 1984). Furthermore, PGE<sub>2</sub> inhibits not only T cell IL-2 production (Minakuchi *et al.*, 1990; Rappaport and Dodge, 1982), but also accumulation of IL-2 mRNA (Minakuchi *et al.*, 1990), IL-2 receptor expression (Rincon *et al.*, 1988) and antigen- and mitogen-induced T cell proliferation (Ellner and Spagnuolo, 1979; Minakuchi *et al.*, 1990).

Host cells are therefore one source of eicosanoids during infection. Pathogens may induce the production of these lipid mediators, which could in turn act in an autocrine manner and suppress some key functions of the secreting cell itself or in a paracrine manner and modulate the biology of immune cells present in the local environment. Another potential source of eicosanoids is the pathogen itself. Many reports have suggested that pathogenic helminths, protozoa and fungi have the ability to produce prostaglandins and prostaglandin-like molecules (Noverr *et al.*, 2003), which may play a role in suppression of the host immune response, inhibiting macrophages, DCs and T and B cell functions or induce inappropriate T cell responses that could affect the severity of the infection (Belley and Chadee, 1995). It is also possible that parasite-derived eicosanoids are directly involved in the pathogenesis of parasitic diseases, regulating physiological processes in host tissues that enable the parasites to disseminate, migrate, reproduce and persist in the body (Belley and Chadee, 1995). Among the parasites known to produce eicosanoids are *T. brucei* (Kubata *et al.*, 2000), *S. mansoni* (Fusco *et al.*, 1985), *B. malayi* (Liu *et al.*, 1992) and *P. falciparum* (Kilunga Kubata *et al.*, 1998).

### 1.2.8.2 Role of peroxisome proliferation-activated receptors on DC function

Some eicosanoids, such as PGJ<sub>2</sub> and 15-deoxy PGJ<sub>2</sub>, are potent ligands of the intracellular receptors peroxisome proliferation-activated receptors (PPARs) (Daynes and Jones, 2002). PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily (Oberfield *et al.*, 1999). They function as regulators of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation and inflammatory responses (Lemberger *et al.*, 1996; Pineda Torra *et al.*, 1999). So far three PPAR isoforms have been identified and are encoded by separate genes on different chromosomes: PPAR $\alpha$ , PPAR $\beta/\delta$  and PPAR $\gamma$  (Daynes and Jones, 2002). There is evidence indicating a role of PPARs in the control of various types of inflammatory responses. These functions are mediated largely through the abilities of PPAR $\alpha$  and PPAR $\gamma$  isoforms to repress the activities of many activated transcription factors such as nuclear factor-kB (NF-kB), signal transducers and activators of transcriptions (STATs), activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT) (Daynes and Jones, 2002).

Recent reports have shown that DCs predominantly expressed the PPAR $\gamma$  isoform and that agonist-induced activation of PPAR $\gamma$  can influence DC maturation (Gosset *et al.*, 2001). Shorter PPAR $\gamma$  activation periods was found to have milder immunological effects on DCs mainly leading to the development of Th2 immune responses due to a down-regulation of IL-12 secretion and to a selective inhibition of Th1 lymphocyte-recruiting chemokines in DCs (Gosset *et al.*, 2001). Sustained PPAR- $\gamma$  activation, achieved by generating human DCs in the presence of 15-deoxy PGJ<sub>2</sub> or synthetic PPAR $\gamma$  agonists, resulted in more profound effects on DC function causing impairment of the expression of costimulatory molecules and cytokine production (including Th2 cytokines) and affecting the ability of DCs to induce lymphocyte proliferation and antigen-specific T cell responses (Nencioni *et al.*, 2002). Therefore, it was suggested that sustained PPAR $\gamma$  activation in DCs might result in induction of anergy/tolerance in T lymphocytes instead of committing them toward type 2 cytokine- secreting cells. Furthermore, the activation of PPAR $\gamma$  was shown to reduce the ability of murine splenic DCs to produce IL-12 in

response to CD40 or LPS stimulation (Faveeuw *et al.*, 2000) and to affect the maturation of human monocyte-derived DCs by reducing the secretion of chemokines, such as CCL3 (Gosset *et al.*, 2001). More recently Appel *et al.* (Appel *et al.*, 2005) reported that TLR ligand-induced activation and migration of human monocyte-derived DCs, as well as their ability to initiate lymphocyte proliferation, was impaired upon PPAR $\gamma$  activation. Activation of PPAR $\gamma$  resulted in down-regulation of costimulatory and adhesion molecules by DCs and reduced secretion of cytokines and chemokines involved in T lymphocyte activation and recruitment. These inhibitory effects on TLR induced DC activation were shown to be mediated *via* inhibition of the NF- $\kappa$ B and MAP kinase pathways (Appel *et al.*, 2005). Therefore, it is reasonable to assume that pathogens may modulate DC function by inducing activation of PPAR $\gamma$ . This could potentially be achieved either by inducing DCs to produce prostaglandins, which in turn can activate PPAR $\gamma$  or in certain cases by prostaglandins produced by the pathogen itself as explained in section 1.2.8.1.

#### 1.2.8.3 Indoleamine 2,3 dioxigenase-regulation of DC function

Recently there has been a growing interest in investigating an immunosuppressive mechanism, involving tryptophan catabolism by DCs through expression of the enzyme indoleamine 2,3-dioxygenase (IDO). It has been reported that PGE<sub>2</sub> induces mRNA expression of IDO in DCs *via* interaction with the EP<sub>2</sub> receptor. However, the enzyme remained inactive (Braun *et al.*, 2005). A second signal through TNF receptor or a TLR was shown to be necessary to activate the enzyme (Braun *et al.*, 2005). Interestingly, the use of TNF- $\alpha$  or LPS alone did not upregulate IDO expression (Braun *et al.*, 2005). Early studies documented the ability of IDO to inhibit the proliferation of intracellular pathogens and tumour cells *in vitro* through consumption of the essential amino acid tryptophan (Taylor and Feng, 1991). Although many microorganisms can synthesize their own tryptophan, some depend on exogenous sources. Such organisms are sensitive to the tryptophan-depleting activity of IDO. Examples include *Chlamydia pneumoniae*, *Toxoplasma gondii* and certain bacteria such as group B streptococci and *mycobacteria* (Carlin *et al.*, 1989; Gupta *et al.*, 1994; Hayashi *et al.*, 2001; MacKenzie *et al.*, 1998;

Pfefferkorn *et al.*, 1986). Furthermore, it has been reported that during viral infection, IDO inhibits the replication of cytomegalovirus and herpes simplex virus *in vitro* (Adams *et al.*, 2004; Bodaghi *et al.*, 1999). Although tryptophan depletion was the first mechanism recognized for IDO, other models suggested that toxic metabolites of tryptophan played an important role in mediating its effects. Mouse thymocytes and mouse CD4<sup>+</sup> T cell clones were shown to be sensitive to apoptosis induced by tryptophan metabolites such as quinolinic acid (Fallarino *et al.*, 2002). In the latter study, Th1 cell clones but not Th2 cell clones were sensitive to metabolite-induced apoptosis, raising the possibility that IDO might alter the Th1/Th2 balance. Human T cells were also found to be sensitive to the anti-proliferative and cytotoxic effects of exogenously added tryptophan metabolites (Frumento *et al.*, 2002; Terness *et al.*, 2002). A third mechanism by which IDO might exert its effects is by altering the biology of the IDO-expressing APCs (Hwu *et al.*, 2000; Munn *et al.*, 1999) It has been reported that pre-activation of CD8 $\alpha$ <sup>+</sup> DCs *in vitro* with IFN- $\gamma$ , which has been shown to be a potent inducer of IDO expression (Taylor and Feng, 1991), rendered them tolerogenic when subsequently injected *in vivo* (Grohmann *et al.*, 2000). This effect required IDO expression during the *in vitro* pre-activation, as addition of a pharmacological inhibitor of IDO, 1-methyl tryptophan (1-MT), (Cady and Sono, 1991) during that time blocked the development of tolerogenic activity (Grohmann *et al.*, 2000). The effect of IDO, however, seemed to be exerted on the DCs themselves rather than on the responding T cells as 1-MT was not present when DCs were subsequently transferred into the recipient host (Grohmann *et al.*, 2000). The nature of this effect is unknown but it has been speculated to be mediated either by intracellular tryptophan depletion, or by some effect of intracellular tryptophan metabolites. IDO might, therefore, functionally alter the DC either by decreasing its APC function or by upregulating the expression of suppressive ligands (such as CD95) or by triggering the secretion of immunoregulatory cytokines (such as IL-10 or TGF- $\beta$ ). Although these notions are speculative in the case of DCs, IDO has been shown to regulate gene expression and cell biology in other cell types (Li *et al.*, 2004b; Marshall *et al.*, 2001).

IDO-dependent suppression of T cell responses has also been reported and might function as a natural immunoregulatory mechanism (Mellor and Munn, 1999). Studies from a range of mouse models demonstrated that IDO regulates adaptive T cell immunity (Mellor and Munn, 2004). In many of these studies 1-MT was used to inhibit IDO activity *in vivo*. Administration of 1-MT to pregnant mice resulted in rejection of allogeneic fetuses (Mellor *et al.*, 2001; Munn *et al.*, 1998). In autoimmune models, 1-MT treatment exacerbated symptoms of experimental autoimmune encephalomyelitis (Sakurai *et al.*, 2002) and markedly increased disease severity and lethality in a model of T cell mediated colitis (Gurtner *et al.*, 2003). By contrast, overexpression of IDO results in immunosuppression and tolerance. IDO-transfected cell lines suppressed antigen-specific T cell responses *in vitro* (Mellor *et al.*, 2002) and IDO overexpression in mouse tumour cell lines rendered them resistant to immune rejection (Uytenhove *et al.*, 2003).

#### 1.2.8.4 Nitric oxide-regulation of DC function

Nitric oxide (NO) is a pleiotropic second messenger, that can exert its effects in an autocrine or paracrine fashion on neighbouring cells (Korhonen *et al.*, 2005). It is generated intracellularly by NO synthases (NOS), which can be constitutive (cNOS) or inducible (iNOS). cNOS can produce only low levels of NO, whereas iNOS is expressed primarily in response to pro-inflammatory cytokines or bacterial products and catalyses the production of much higher amounts of NO (Korhonen *et al.*, 2005). It is known that NO can exert beneficial or detrimental effects on immune responses depending on the timing and amounts of its production and the biological milieu in which it is released (Korhonen *et al.*, 2005). In activated macrophages, NO and its metabolites have been shown to mediate a number of host defence functions that include anti-microbial and tumoricidal activity (Bogdan, 2001). Due to its anti-microbial effects, NO is an important molecule of the innate immune system. Nevertheless, suppression of host immune responses by NO and other reactive nitrogen intermediates (RNI) has also been reported during many infections. They were shown to be involved in the immunosuppression of T cell proliferative response in mice infected with *T. brucei* (Sternberg and McGuigan, 1992) and *L. monocytogenes* (Gregory *et al.*, 1993). RNI were also reported to be

responsible for the decreased proliferative responses of splenocytes observed in a murine model of filariasis (O'Connor *et al.*, 2000) and toxoplasmosis (Candolfi *et al.*, 1994).

Evidence obtained from murine models, indicates that NO may also modulate DC function. Bone marrow-derived DCs were shown to produce NO following IFN- $\gamma$  and LPS stimulation *in vitro* and inhibited T cell proliferation (Bonham *et al.*, 1996). In addition to its ability to inhibit T cell proliferation, NO was also shown to induce programmed cell death in DCs (Bonham *et al.*, 1996; Lu *et al.*, 1996). The percentage of DC apoptosis correlated with the level of NO in the cultures and was blocked by using inhibitors of NOS (Bonham *et al.*, 1996). Other studies showed that NO, either exogenous or produced by iNOS in DCs themselves, inhibited the antigen presenting function of DCs (Holt *et al.*, 1993; Lu *et al.*, 1996). More recent studies have confirmed the inhibitory role that NO may play on DC function. NO has been reported to selectively downregulate the expression of the costimulatory molecule CD86 on monocyte-derived DCs in response to LPS or soluble CD40 ligand and led to a decreased capacity of DCs to stimulate T cells *in vitro* (Corinti *et al.*, 2003). Furthermore, NO inhibited the release of IL-10 and IL-12p40 by mature DCs as well as the secretion of chemokines crucially involved in T cell recruitment (IP-10/CXCL10 and RANTES/CCL5) (Corinti *et al.*, 2003). A subsequent study confirmed the involvement of NO in the suppression of IL-12p40 production by murine macrophages and dendritic cells (Xiong *et al.*, 2004). Splenocytes derived from iNOS-deficient mice were shown to produce increased IL-12p40 protein and mRNA compared to wild type mice of the same strain. The inhibitory effects of NO on IL-12 production were reported to be independent of IL-10 (Xiong *et al.*, 2004). Furthermore, NO was shown to inhibit TLR signal transduction through inhibition of IRAK activity and consequent attenuation of the molecular interaction between TRAF6 and IRAK (see Figure 1.3 for TLR signaling pathway) (Xiong *et al.*, 2004). These experiments suggest a role for NO in the regulation of Th1-type immunity through inhibition of IRAK activity, NF- $\kappa$ B and consequently IL-12. The effects that NO exert on plasmacytoid DCs has also been recently examined (Morita *et al.*, 2005). NO was reported to inhibit IFN- $\alpha$ , IL-6 and TNF- $\alpha$  production by human plasmacytoid DCs and polarized them toward a Th2-promoting phenotype partly *via* a Guanosine 3', 5'-

Cyclic Monophosphate (cGMP)-dependent pathway (Morita *et al.*, 2005). All the above studies clearly indicate that NO can affect the outcome of the host immune response by interfering with DC function and may therefore represent a potential mechanism used by pathogens to subvert protective immune responses directed against them.

#### 1.2.8.5 TGF- $\beta$ regulation of DC function

TGF- $\beta$  is a potent regulatory cytokine with a pivotal role in maintaining tolerance *via* the regulation of lymphocyte proliferation, differentiation and survival (Li *et al.*, 2005). In addition, TGF- $\beta$  controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis, activation and survival of lymphocytes, NK cells, DCs, macrophages, mast cells and granulocytes (Li *et al.*, 2005). It is produced by a wide range of cells including macrophages, DCs, NK cells and T and B cells and is secreted in a biologically inactive or latent form that must be cleaved and activated before interaction with its receptor (Letterio and Roberts, 1998).

Many studies have reported the role of TGF- $\beta$  in affecting DC function. It regulates the maturation of differentiated DCs and DC-mediated T cell responses (Li *et al.*, 2005). DCs differentiated from human haematopoietic progenitor cells or monocytes in the presence of TGF- $\beta$  have been shown to express predominantly intracellular MHC class II, low levels of CD1d and costimulatory molecules CD80, CD83 and CD86 (Geissmann *et al.*, 1999; Ronger-Savle *et al.*, 2005). These observations are consistent with murine studies in which TGF- $\beta$  inhibits maturation of DCs differentiated from bone marrow cells with GM-CSF (Yamaguchi *et al.*, 1997), demonstrating that TGF- $\beta$  promotes the generation of DCs with an immature phenotype. TGF- $\beta$  also regulates the antigen presenting function of differentiated DCs *in vitro*. The presence of TGF- $\beta$  in LPS-stimulated DC cultures inhibits the expression of MHC class II and costimulatory molecules, which attenuates the antigen presenting function of DCs (Geissmann *et al.*, 1999). In addition, DC maturation and IL-12 production induced by inflammatory cytokines such as IL-1 and TNF- $\alpha$  are inhibited by TGF- $\beta$  (Geissmann *et al.*, 1999). In contrast, CD40L-induced DC maturation or cytokine production is not affected by TGF- $\beta$  (Geissmann *et al.*, 1999).

CD40L represents a key T helper signal for DC maturation as explained earlier in section 1.2.5.1. Lack of TGF- $\beta$  inhibition of CD40L-induced DC maturation implies that TGF- $\beta$  primarily suppresses non-cognate DC activation.

There is evidence that pathogens have evolved mechanisms to induce infected host cells to secrete active TGF- $\beta$ , which in turn suppresses APC function, enhancing pathogen survival. For example, infection of macrophages with the intracellular bacteria *M. avium* increases macrophage production of active TGF- $\beta$  and suppresses their antibacterial activity (Champsi *et al.*, 1995). Parasites have also developed similar mechanisms for their survival and proliferation in the mammalian host. For instance, *T. cruzi* directly uses the host TGF- $\beta$  signaling pathway to invade host cells (Ming *et al.*, 1995). *T. cruzi* is in fact unable to invade cells lacking TGF- $\beta$  receptors or cells in which the downstream signaling of these receptors is interrupted (Ming *et al.*, 1995). Furthermore, *T. cruzi* has been shown to activate the TGF- $\beta$  signaling pathway directly to facilitate its entry into mammalian cells (Ming *et al.*, 1995). *Leishmania* species have also been reported to be potent inducers of TGF- $\beta$  (Barral-Netto *et al.*, 1992) and are able to activate latent TGF- $\beta$  directly to its bioactive form. Activation of TGF- $\beta$  was linked to enhanced parasite survival within macrophages (Gantt *et al.*, 2003).

### 1.2.9 Pathogen-induced T cell anergy

The ability of pathogens to induce T cell anergy has been extensively reported. In a recent study human rhinovirus was shown to significantly down-regulate the T cell stimulatory capacity of DCs through the induction of a novel DC activation program (Kirchberger *et al.*, 2005). It was demonstrated that co-cultivation of DC with the virus induces the expression of inhibitory receptors B7-H1 (PDL-1) and sialoadhesin (Sn, Siglec-1, CD169) without affecting stimulatory receptors such as CD80 or CD86. The consequence of this altered accessory repertoire on rhinovirus-treated DCs is that co-cultured T cells acquire a deep anergic state, which is antigen-nonspecific and not reversible by exogenous IL-2 (Kirchberger *et al.*, 2005). Hepatitis C virus provides another example of a virus, which induces T cell anergy characterized by a decreased IL-

2 response and impaired activation of the JNK signaling pathway (Sundstrom *et al.*, 2005). Similarly Helminth parasites have been shown to secrete substances, such as ES-62, that render lymphocytes anergic (Harnett *et al.*, 1998) and Leishmania antigens were found to induce T cell anergy due defective antigen presentation (Pinheiro *et al.*, 2004).

Although induction of regulatory T cells, anergy and apoptosis play important roles in the maintenance of central and peripheral tolerance by providing mechanisms to avoid inappropriate immune responses to 'self' or to harmless foreign antigens, it has become clear that many pathogens have evolved the ability to use these mechanisms to their advantage. Altering DC function is particularly advantageous to pathogens as these cells have the unique ability to induce primary immune responses by efficient activation of naïve T cells. By interfering with DC functions, microbes can therefore avoid the induction of immune responses directed against them, thus prolonging their survival and increasing the possibility of transmission to other hosts.

#### 1.2.10 Pathogen-induced apoptosis

Interaction of some pathogens with DCs has been reported to result in T cell apoptosis. A recent report demonstrated that both inactivated and infectious HIV-1 stimulated plasmacytoid DCs to secrete IFN- $\alpha$ , which in turn regulated the expression of TRAIL (TNF-related apoptosis-inducing ligand) by primary CD4<sup>+</sup>T cells, leading to T cell apoptosis (Herbeuval *et al.*, 2005). This observation provided a unique role for plasmacytoid DCs in TRAIL-mediated apoptosis of CD4<sup>+</sup>T cell in HIV-1 infection and progression to AIDS.

DCs have also been shown to acquire killer functions *via* expression of TRAIL during Measles infection, hence becoming cytotoxic effector cells with the ability to kill facing activated T cells (Vidalain *et al.*, 2001). Furthermore, Measles virus-infected DCs have been reported to undergo Fas ligand (FasL)-dependent apoptosis by up-regulation of Fas following interaction with the virus. As activation up-regulates FasL expressed by T cells, early apoptosis of Fas-sensitive infected DCs would prevent subsequent T cell

activation and could account for the *in vivo* suppression of cell-mediated immunity (Vidalain *et al.*, 2001). Therefore, besides inducing the development of DCs with 'regulatory' phenotype and inhibiting naïve or activated T cell proliferation, Measles virus induces the synthesis of TRAIL death factor in DCs, leading to activated T cell apoptosis and finally induces FasL-dependent apoptosis of the DCs themselves (Vidalain *et al.*, 2001).

The ability to induce DC apoptosis is not unique to Measles virus. Vaccinia virus and canarypox virus have both been associated with inhibition of DC maturation and DC apoptosis (Engelmayer *et al.*, 1999; Ignatius *et al.*, 2000). Likewise, *Streptococcus pneumoniae* has been reported to induce DC apoptosis through two distinct mechanisms: a rapid caspase-independent mechanism, critically dependent on bacterial expression of pneumolysin, and a delayed-onset caspase-dependent mechanism associated with terminal DC maturation (Colino and Snapper, 2003). The latter mechanism was found to be independent of Fas/FasL interactions and TNF- $\alpha$  and did not require bacterial internalisation. Instead it was dependent upon interaction between bacterial subcapsular components with a cell-surface receptor on DCs (likely a TLR) acting in a MyD88-dependent manner (Colino and Snapper, 2003).

### 1.3 *Plasmodium* and Dendritic cells

As previously described in section 1.2, DCs are professional antigen presenting cells with the ability to induce primary immune responses by efficient activation of naïve T cells (Adams *et al.*, 2005). They are generally the first cells of the immune system to encounter foreign organisms and their subsequent interaction with other cells of the immune system is a major component of the immune response (Adams *et al.*, 2005). Therefore, it is reasonable to assume that DCs also play a critical role in initiating immune responses to malaria.

Urban *et al.* first showed that *P. falciparum* infected erythrocytes could prevent up-regulation of MHC class II and co-stimulatory molecules CD83 and CD86 on human DCs in response to LPS treatment *in vitro* (Urban *et al.*, 1999). In addition to affecting maturation, they impeded the ability of DCs to induce antigen-specific primary and secondary T cell responses (Urban *et al.*, 1999). CD36 and CD51 were identified as the receptors on DCs responsible for this inhibitory effect (Urban *et al.*, 2001b). These same receptors were found to mediate the inhibitory effect of macrophages by decreasing TNF- $\alpha$  and IL-1 secretion during malaria infection (Schwarzer *et al.*, 1992). It is interesting that these molecules are responsible for the recognition of apoptotic cells by phagocytic cells, which can also have suppressive effects on DC function and maturation (Voll *et al.*, 1997). Interaction of infected erythrocytes with human DCs also resulted in decreased IL-12 secretion by DCs, which would otherwise promote adequate effector T cell proliferation and differentiation towards a Th1 phenotype as described in section 1.2.5.1. Instead, an increase in IL-10 was observed, which could potentially directly suppress the stimulatory function of DCs (Haase *et al.*, 2002; McBride *et al.*, 2002) as well as promote the generation of T regulatory cells as previously explained in section 1.2.5.2. Moreover, a study in malaria-exposed children showed that DCs in peripheral blood may have down-regulated MHC class II expression (Urban *et al.*, 2001a), suggesting that DC function can be impaired *in vivo* during the acute stages of malaria infection.

These human studies provoked the generation of a number of animal models investigating the function of DCs during malaria infection *in vivo*. Although there are less than a handful published studies, there are already inconsistencies in the literature on the effect of DC function after interaction with malaria parasites. Seixas and colleagues reported that bone marrow-derived DCs up-regulated the expression of MHC class II, CD40 and CD86 after exposure to *P. chabaudi* infected erythrocytes (Seixas *et al.*, 2001). Their ability to stimulate T cell responses was maintained and increased production of TNF- $\alpha$ , IL-12 and IFN- $\gamma$  was observed (Seixas *et al.*, 2001). Similarly Pichyangkul *et al.* reported activation of murine bone marrow-derived and human plasmacytoid DCs following interaction with *P. falciparum* schizonts *in vitro* as observed by the up-regulation of CD86 expression and IFN- $\alpha$  production (Pichyangkul *et al.*, 2004). In this particular study the schizont immune stimulatory effects were shown to require the TLR9-MyD88 signalling pathway (Pichyangkul *et al.*, 2004).

Luyendyk and colleagues focused on analysing splenic CD11b<sup>+</sup> and CD11c<sup>+</sup> DC subtypes after acute infection with *P. yoelii* infected mice. They found that MHC class II and CD80 molecules on DCs were up-regulated and supported IFN- $\gamma$  production (Luyendyk *et al.*, 2002). A similar study carried out by Perry and colleagues confirmed these results as they showed that DCs taken from *P. yoelii* infected animals expressed high levels of co-stimulatory molecules, activated naïve T cells to produce IL-2 and supported high levels of IFN- $\gamma$  and TNF- $\alpha$  production in an IL-12-dependend manner (Perry *et al.*, 2004). Furthermore, in a subsequent study, Perry *et al.* showed that as the infection progressed, DCs became refractory to TLR-mediated IL-12 and TNF- $\alpha$  production, while increasing their ability to produce IL-10 and retaining their capacity for activation of naïve T cells (Perry *et al.*, 2005).

The above observations contrast human studies with *P. falciparum* (Urban *et al.*, 1999) as well as a study in mice carried out by Ocana-Morgner and colleagues who showed that *P. yoelii* infected erythrocytes inhibited the LPS-induced maturation of bone marrow-derived DCs *in vitro* (Ocana-Morgner *et al.*, 2003). Thus, there is disagreement in the literature on the role of DCs in protective immunity to blood-stage malaria. Since the

*Plasmodia* parasites have been shown to impair human (Urban *et al.*, 1999) and murine (Ocana-Morgner *et al.*, 2003) DC maturation *in vitro*, it has been suggested that DCs cannot prime protective immunity during infection. However, Bruna-Romero and colleagues have shown that DCs presenting *P. yoelii* sporozoite antigens can induce protective immunity against liver-stage malaria in mice and can stimulate both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses (Bruna-Romero and Rodriguez, 2001). Furthermore Pouniotis *et al.* have directly addressed the hypothesis that DCs can prime blood-stage malaria immunity (Pouniotis *et al.*, 2004). Maturation-arrested DCs *in vitro* were used to immunise animals, which were then challenged 10 days later with freshly isolated infected erythrocytes. DCs pulsed with *P. yoelii* or *P. chabaudi* infected erythrocytes were shown to be able to induce antigen-specific T cell responses, antibody responses and potent protection against lethal blood-stage malaria challenge *in vivo* despite an *in vitro* parasite-induced maturation defect (Pouniotis *et al.*, 2004). It was suggested that one possibility to explain the efficacy of the parasite-treated DCs immunisation was that, although a maturation defect was evident *in vitro*, once returned to the *in vivo* environment, these DCs can eventually develop into mature DCs (Pouniotis *et al.*, 2004). Thus, there may be a delay in maturation rather than a maturation arrest. These results suggest that DCs may be involved in the induction of immunity against blood-stage malaria parasites and further indicate that targeting of parasite antigens to DCs may provide an attractive approach for the development of blood-stage malaria vaccines.

### 1.3.1 Effect of haemozoin on phagocytic cells

Besides reports describing contrasting effects of *Plasmodium*-infected erythrocytes on DC function, different outcomes have also been reported regarding the effects of haemozoin on phagocytic cells.

The malaria parasite resides inside the erythrocytes of the infected host during the asexual blood-stage of its life cycle and uses haemoglobin as a nutrient (Goldberg, 1993). The parasite digests about 75% of the host haemoglobin but is unable to catabolize the toxic free haeme, which is formed as a byproduct of haemoglobin degradation (Francis *et*

*al.*, 1997). To protect the parasite membranes against the toxic effect of haeme molecules, the parasite converts the free haeme into haemozoin (malaria pigment) using a unique pathway referred to as the haeme polymerisation or biocrystallisation (Slater *et al.*, 1991).

Haemozoin enters phagocytes either within parasitized erythrocytes or, after schizont rupture, as a residual body and it has been reported to influence the immune responses of the host. Arese and colleagues have extensively studied the effects of haemozoin on human monocyte/macrophage function. *In vitro* studies have shown that haemozoin-fed monocytes are viable but functionally impaired (Arese and Schwarzer, 1997). They are unable to digest haemozoin or to repeat phagocytosis (Schwarzer *et al.*, 1992), to generate oxidative burst upon appropriate stimulation (Schwarzer and Arese, 1996) or to kill ingested bacteria, fungi or tumor cells (Fiori *et al.*, 1993). Haemozoin-fed human and murine monocytes/macrophages were found to release large amounts of TNF- $\alpha$  (Pichyangkul *et al.*, 1994; Prada *et al.*, 1995; Sherry *et al.*, 1995), nitric oxide (Prada *et al.*, 1996), macrophage-inhibitory protein 1 $\alpha$  and macrophage-inhibitory protein 1 $\beta$  (Sherry *et al.*, 1995) and reduced amounts of IL-6 (Prada *et al.*, 1995). In addition, monocyte protein kinase C, an effector of phagolysosome formation, lysosomal acidification and lysosomal enzyme production (Gewert *et al.*, 1995; Tapper and Sundler, 1995), was found to be inhibited after haemozoin phagocytosis (Schwarzer *et al.*, 1993). Inhibition of protein kinase C activity was suggested to be responsible for the increased levels of 4-hydroxy-nonenal in haemozoin-loaded monocytes, which was found to depress expression of MHC class II proteins in monocytes (Schwarzer *et al.*, 1998; Schwarzer *et al.*, 1996).

More recent studies have confirmed the potent pro-inflammatory effects of haemozoin *in vivo* (Jaramillo *et al.*, 2004) as well as demonstrating its involvement in the generation of large amounts of bioactive peroxidation derivatives of polyunsaturated fatty acids that inhibit monocyte function (Schwarzer *et al.*, 2003). Furthermore haemozoin has been reported to inhibit the proliferative response of mitogen-stimulated human peripheral blood mononuclear cells *via* an IL-10-dependent mechanism (Deshpande *et al.*, 2004) as

well as affecting the ability of monocytes to differentiate and mature to functioning DCs (Skorokhod *et al.*, 2004).

The effects that haemozoin exert on DC function have not been extensively investigated and there are only two published studies to date on this subject. Coban and colleagues first reported that purified haemozoin from *P. falciparum* enhanced the maturation of myeloid DCs *in vitro* as observed by the up-regulation of DC surface markers CD83, CD86 and CD1a (Coban *et al.*, 2002). Subsequently, the ability of haemozoin to activate innate immune responses, resulting in the production of cytokines, chemokines and up-regulation of costimulatory molecules, was confirmed *in vivo* and demonstrated that haemozoin exerted its effects through TLR9 (Coban *et al.*, 2005).

All of the above studies clearly indicate that when ingested by phagocytic cells, haemozoin is not inert but can either impair a number of functional parameters in the case of monocytes and macrophages or activate immune responses by inducing DC maturation and cytokine production. A better understanding of the mechanisms by which haemozoin exerts its effects on immune cells may aid in the development of novel therapeutic approaches and offer a new chance to control pathogenic mechanisms in malaria.

## 1.4 Aim of project

The present study aims at examining the effects that *P. chabaudi* AS infection have on DC function *in vitro*, with the view that a better understanding of how the malaria parasite affects DC function could have important implications for vaccine design and immunotherapy and might aid in the development of effective DC-based vaccines. Moreover, as the morbidity and mortality associated with malaria are derived exclusively from the asexual erythrocytic stages, I examined whether intact, trophozoite-infected erythrocytes modulate key functions of DCs that may potentially lead to or contribute to more generalised immunosuppression in the host. In particular, the erythrocytic trophozoite stage was selected for this study first because the majority of the parasite induced changes in the infected erythrocytes have been shown to occur during this stage (Newbold, 1984); secondly because trophozoite-infected erythrocytes are less fragile compared to cells harbouring the schizont stage of the parasite (Newbold *et al.*, 1982), limiting the possibility that various metabolites released in the culture following schizont rupture might be responsible for any effects observed on DC function.

## 2. Materials and Methods

## **2.1 Tissue culture**

### **2.1.1 Animals**

Female BALB/c mice were purchased from *Harlan Olac* (Bicester, U.K.). DO11.10 SCID mice, with CD4<sup>+</sup> T cells specific for OVA<sub>323-339</sub> peptide in the context of I-A<sup>d</sup> recognised by the KJ1.26 clonotypic antibody (Haskins *et al.*, 1983; Murphy *et al.*, 1990) were bred in-house at the Central Research Facility, University of Glasgow. All mice were kept under specific pathogen-free conditions and first used between 6 and 8 weeks of age in accordance with local and U.K. Home Office ethical regulations.

### **2.1.2 Parasites**

*Plasmodium chabaudi chabaudi* AS strain was originally isolated from adult thicket rats (*Thamnomys rutilans*) in 1967 by Professor David Walliker (University of Edinburgh) from La Maboque, Central African Republic. The strain was subsequently established in laboratory mice and cloned by limiting dilution (Walliker *et al.*, 1971). Stabilates of parasites, derived from the original AS parent clone, were maintained by cryopreservation in liquid nitrogen and subpassage through mice.

### **2.1.3 Cryopreservation of blood**

Infected blood, at a parasitaemia of 20-25% containing ring stage parasites, were collected by cardiac puncture from mice sacrificed in a CO<sub>2</sub> chamber into a syringe containing sodium heparin (1000 i.u./ml) in phosphate buffer saline (PBS, pH 7.2) as an anticoagulant at 10 i.u. heparin per ml of blood. The infected blood was diluted with an equal volume of a sorbitol-glycerol solution (38% glycerol, 2.9% sorbitol, 0.63% NaCl). 200µl aliquots were dispensed into 1.2 ml cryopreservation vials (Nunc, Hereford, UK). The vials were snap frozen by immersion into liquid nitrogen and stored until needed.

### **2.1.4 Maintenance of *P. chabaudi chabaudi* AS**

For long term preservation, cryopreservation vials of blood infected with *P. chabaudi* AS parent were kept in liquid nitrogen (-196°C). Infected blood was recovered for experimental use by allowing the vials to thaw at room temperature. Once defrosted, an equal volume of a 17.5% sorbitol solution was added slowly with frequent mixing (Gray and Phillips, 1981). The diluted blood was administered by i.p. injection into one or two recipients naïve BALB/c mice from which the experimental groups were infected once the parasitaemia reached 20-30%.

### **2.1.5 Determination of parasitaemia**

The level of parasitaemia of infected mice was determined by daily examination of stained thin blood smears. Samples were taken between 8:00 and 9:00 each day by piercing the tip of the tail with a lancet (BD, Oxford, UK). A new lancet was used for each experimental group. A drop of blood was placed at one end of a glass microscope slide (BDH, Poole, Dorset, UK), 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 (Gurr, BDH Ltd) (1:10 of Giemsa's stain in Giemsa's phosphate buffer (3g Na<sub>2</sub>HPO<sub>4</sub>, 0.6 KH<sub>2</sub>PO<sub>4</sub>, 1l distilled water, pH 7.2) for 15 minutes. 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 (Nutley, New Jersey, USA) binocular microscope. Percentage parasitaemia was determined by counting total infected and non-infected erythrocytes in a total of at least 10 fields containing around 300 RBC/field.

### **2.1.6 Challenge infections**

Parasites from frozen stock were injected i.p. into BALB/c mice and the parasitaemia monitored daily by examination of Giemsa's stained thin blood smears. *Plasmodium chabaudi* AS asexual erythrocytic stages grow synchronously. When a patent parasitaemia developed, infected blood was recovered in heparin (10 i.u./ml) by cardiac puncture and diluted in PBS (Invitrogen, Paisley, UK) to the required concentration of parasitised erythrocytes (pRBCs). Experimental mice were infected with pRBCs

administered i.p. as a 0.25 ml inoculum. Infected mice were held in reverse light/dark cycle so that parasites harvested at 08:00 hours were at the late trophozoite stage. Blood was collected when parasitaemia was 30-40 %. In all experiments the ratio of pRBC to DC was 100:1 unless otherwise stated.

### 2.1.7 Generation of bone marrow-derived DCs

GM-CSF was made by growing the x63 GM-CSF myeloma cell line transfected with mouse GM-CSF cDNA (Lutz *et al.*, 1999) for 3-5 days in complete RPMI (cRPMI: RPMI 1640 supplemented with 2-mercaptoethanol (2-ME, 50  $\mu$ M), L-Glutamine (2 mM), penicillin (100  $\mu$ g/ml), streptomycin (100  $\mu$ g/ml) (all from Invitrogen) and 10% foetal calf serum (FCS, Labtech International, East Sussex, UK)) supplemented with 0.8 mg/ml G418 (Sigma-Aldrich, Poole, UK) and then harvesting the supernatant.

Bone marrow-derived DCs were generated as previously described (Lutz *et al.*, 1999). Briefly, femurs and tibiae of BALB/c mice were removed aseptically and separated from the surrounding muscle. The end of each bone was removed and the bone marrow flushed out using a 26 gauge needle (BDH Microlance) and a syringe filled with RPMI 1640 (Invitrogen). Cell clusters were disaggregated by vigorous pipetting before passing the cell suspension through sterile Nitex mesh (Cadisch & Sons, London, UK). Cells were centrifuged at 450g for 10 minutes and resuspended in cRPMI supplemented with 10% GM-CSF. The cell concentration was adjusted to  $5 \times 10^5$  cells/ml. 1ml aliquots were distributed in six-well plates (Corning/Costar, Cambridge, MA) containing 1ml of cRPMI supplemented with 10% GM-CSF and cultured at 37°C, 5%CO<sub>2</sub> in air (37°C, 5%CO<sub>2</sub>). Fresh medium (2ml) was added to the cell cultures every three days. On day 6, DCs were harvested and cultured at the required concentration with RBCs or pRBCs for each individual experimental procedure as described below.

For DC maturation, 1  $\mu$ g/ml of LPS (from *Salmonella abortus equi*; Sigma-Aldrich) or 3 $\mu$ M of CpG (Sweet *et al.*, 2002) (Sigma-Genosys, Cambridge, UK) were added to the culture and incubated for 18h at 37°C, 5%CO<sub>2</sub>.

In some experiments, 1  $\mu$ M ONO-AE3-208 (PGE<sub>2</sub>-EP<sub>4</sub> antagonist) (Kabashima *et al.*, 2003b), 10  $\mu$ M GW9662 (PPAR- $\gamma$  antagonist) (Calbiochem, San Diego, CA) (Hammad *et al.*, 2004), 1 mM L-NIL (L-NIL) (Calbiochem) (Moore *et al.*, 1994), 5  $\mu$ g/ml of anti-TGF- $\beta$  antibody (R&D Systems, Minneapolis, USA) and 2 nM 1-MT (a pharmacological inhibitor of the enzyme indoleamine 2,3-dioxygenase) (Sigma-Aldrich) (Cady and Sono, 1991) were cultured with DCs at the time of RBC or pRBC addition.

### **2.1.8 *In vitro* culture of CD40L-transfected fibroblasts with DCs**

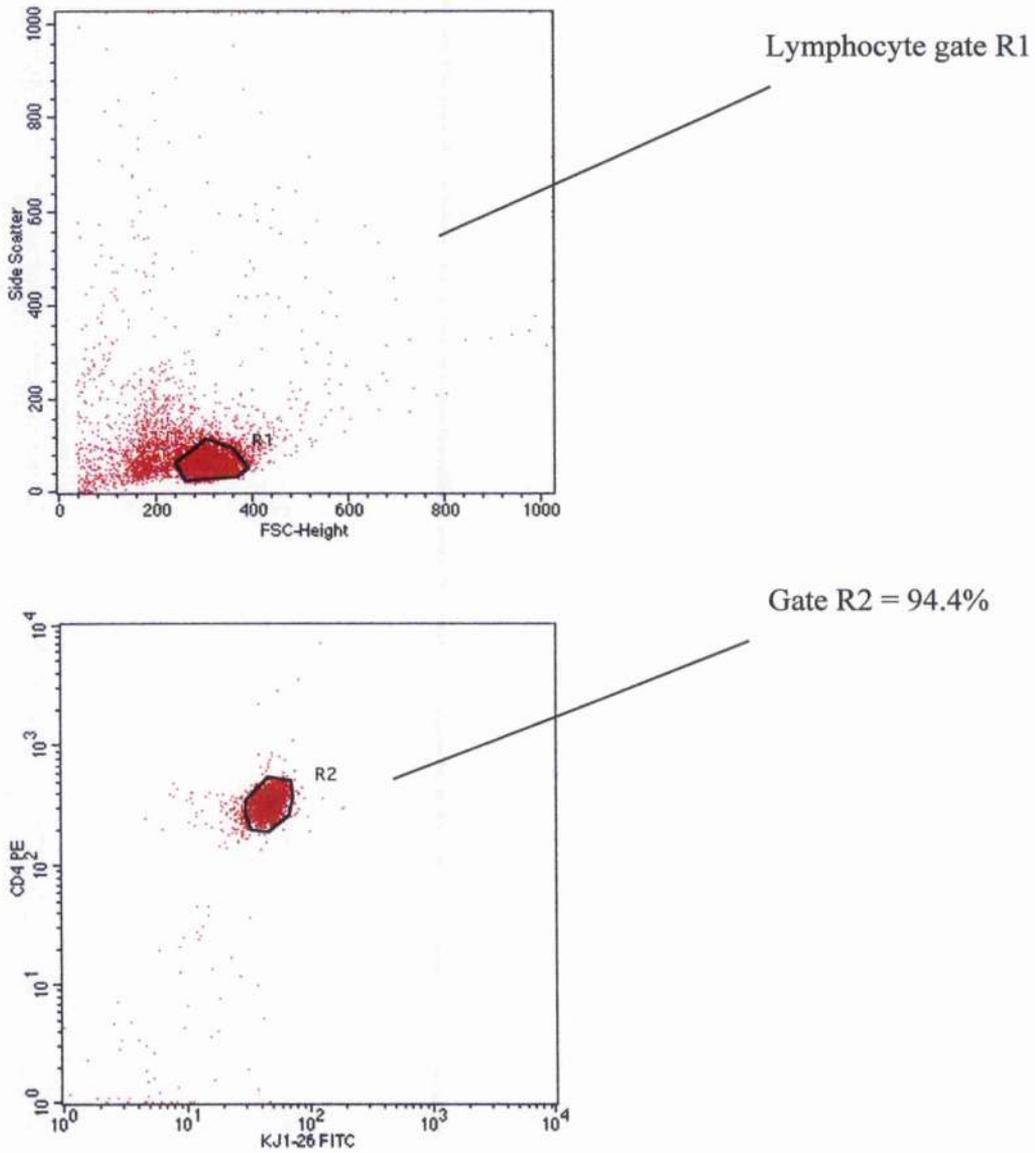
The cell lines 3T3-CD40L and 3T3-SAMEN (Schulz *et al.*, 2000) were kind gifts from Dr P. Hwu (NCI, Bethesda, MD). Cells were grown in cRPMI in T75 tissue culture flasks (Helena Biosciences, Gateshead, UK) and, when confluent, harvested and distributed in six-well plates (Corning Costar) at 2.5 x 10<sup>5</sup> cells/ml of cRPMI. Bone marrow-derived DCs were prepared as previously described and cultured with infected (pRBC) or non-infected erythrocytes (RBC) at a ratio of 1:100. After 24 h, DCs were harvested, resuspended at 1x10<sup>6</sup> cells/ml and cultured in a 1:1 ratio with either 3T3-CD40L or 3T3-SAMEN for a further 24 h. The level of CD40 expression on DCs was analysed by flow cytometry and culture supernatants collected for IL-12 cytokine analysis.

### **2.1.9 *In vitro* culture of DCs with fixed infected or non-infected erythrocytes**

Blood from *Plasmodium chabaudi* AS-infected mice was washed twice in PBS before being resuspended in cRPMI for addition to DCs. For fixation, infected blood was washed three times in PBS and resuspended in 0.5 % paraformaldehyde for 30 minutes at 4°C. Fixed erythrocytes were then washed in PBS, resuspended in 0.06% gly-gly (Sigma-Aldrich) for 5 minutes at 4°C and washed twice more in PBS before being resuspended in cRPMI for addition to DCs. After 24 h of culture, DCs were stimulated with 1  $\mu$ g/ml of LPS (Sigma-Aldrich) and the expression of cell surface molecules on DCs analysed 18 h later by flow cytometry. To confirm complete fixation, 2x10<sup>7</sup> fixed, infected erythrocytes could not establish infection when injected i.p into a female BALB/c mouse.

### **2.1.10 Lymph node single cell suspension**

Peripheral and mesenteric lymph nodes were aseptically removed from DO11.10 transgenic mice (Murphy *et al.*, 1990) on the SCID background (Saparov *et al.*, 1999). They were made into a single cell suspension by rubbing through Nitex mesh (Cadisch & Sons, London, UK) in cRPMI medium using a 2.5ml syringe plunger. Cells were centrifuged for 5 minutes at 450g and resuspended in cRPMI. The proportion of transgenic T cells in a lymph node preparation from these mice is approximately 95% as measured by flow cytometry (Figure 2.1). Cells were counted on a Neubauer Haemocytometer (Hawksley & Sons, Sussex, UK) using a phase contrast microscope. Viable cells were determined by Trypan Blue exclusion (see section 2.2.2 for details).



**Figure 2.1** Percentage of transgenic CD4<sup>+</sup>T cells in aDO11.10 SCID donor

The plots are of a lymph node preparation from a naïve DO11.10 SCID mouse. Gate R1 is set around the population of viable lymphocytes. Gate R2 is set around transgenic T cells (CD4<sup>+</sup>KJ<sup>+</sup>), after gating the plot on R1, and represent 94.4% of total cells.

### 2.1.11 Antigen presentation assay

Bone marrow-derived DCs were centrifuged at 450g, resuspended at  $1 \times 10^6$  cells/ml and 500 $\mu$ l aliquots were distributed into 24-well tissue culture plates (Corning Costar) with pRBCs or RBCs. After 24 h incubation at 37 °C in 5 % CO<sub>2</sub>, DCs were antigen pulsed for 6 h with 5 mg/ml of LPS-free OVA (Worthington Biochemical, Freehold, NJ). An OVA-specific T cell hybridoma (DO11-GFP; supplied by Dr D. Underhill, Institute for Systems Biology, Seattle, WA) expressing green fluorescent protein (GFP) under the control of NFAT (Underhill *et al.*, 1999), was added to the cultures to achieve 1:1 DC: T cell ratio. A set of cells was pulsed with an equimolar concentration of OVA peptide (aa. 323-339) (40 $\mu$ g/ml)(Sigma-Genosys) prior to the addition of T cells. GFP expression by CD4<sup>+</sup> T cells identified with PE-rat anti-mouse CD4 (BD Pharmingen, Oxford, UK) was determined by flow cytometry.

### 2.1.12 Thymidine incorporation assay

$2 \times 10^5$  DCs were cultured with  $2 \times 10^7$  pRBCs or RBCs in a 96 well plate (Corning Costar) for 24h at 37°C, 5%CO<sub>2</sub>. OVA-specific T cells were isolated from the mesenteric and peripheral lymph nodes of DO11.10 transgenic mice (Murphy *et al.*, 1990) on the SCID background (Saparov *et al.*, 1999) and cultured at a 1:1 ratio with DCs. T cell proliferation was analysed after 48, 72, 96 and 120 h of culture and assessed by incorporation of [<sup>3</sup>H] thymidine (0.5  $\mu$ Ci/well) (Radionucleotide Dispensary, Western Infirmary, Glasgow) for the last 24 h of culture. In some experiments, 10ng/ml of rIL-2 or 5 $\mu$ g/ml of anti-TGF- $\beta$  (both from R&D Systems) were added at the beginning of the proliferation assay. Cells were harvested onto filter mats (Wallac, Boston, USA) using a Betaplate 96 well harvester (Wallac) and [<sup>3</sup>H] thymidine incorporation measured on a Betaplate liquid scintillation counter (Wallac).

## **2.2 Cell viability/Apoptosis assay**

### **2.2.1 Quantitation of viable DCs**

DCs were co-cultured with RBCs or pRBCs in 6-well plates (Corning Costar) for 24h at 37°C, 5% CO<sub>2</sub>. Cells were harvested following several washes with ice cold PBS (Invitrogen). RBCs were lysed using Boyles' solution (Tris-NH<sub>4</sub>Cl buffer) (Fonseca *et al.*, 2001) and the number of viable DCs recovered from each well quantified by Trypan blue (Sigma-Aldrich) exclusion.

### **2.2.2 Trypan Blue Exclusion Assay**

Cell viability was analysed by using the trypan blue exclusion assay since trypan blue is excluded from viable cells. 50µl of cell suspensions were diluted with 50µl of 0.4% trypan blue (Sigma-Aldrich). The number of cells excluding trypan blue was assessed using a Neubauer Haemocytometer (Hawksley & Sons).

### **2.2.3 Annexin V/PI Assay**

The Annexin V/Propidium Iodide (PI) assay was used to detect cell apoptosis. In apoptotic cells, the inner membrane phospholipid phosphatidylserine (PS) is exposed to the external cellular environment. Annexin V, a phospholipid-binding protein conjugated to FITC, binds to cells with exposed PS. Viable cells exclude PI (Vermes *et al.*, 1995). An Annexin V-FITC apoptosis detection kit was purchased from BD Pharmingen. Cells to be tested for apoptosis were washed twice in cold PBS (Invitrogen) and resuspended in binding buffer provided at a concentration of  $1 \times 10^6$  cells/ml. 100µl aliquots of the cell suspension were incubated with 5µl of Annexin V-FITC and 5µl of PI at room temperature for 15 minutes prior to analysis by flow cytometry.

### 2.3 Flow cytometry

Approximately  $5 \times 10^5$  cells were transferred into 12 x 75mm polystyrene tubes (Falcon BD, Oxford, UK), centrifuged at 450g and resuspended in 100 $\mu$ l FcR blocking buffer (anti-CD16/32, clone 2.4G2, hybridoma supernatant, 10 % mouse serum (Alba Bioscience, Edinburgh, UK) and 0.1 % sodium azide (Sigma-Aldrich)), to reduce non-specific binding by Fc receptors, and the appropriate fluorochrome-conjugated or biotinylated primary antibodies. Details of antibodies used are described in table 2.1. All samples were incubated at 4°C in the dark for 20-30 minutes, washed with 2ml FACS buffer (PBS, 2 % FCS, and 0.05 % sodium azide) and, where appropriate, biotinylated antibodies were detected by incubation with 100 $\mu$ l of a 1/200 dilution of fluorochrome-conjugated streptavidin (Pharmingen) at 4°C in the dark for 20-30 minutes. The cells were then washed in FACS buffer and resuspended in 300 $\mu$ l FACSflow (BD) and data acquired using a FACSCalibur flow cytometer equipped with a 488 nm Argon laser and a 635 nm red diode laser and analysed using CellQuest software (both from BD Biosciences).

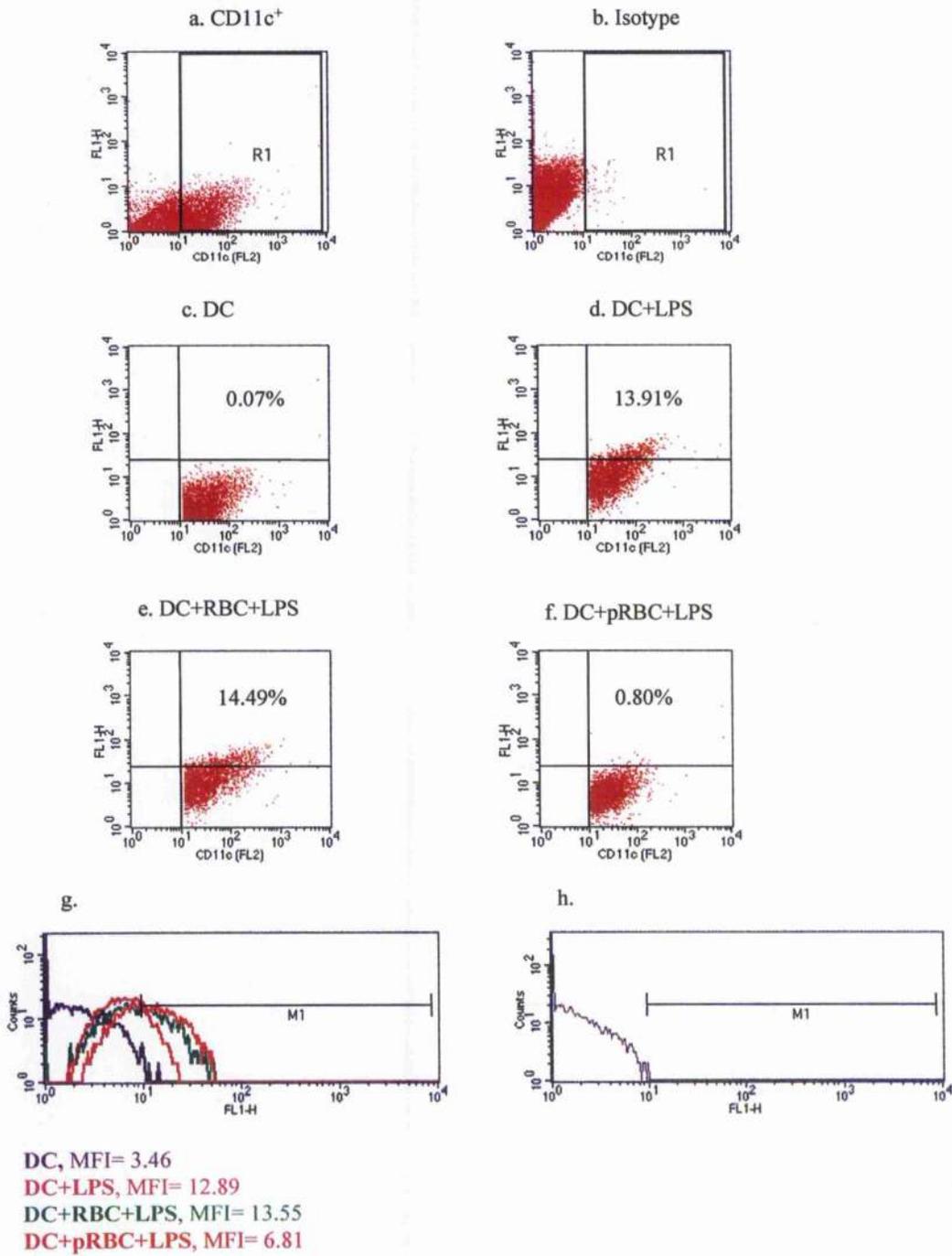
Prior to analysing labelled cells, forward scatter (FSC) and side scatter (SSC) were adjusted so cells of interest could be gated on the screen. Unstained samples were used as auto-fluorescence controls allowing settings to be adjusted so that auto-fluorescence background was roughly within the first decade of the log scale of the fluorescence intensity histogram. Unstained samples were compared with isotype and positively stained controls to confirm the correct scale. Compensation settings were set such that on an FL-1 vs FL-2 dot plot, an FL-1 positive population was negatively aligned with negative population, and an FL-2 positive population was horizontally aligned with the FL-2 negative population.

Antigen	Conjugate	Clone	Isotype
CD4	PE	GK1.5	Rat IgG2ak
CD4	FITC	L3T4	Rat IgG2ak
CD40	PerCP	RM4-5	Rat IgG2ak
CD69	FITC	H1.2F3	Hamster IgG1 $\lambda$
I-A/I-E	FITC	2G9	Rat IgG2ak
CD40	FITC	3/23	Rat IgG2ak
CD80	FITC	B7.1	Hamster IgG1 $\lambda$
CD86	FITC	B7.2	Rat IgG2ak
CD11c	PE	HL3	Hamster IgG1 $\lambda$

**Table 2.1** Monoclonal antibodies used for flow cytometry

All antibodies were purchased from BD Pharmingen and used according to manufacturers' guidelines.

### 2.3.1 Flow cytometric analysis



**Figure 2.2** Flow cytometric analysis of CD40 expression on bone marrow-derived DCs. Gate R1 is set around CD11c<sup>+</sup> cells (a), drawn using the isotype control (b). Dot plots (c-f) and histograms (g-h) are gated on R1 to obtain the percentage of CD11c<sup>+</sup> cells

expressing CD40 (c-f) or the Mean Fluorescence Intensity (MFI) values (g). The marker M1 is set using the isotype control (h). FL1=CD40 expression. Plots are representative of three independent cultures. The obtained MFI and percentage positive values are used to plot the bar graphs shown throughout this thesis.

## **2.4 Preparation of erythrocyte ghosts from infected and non-infected mice**

Ghosts from infected and non-infected erythrocytes were generated as previously described (Wunderlich *et al.*, 1985). Briefly, blood was collected by cardiac puncture and washed three times in PBS. Infected and non-infected erythrocytes were concentrated in PBS supplemented with 113 mM of Glucose (Sigma-Aldrich) and 3% FCS. Infected erythrocytes were incubated in an equal volume of glycerol buffer (i.e. 10 % glycerol (Sigma-Aldrich) supplemented with 5 % FCS in PBS) for 1h at 4°C. Parasites and ghosts were separated in a continuous Percoll (Amersham Biosciences, Buckinghamshire) gradient ( $\rho$ : 1.02-1.10 g/cm<sup>3</sup>) in intracellular medium buffer (IM: 20 mM NaCl, 120 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM Glucose, 5 mM Hepes, pH 6.7) and centrifuged at 5000 g for 30 minutes. Ghosts were then washed in IM buffer and layered on a two-step Percoll gradient ( $\rho$ : 1.01+1.02 g/cm<sup>3</sup>) to separate them from possible ghosts that still contained parasites. Ghosts from non-infected erythrocytes were obtained by adding a 40-fold volume of phosphate buffer (5 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 1 mM PMSF, 0.01 % Azide, pH 8.5). The suspension was centrifuged at 32000g for 30 minutes. Ghosts from infected and non-infected erythrocytes were then washed three times in PBS before being resuspended in cRPMI for addition to DCs at a ratio of 100:1.

## **2.5 Haemozoin preparation and quantitation**

Haemozoin was purified from supernatants obtained from cultures of *Plasmodium falciparum* gametocytes, which were kindly provided by Dr Lisa Ranford-Cartwright, (Division of Infection and Immunity, IBLS, University of Glasgow, U.K) and endotoxin-

free buffers and solutions used throughout. Supernatants were centrifuged for 20 minutes at 450g. The pellet was washed three times in 2% SLS and resuspended in 6M Guanadine HCl. Following five to seven washes in PBS, the pellet was resuspended in PBS and sonicated for 90 minutes using Soniprep 150, (Sanyo Scientific, Bensenville, IL) at an amplitude of 5-8 microns to minimize aggregation and maintain the haemozoin in suspension. Total haeme content was determined as previously described (Sullivan *et al.*, 1996) by depolymerising haeme polymer in 1 ml of 20 mM NaOH/2 % SDS, incubating the suspension at room temperature for 2h and then reading the OD at 400 nm using UV visible spectrophotometer (Thermo Spectronic, Heλios, Cambridge, UK).

## 2.6 Cytokine analysis

For the detection of IL-12 (p40) and IL-10 produced by DCs, OptEIA™ ELISA kits (BD) were purchased and standards and antibody pairs specific for the corresponding cytokine were used according to manufacturer's instructions. Briefly, 96 well plates (Immulon-4 HBX, Dynex (West Sussex, UK)) were coated overnight at 4°C with 100µl/well of Anti-mouse IL-12 or Anti-mouse IL-10 monoclonal antibody diluted in 0.2M Sodium Phosphate (1:250 dilution for IL-12 and 1:125 for IL-10), pH 6.5. After 3 washes with PBS/0.05% Tween 20, non-specific binding sites were blocked for 1 hour at room temperature by adding 200µl/well of PBS/10% FCS. After 3 washes with PBS/0.05% Tween 20, 100µl of samples or serial dilutions of recombinant cytokine standards (rIL-12 dilutions starting at 4000pg/ml, rIL-10 dilutions starting at 2000pg/ml) were added to each well and incubated for 2 hours at room temperature. After incubation, the plates were washed 6 times and incubated with 100µl of the appropriate anti-cytokine detection monoclonal antibody + Avidin-horseradish peroxidase conjugate for 1 hour at room temperature. Plates were washed a further 6 times before the addition of 100µl/well of substrate solution (TMB). 50µl/well of stop solution (Sulphuric acid, 0.4M) were added before measuring the optical density (OD) at 450/630nm (dual wavelength mode) using a Dynex microplate reader. The OD values were converted to pg/ml of cytokine in supernatants by using Revelation software to analyse the standard curves obtained from

serial dilutions of the cytokine standards. Detection limits were 31.3 pg/ml for IL-12 and 15.6 pg/ml for IL-10.

For the detection of T cell cytokines (IL-4, IL-5, IL-10, IL-2, IFN- $\gamma$  and IL-12), Mouse Th1/Th2 6-Plex Antibody Bead kit (Biosource) was used according to manufacturer's instructions. Briefly, all washing steps were performed by placing a 96 well filter-bottom microplate provided on a filtration apparatus and applying a vacuum sufficient to gently empty the wells, which have been pre-filled with 200 $\mu$ l of a Working Wash Solution provided. All incubation steps were carried out in the dark at room temperature and by placing the microplate on an orbital shaker. 25 $\mu$ l of the antibody-coated beads were added to each well. After washing the plate twice, serial dilutions of cytokine standards (dilutions starting at 5.78ng/ml for IL-5, 11.47ng/ml for IL-4, 3.39ng/ml for IL-10, 4.75ng/ml for IL-12, 7.92ng/ml for IL-2 and 5.43ng/ml for IFN- $\gamma$ ) and samples were added to the appropriate wells and incubated for 2h. Following the incubation time, the plate was washed twice and 100 $\mu$ l of diluted biotinylated detection antibody added into each well and incubated for 1h. The wells were then washed twice before adding 100 $\mu$ l Streptavidin-RPE (fluorescent), which was incubated for 30 minutes. Finally, the plate was washed three times before addition of 100 $\mu$ l of Working Wash Solution and then loaded into the Luminex XY<sup>TM</sup> platform to read the analyte concentration.

## **2.7 Microscopic analysis**

### **2.7.1 Bright field microscopy**

$5 \times 10^5$  DCs were dispensed onto 13mm polylysine (Sigma-Aldrich) coated coverslips (BDH) in 24 well plates (Corning Costar) and left to adhere overnight at 37°C, 5% CO<sub>2</sub>.  $5 \times 10^7$  RBC or pRBC were added to DCs and incubated for a further 24h. Coverslips were mounted onto glass slides (BDH) and examined by bright field microscopy using an Axiovert S-100 Zeiss microscope (Carl Zeiss Ltd, Hertfordshire, UK) using a 63x oil immersion lens.

### 2.7.2 Fluorescence microscopy

DCs and pRBC co-cultures were set up as described above. After 3h incubation at 37°C, 5% CO<sub>2</sub> DCs were stained with 0.5µg/ml of CTxB conjugated to Alexa Fluor 488 (Molecular Probes, Oregon, USA) and incubated for 15 minutes at 37°C in 5% CO<sub>2</sub>. Cells were then fixed with 0.5% paraformaldehyde for 20 minutes at 4°C. RBCs were stained with 5µg/ml of biotin-conjugated rat anti-mouse TER-119 monoclonal antibody (Pharmingen) for 10 minutes at 4°C. The biotinylated antibody was detected using streptavidin-FITC (Vector Laboratories, CA, USA). Vectashield containing DAPI (Vector Laboratories) was used to stain cell nuclei. Coverslips were mounted onto glass slides (BDH) and fluorescence microscopy performed using an Axiovert S-100 Zeiss microscope using a 63x oil immersion lens.

### 2.8 Total RNA extraction from bone marrow-derived DCs

RNeasy Midi kit was purchased from Qiagen (West Sussex, UK) and used according to the manufacturer's instructions. Briefly,  $2 \times 10^6$  bone marrow-derived DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected or non-infected RBCs for 6h at 37°C, 5%CO<sub>2</sub>. Cells were then harvested and centrifuged for 5 minutes at 300g. Supernatants were carefully removed by aspiration and cells disrupted by adding 2ml of buffer RLT provided. Cells were homogenised by passing the lysate through a 20 gauge needle (BDH) fitted to an RNase-free syringe (BDH). 2ml of 70% ethanol (Sigma-Aldrich) was added to the homogenised lysate and mixed thoroughly. Samples were then applied to an RNeasy midi column and centrifuged for 5 minutes at 3000g. 2ml of buffer RW1 supplied, was used to wash the column for 5 minutes at 3000g before carrying out on-column DNase digestion. 160µl of DNase I incubation mixture provided was added directly onto the RNeasy silica-gel membrane and incubated for 15 minutes at room temperature. 2ml of buffer RW1 was then added to the column and incubated for 5 minutes at room temperature, before centrifuging it for 5 minutes at 3000g. The column was washed twice for 5 minutes at 3000g with 2.5ml of buffer RPE supplied. To elute, the RNeasy column was transferred to a new 15ml collection tube and 150µl of RNase-free water (Invitrogen) added directly

onto the RNeasy silica-gel membrane and incubated for 5 minutes at room temperature. The column was then centrifuged for 3 minutes at 3000g and the elution step carried out one more time using the same amount of RNase-free water. The total RNA extracted was stored at  $-20^{\circ}\text{C}$  for gene array analysis. Mouse Genome 430 2.0 Array was purchased from Affymetrix and experiments performed at the Sir Henry Wellcome Functional Genomics Facility at the University of Glasgow.

## **2.9 Statistical analysis**

Results are expressed as mean  $\pm$  standard error. Significance was determined by one-way ANOVA in conjunction with the Tukey test, using Minitab. A p-value of  $p \leq 0.05$  was considered significant.

### **2.9.1 Statistical methods for identifying differentially expressed genes in microarray data.**

Microarray technology certainly has the potential to greatly enhance our knowledge about gene expression. Due to the ability of this technique to monitor the expression levels of thousands of genes simultaneously, microarray experiments generate massive amounts of data. Therefore methods are needed to determine whether changes in gene expression are experimentally significant. Data obtained from microarray analysis has to be interpreted cautiously. Fluorescence intensities from replicate microarrays cannot generally be compared directly, but only after appropriate calibration (or normalization) (Huber *et al.*, 2002), due to variation in sample treatment, labelling, dye efficiency and detection. Furthermore, since different laboratories may use different array technologies to profile the same genes, it would be beneficial to be able to combine expression measurements within a single analysis, as utilization of data from different technologies has the potential to reduce the need to duplicate experiments. However, this will require the different measurements to be comparable. For instance, expression levels measured by cDNA microarrays are usually reported as a ratio of the signal from a target mRNA sample relative to one from a co-hybridized reference mRNA sample (Schena *et al.*,

1995). This method complicates a direct comparison with results from other technologies as the reported ratios depend on the chosen mRNA reference. It is therefore extremely desirable that a researcher has the best possible analytical tools available to make the most of the information that this powerful technology has to offer.

As microarray data is often noisy and not normally distributed (Hunter *et al.*, 2001), it is challenging to construct a statistical data model applicable to all microarray data sets. In this context, non-parametric methods that do not assume a specific distribution of data are particularly attractive. Recently, a powerful new test statistics, based on calculating rank products (RP) from replicate experiments to define differentially expressed genes, has been successfully implemented and is now used as the standard method for microarray data analysis at the Sir Henry Wellcome Functional Genomics Facility at the University of Glasgow (Breitling *et al.*, 2004b). A major advantage of this new technique is the ease with which results from different array technologies can be combined in one analysis. As long as the results can be expressed as rank lists, it does not matter whether they are produced by two-colour cDNA arrays or Affymetrix oligonucleotide chips.

The strength of the RP approach relies on making relatively weak assumptions about the data. In an experimental setup, only a minority of the genes are assumed to significantly change their expression levels (Breitling *et al.*, 2004b). This may seem relatively restrictive, as it is easy to imagine experimental setups where the majority of the genes are differentially expressed. However, in these circumstances, one will usually want to know the most important genes. In this context, relevant changes are considered to be always large, while small changes may have statistically but rarely biological significance; thus significant gene regulation is almost a 'switch-like' process (Breitling *et al.*, 2004b). Equal variance for all genes, changes being independent of each other and measurements being independent between replicate arrays are among the other weak assumptions that makes this method a powerful statistical test for identifying differentially expressed genes under two experimental conditions (Breitling *et al.*, 2004b). The principle behind the RP method is briefly described in Figure 2.3

## Rank Products

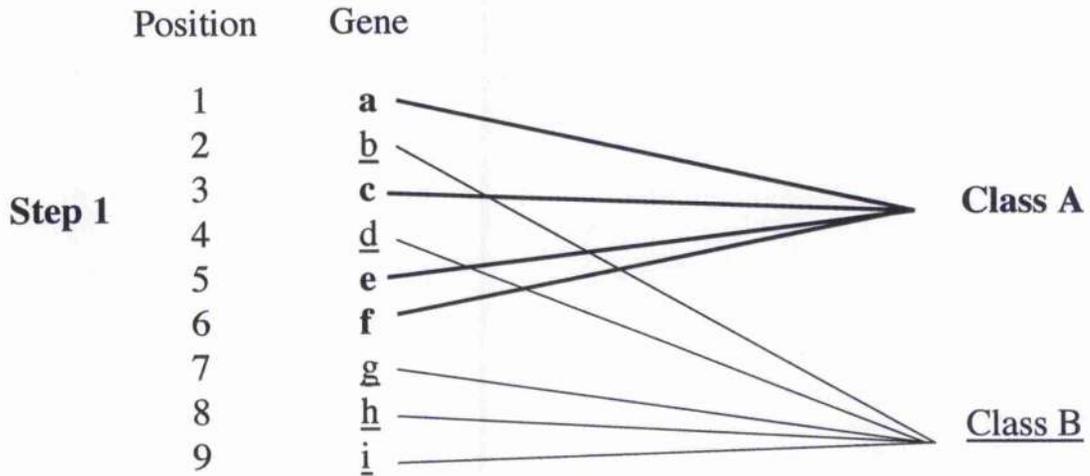
Replicate A		Replicate B					
Gene	Position	Gene	Position	Gene	RP	position	
↑ FC ↓	a	1	a	1	b	0.1	1
	b	2	g	2	d	0.2	2
	c	3	c	3	f	0.3	3
	d	4	b	4	h	0.4	4
	e	5	e	5	i	0.5	5
	f	6	f	6	a	0.6	6
	g	7	d	7	c	0.7	7
	h	8	i	8	e	0.8	8
	i	9	h	9	g	0.9	9

$$\text{Calculate RP} = \frac{\text{gene position in A}}{n} \times \frac{\text{gene position in B}}{n}$$

**Figure 2.3 Principle of Rank Product Analysis.** Genes are sorted by increasing/decreasing Fold Change (FC). The probability ( $p$ ) of a gene to be found at the top of the list if the lists were random is  $p = 1/n^k$ , where  $n$  is the total number of genes and  $k$  the number of replicates. This probability can be calculated as a Rank Product (RP). The RP values can then be used to sort the genes in a new list where genes with the smallest RP values are the most interesting candidates. In the example shown above the RP for gene  $d$  is  $RP_d = 4/9 \times 7/9$ . The significance level ( $p'$ ) is then calculated using a permutation-based procedure and determines the likelihood of observing each gene so high on the lists just by chance. For example,  $p'$  for gene  $d$  is the probability that all the other genes in the list are not at position 2. Once  $p'$  has been calculated, the False Discovery Rate (FDR) is determined, which identifies whether a gene has been randomly regulated. For gene  $d$ , the FDR will define whether its position in the list is just an artefact of the experimental procedure.

Another major advantage of the RP method is that it provides data that are suitable to be used in the Iterative Group Analysis (iGA) approach (Breitling *et al.*, 2004a) (Figure 2.4). This method is based on the idea that a concerted expression change of some members of the same functional class is physiologically relevant (Breitling *et al.*, 2004a). Therefore, iGA identifies functional classes of genes that are significantly changed and also which of the class members are most likely to be differentially expressed (Breitling *et al.*, 2004a), thus facilitating, improving and accelerating the biological interpretation of microarray experiments (Breitling *et al.*, 2004a).

## Iterative Group Analysis

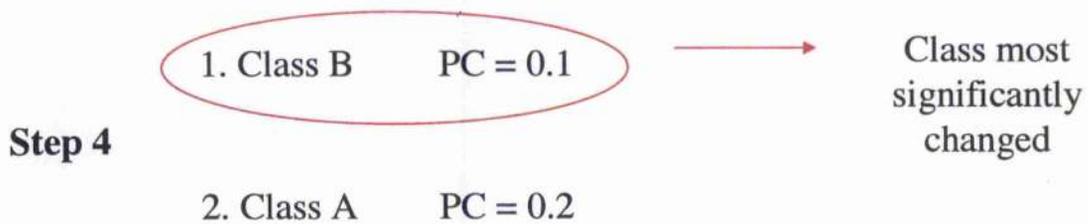
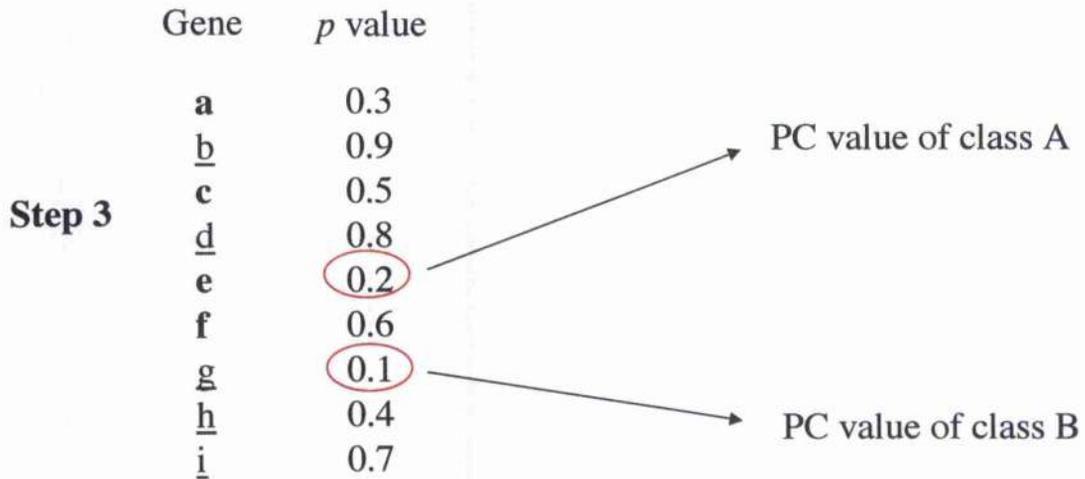


**Step 2**

Gene	Position	<i>p</i> value	
<b>a</b>	1	0.3	
<u>b</u>	2	0.9	
<b>c</b>	3	0.5	
<u>d</u>	4	0.8	
<b>e</b>	5	0.2	←
<b>f</b>	6	0.6	
<u>g</u>	7	0.1	←
<u>h</u>	8	0.4	
<u>i</u>	9	0.7	

Members of class A above position 5 are differentially expressed

Members of class B above position 7 are differentially expressed



**Figure 2.4** Principle of Iterative Group Analysis. **Step 1.** Genes are sorted by increasing Fold Change (FC) values (or any other choice of metrics of differential expression) and assigned to functional classes based on their GeneOntology assignments. **Step 2.** The *p* value (i.e. the probability of observing members of a given class at a particular position in the list by chance) for each gene belonging to a specific functional class is determined. The position in the list that yields the smallest *p* value is used to determine the cut-off of the class. All class members above that position are considered as ‘potentially differentially expressed’. **Step 3.** The smallest *p* value determined is assigned as the PC (Probability of Change) value of the class. **Step 4.** All classes are sorted by their PC values and the classes with the lowest value are the most significantly changed.

**3. Assessing the effects of *P. chabaudi* AS-infected erythrocytes  
on DC function *in vitro*.**

### 3.1 Introduction

Host resistance against infectious organisms requires the development of an appropriate immune response. As previously mentioned in section 1.2.5, DCs play a key role in determining the type of induced response. These cells have evolved to monitor the environment, detect pathogens and trigger T cell activation providing a link between the innate and adaptive immune systems (Adams *et al.*, 2005). In turn, many pathogens have evolved mechanisms to subvert the function of these important cells thereby modulating the host's immune response to their advantage (Rescigno, 2002; Rescigno and Borrow, 2001).

It is now widely accepted that encounter with parasites can strongly modify DC function and lead to altered T cell responses (Sher *et al.*, 2003). Some parasites may lead to DC activation. Nevertheless there is evidence indicating that although activated by pathogens, mature DCs may either fail to respond or are functionally suppressed. For instance Langerhans cells in the skin of mice infected with *S. mansoni* become activated but are unable to migrate from the epidermis. This inhibition appears to be mediated by prostaglandin D2 produced by the parasite and may help delay the subsequent adaptive response (Angeli *et al.*, 2001). Similarly, infection with *T. gondii* selectively activates human DCs by increasing MHC class II, CD86 and CD83 but not CD40, CD54 or CD80 expression. However, these DCs induce apoptosis of T cells (Wei *et al.*, 2002).

Other parasites may instead avoid activating DCs in the first place. For instance, helminth antigens do not appear to be able to directly induce DC maturation, at least as far as increased expression of classical DC maturation markers such as MHC class II, CD80 and CD86 is concerned (MacDonald *et al.*, 2001; Whelan *et al.*, 2000). Particularly interesting is the inhibition of DC maturation induced by the malaria parasite *P. falciparum* (Urban *et al.*, 1999). Malaria-infected erythrocytes were found to bind to the surface of myeloid DCs *in vitro* and to markedly suppress the normal upregulation of MHC class II molecules, adhesion molecules (e.g. ICAM-1) and costimulatory molecules (CD83 and CD86) on DCs following LPS stimulation. The resulting DCs were severely

impaired in their capacity to induce antigen-specific primary and secondary T cell responses (Urban *et al.*, 1999). The receptors on the surface of DCs mediating this inhibitory effect were shown to be the integrins CD36 and CD51 (Urban *et al.*, 2001b). The major parasite molecule interacting with these integrins appeared to be a functionally conserved domain of *P. falciparum* erythrocyte membrane protein 1 (pfEMP1) (Urban and Roberts, 2002), a molecule that undergoes antigenic variation (Biggs *et al.*, 1991).

DCs exposed to blood-stage malaria parasites were also shown to suppress CD8<sup>+</sup> T cell responses against the liver stages of this parasite and inhibit CD8<sup>+</sup> T cell priming following vaccination with irradiated sporozoites (Ocana-Morgner *et al.*, 2003). As effective liver-stage immunity is essential to protect against new infection, this method of immunosuppression may benefit the parasite by leaving individuals open to constant re-infection (Ocana-Morgner *et al.*, 2003). The ability of malaria parasites to inhibit the maturation of DCs could be involved not only in parasite-specific immunosuppression (Ho *et al.*, 1986), but also in the suppression of responses to heterologous antigens such as vaccines as well as unrelated pathogens (Cook, 1985; Enwere *et al.*, 1999; Greenwood *et al.*, 1972; Mabey *et al.*, 1987; McGregor, 1962; Thursz *et al.*, 1995; Williamson and Greenwood, 1978).

In this study I examined whether *P. chabaudi* AS-infected erythrocytes modulated key functions of DCs that may potentially lead, or contribute to more generalised immunosuppression. A better understanding of how the malaria parasite interferes with DC biology may therefore contribute to the development of new therapeutic approaches and aid in the development of a much needed malaria vaccine.

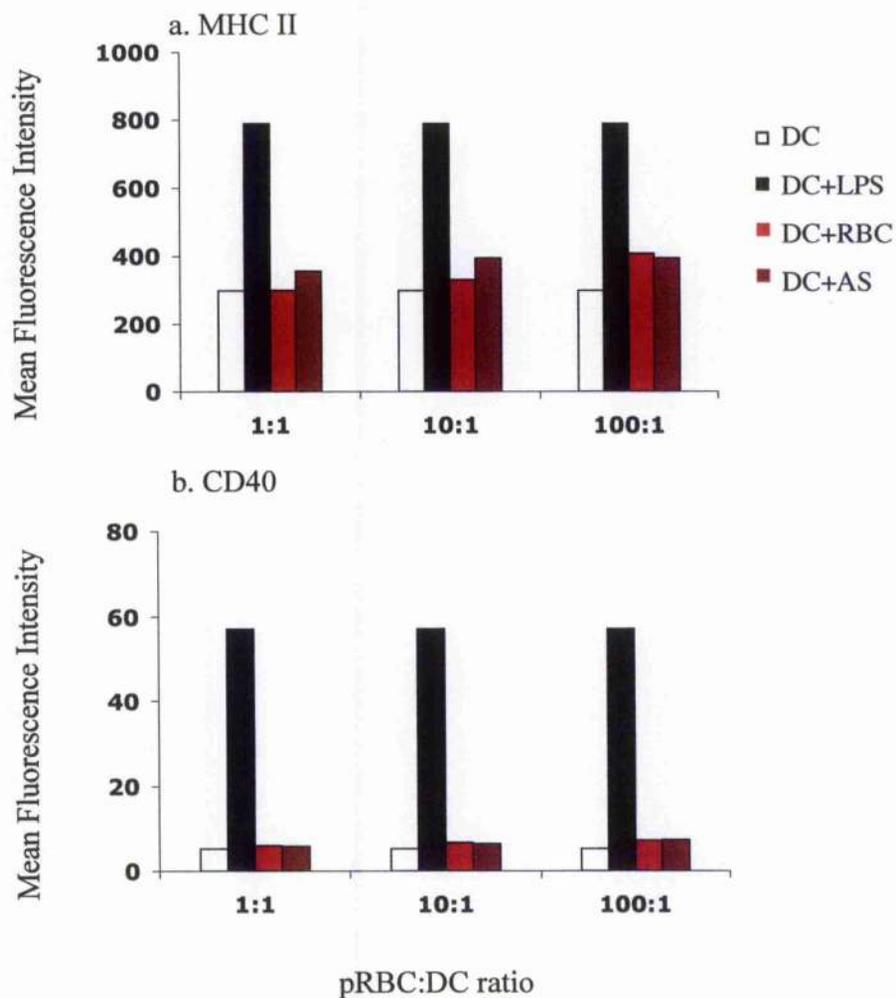
## 3.2 Results

### 3.2.1 Optimisation of culture conditions

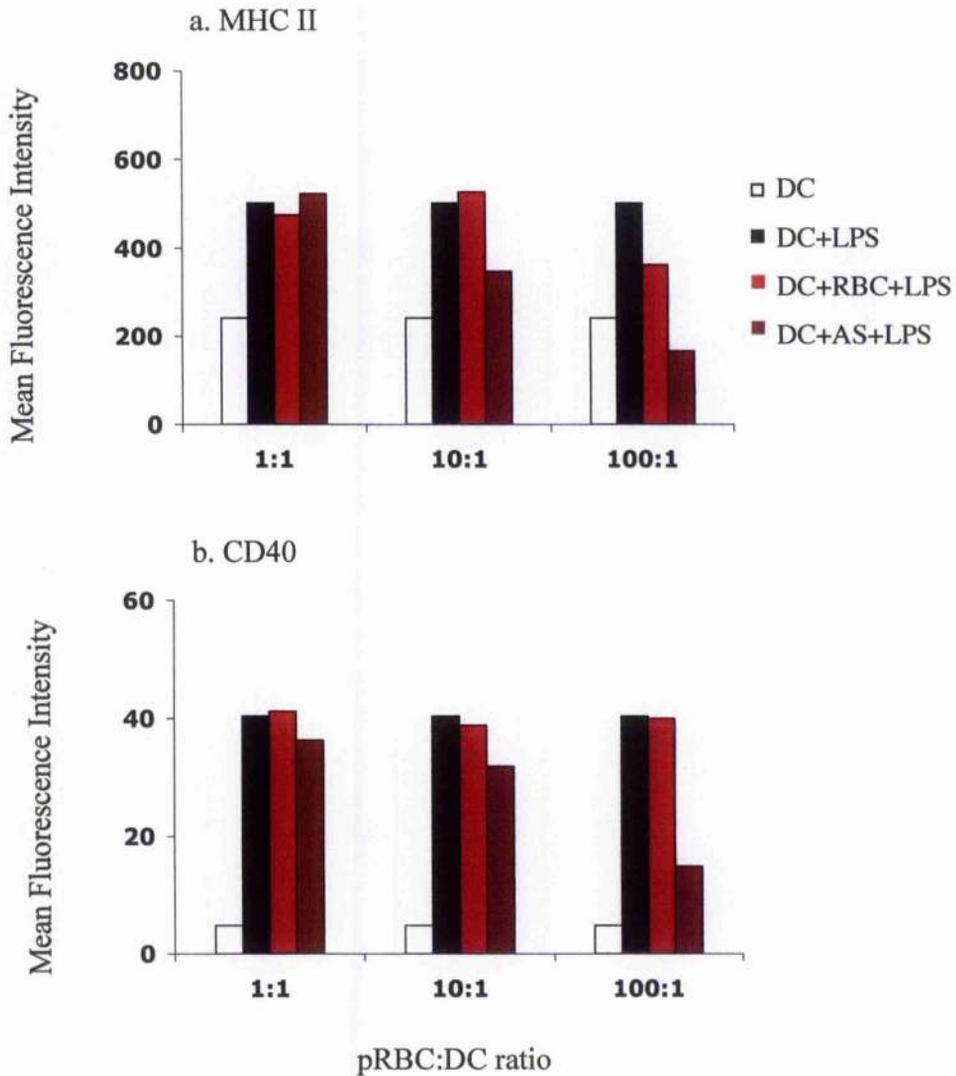
In order to investigate the effects that infected erythrocytes have on DC function, preliminary experiments were carried out to determine the correct ratio of pRBC:DC in co-culture systems. For this purpose, pRBCs or control uninfected RBC, were incubated with bone marrow-derived DCs at a ratio of 1:1, 10:1 and 100:1. After a 24h incubation, one set of cells was analysed for the expression of the surface markers MHC class II and CD40. Another set of cells was challenged with LPS and the levels of costimulatory molecules analysed 18h later. DCs cultured in growth medium alone and those challenged with LPS served as negative and positive controls respectively.

The results show that pRBCs did not induce DC maturation at any of the ratios tested (Fig 3.1) as the levels of MHC class II and CD40 were the same as those of the untreated control DCs and DCs treated with RBCs. However pRBCs were able to induce a dose-dependent suppression of the ability of pRBC-treated DCs to respond to LPS stimulation *in vitro* as seen by the reduced levels of MHC class II and CD40 (Fig 3.2). To ensure that the viability of DCs was not affected by the presence of pRBCs in the culture system, the number of viable DCs was quantified by trypan blue exclusion after 24h incubation with pRBCs. DCs alone and DCs incubated with RBCs served as controls. The results show pRBC and RBC did not have an effect on the number of viable DCs recovered after each treatment (Fig 3.3).

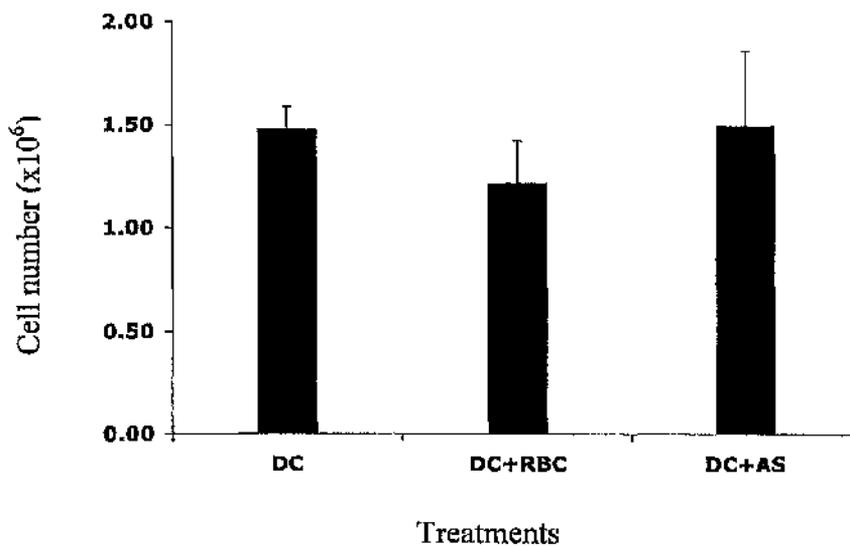
The ability of pRBC-treated DCs to respond to LPS stimulation *in vitro* was consistently found to be affected when using 100 pRBC : 1 DC hence this ratio was used in all the experiments described in this thesis to further investigate the role of pRBCs in the modulation of DC function *in vitro*.



**Figure 3.1** Surface expression of MHC class II (a) and CD40 (b) on bone marrow-derived DCs after 24h of culture with non-infected (RBC) or *P. chabaudi* AS-infected erythrocytes (pRBC).  $5 \times 10^5$  DCs were incubated with  $5 \times 10^5$ ,  $5 \times 10^6$  or  $5 \times 10^7$  pRBC to achieve a ratio of pRBC:DC of 1:1, 10:1 and 100:1 respectively. An equal number of RBCs were used as controls. DCs treated with  $1 \mu\text{g/ml}$  of LPS (DC+LPS) and those grown in medium only (DC) served as positive and negative controls respectively. Results are expressed as the mean fluorescence intensity of duplicate cultures as determined by flow cytometric analysis of gated  $\text{CD11c}^+$  cells.



**Figure 3.2** Surface expression of MHC class II (a) and CD40 (b) on bone marrow-derived DCs following LPS stimulation. DCs were cultured for 24h with non-infected (RBC) or *P. chabaudi* AS infected erythrocytes (pRBC) as described in figure 3.1, then treated with  $1\mu\text{g/ml}$  of LPS for a further 18h. Results were analysed as described in figure 3.1.

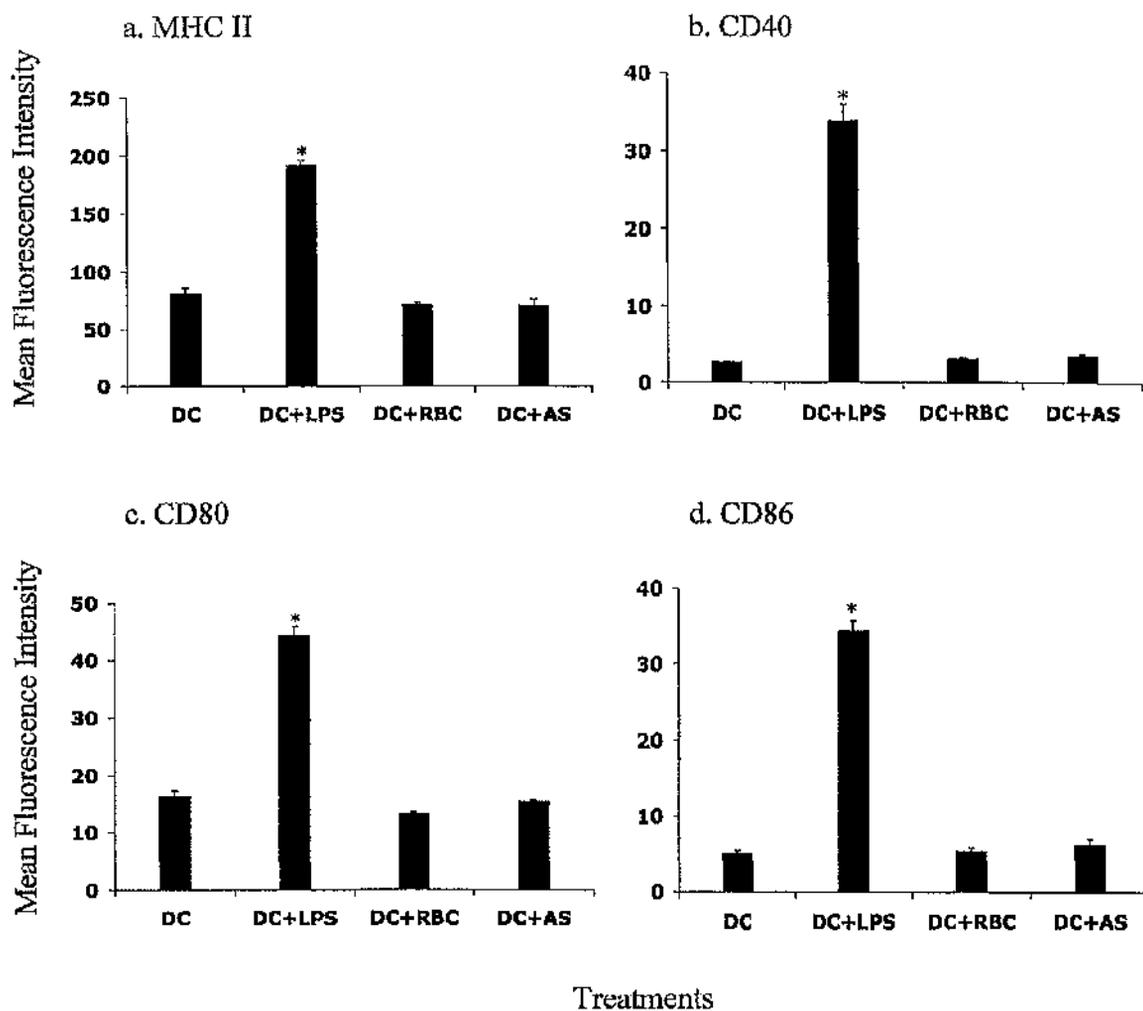


**Figure 3.3** DC viability after incubation with *P. chabaudi* AS infected erythrocytes.  $2 \times 10^6$  bone marrow- derived DCs were cultured either in growth medium alone (DC) or in the presence of non-infected (DC+RBC) or *P. chabaudi* AS infected erythrocytes (DC+AS) at a ratio of 1:100. After 24h incubation, DC viability was assessed by trypan blue exclusion. Results show the mean value of viable cells  $\pm$  standard error of triplicate cultures per group.

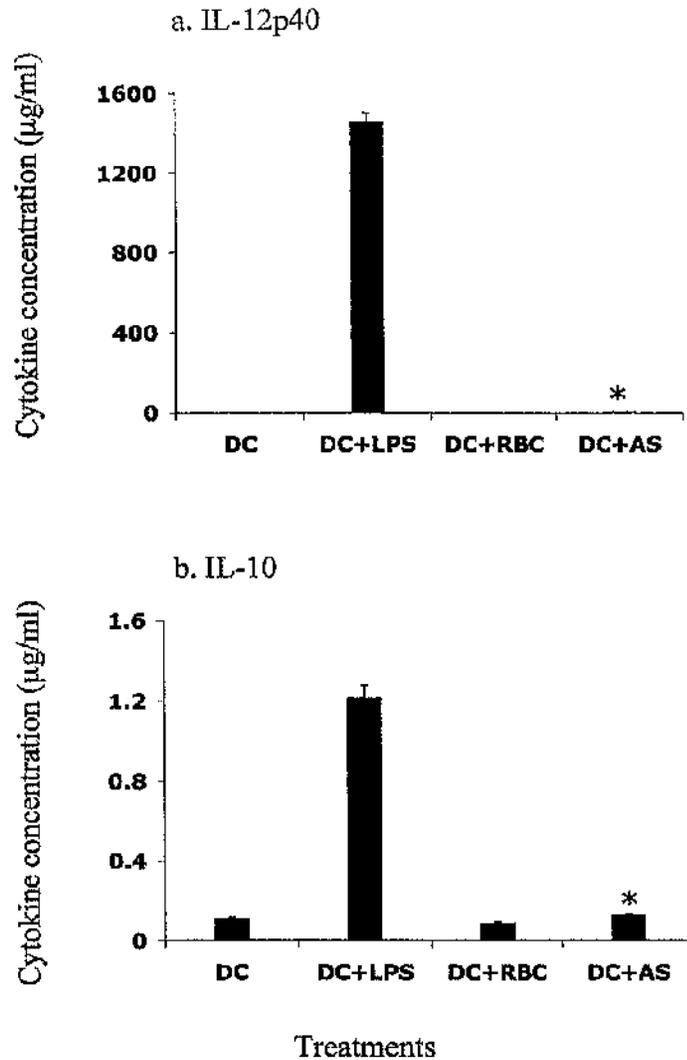
### 3.2.2 Effect of *P. chabaudi* AS-infected erythrocytes on resting DCs

Having established that pRBCs did not induce DC maturation as observed by the levels of MHC class II and CD40, I wanted to extend this observation to other costimulatory molecules by examining the expression of CD80 and CD86 as well as MHC class II and CD40 in order to confirm previous results. DCs were pre-incubated with pRBCs and the expression of surface markers examined 24h later by flow cytometry. The results show that DCs expressed very low levels of surface MHC class II, CD40, CD80 and CD86 when cultured in growth medium alone, confirming their immature state in culture (Fig. 3.4). Stimulation with LPS promoted a significant increase in the expression level of all of the costimulatory molecules tested. However, DCs incubated for 24h with RBCs or pRBCs did not increase expression of MHC class II, CD40, CD80 and CD86, indicating that pRBCs did not induce DC activation directly.

Cytokine production by DCs was also examined and the results show that DCs exposed to RBCs or pRBCs produced small though detectable amounts of both IL-12 and IL-10 that were considerably lower than observed after LPS stimulation but not significantly different than RBC-treated controls or untreated DCs (Fig. 3.5).



**Figure 3.4** Surface expression of MHC class II (a), CD40 (b), CD80 (c) and CD86 (d) on bone marrow-derived DCs following incubation with non-infected (RBC) or *P. chabaudi* AS infected erythrocytes (AS).  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  infected erythrocytes (DC+AS) or with an equal number of non-infected erythrocytes (DC+RBC) for 24h. Control DCs remained unstimulated (DC) or were stimulated with  $1 \mu\text{g/ml}$  of LPS (DC+LPS). Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group as determined by flow cytometric analysis of gated CD11c<sup>+</sup> cells. \*  $p \leq 0.05$  significant difference between DC+LPS and DC.



**Figure 3.5** Cytokine production by DCs following incubation with infected and non-infected erythrocytes. Concentration of IL-12p40 (a) and IL10 (b) secreted by DCs alone (DC), DCs stimulated with 1µg/ml of LPS (DC+LPS), DCs incubated with non-infected erythrocytes (DC+RBC) or with *P. chabaudi* AS infected erythrocytes (DC+AS).  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  infected erythrocytes or with an equal number of non-infected erythrocytes. DCs treated with LPS and those grown in medium only served as positive and negative controls respectively. After 24h of culture, supernatants were harvested for cytokine analysis. Error bars indicate standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between DC+AS and DC+LPS.

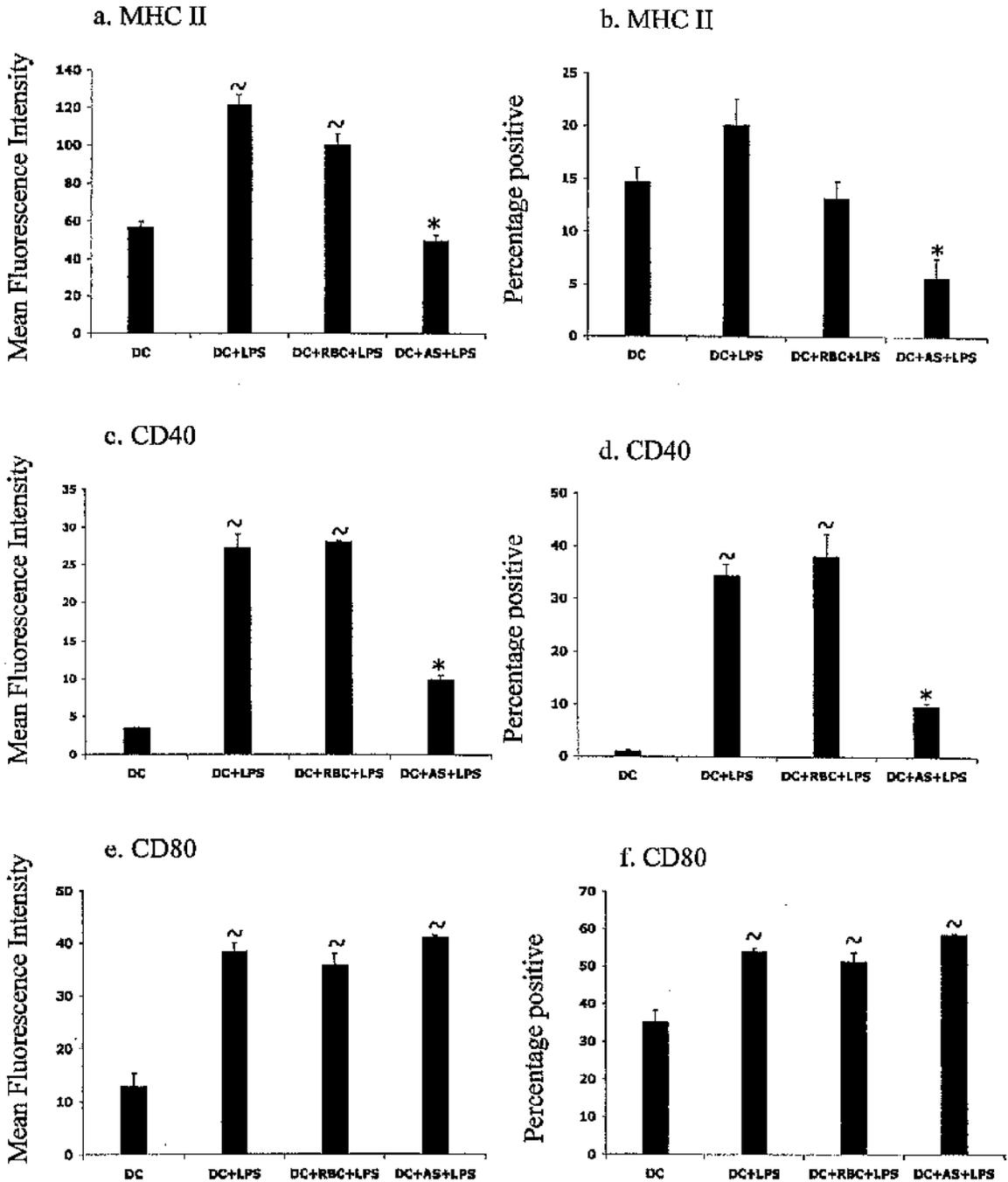
### 3.2.3 Effect of *P. chabaudi* AS-infected erythrocytes on DC responses to LPS *in vitro*

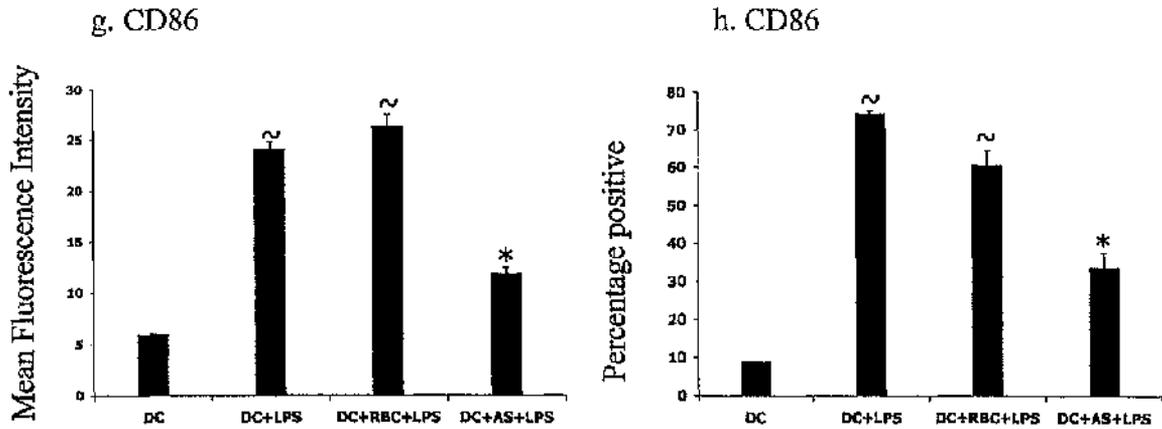
Having demonstrated that pRBCs did not directly induce DC maturation, I examined whether pRBC- treated DCs retained their ability to mature in response to LPS treatment *in vitro* by examining the levels of MHC class II, CD40, CD80 and CD86. DCs were exposed to RBCs or pRBCs for 24 h and subsequently challenged with LPS. After 18 h of LPS stimulation, the expression levels of all of the above molecules increased significantly (Fig. 3.6). However, DCs pre-incubated with pRBCs and subsequently challenged with LPS, showed significantly lower levels of expression of MHC class II, CD40, and CD86 compared to those observed when cells were not treated with pRBCs or were pre-incubated with RBCs prior to LPS challenge.

Cytokine production following treatment with LPS was also analysed and the results show that DCs treated with pRBCs were still able to produce appreciable levels of IL-12 and IL-10 in response to LPS although the amount of IL-12 produced was significantly lower compared with the amount detected when DCs were pre-incubated with RBCs (Fig. 3.7). To exclude the possibility that the reduced levels of costimulatory molecules and cytokine production by pRBC-treated DCs was due to DC death following LPS treatment, an Annexin V/ Propidium Iodide assay was carried out for each experimental group. The results show that similar levels of necrotic and apoptotic DCs were detected in all the conditions tested (Fig. 3.8).

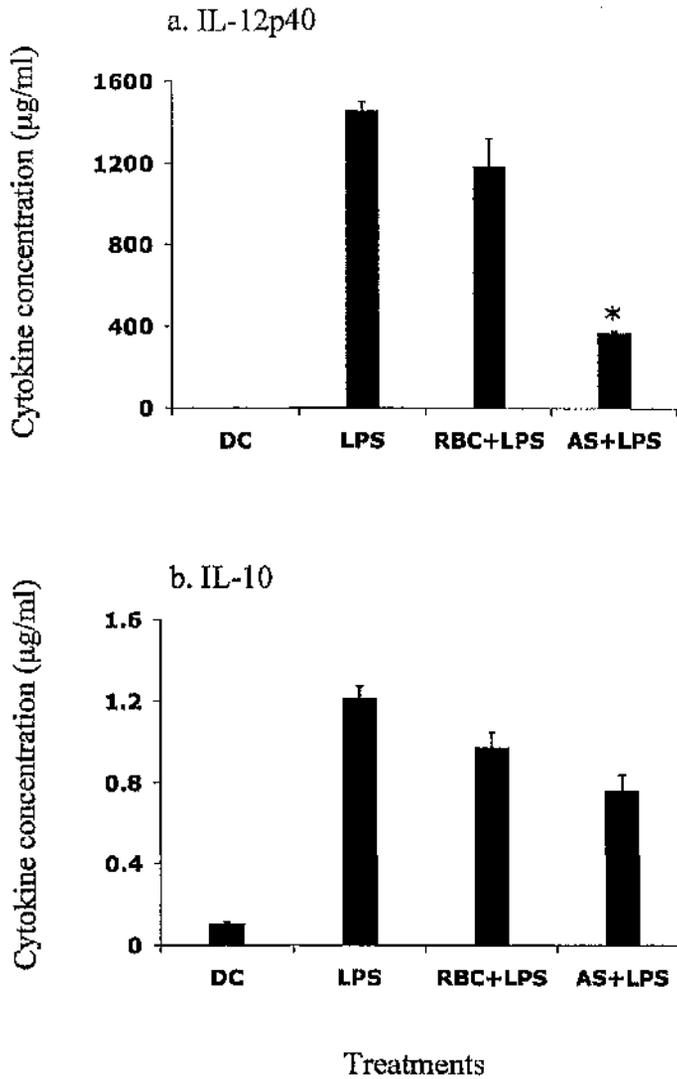
Having established that activation of DCs in response to LPS treatment *in vitro* is suppressed by infected erythrocytes, I examined whether the observed result was specific to the LPS response alone. I therefore decided to challenge pRBC-treated DCs with CpG, which induces DC maturation through the TLR9 signalling pathway as opposed to LPS, which has been shown to mediate signalling through TLR4 (Akira *et al.*, 2001). DCs were exposed to RBCs or pRBCs for 24 h and subsequently challenged with CpG. After 18 h of CpG stimulation, the level of surface CD40 increased in all control groups (Fig. 3.9). However, DCs pre-incubated with pRBCs and subsequently challenged with CpG,

showed reduced CD40 expression compared to DCs, which were not treated with pRBCs or were pre-incubated with RBCs prior to CpG challenge.



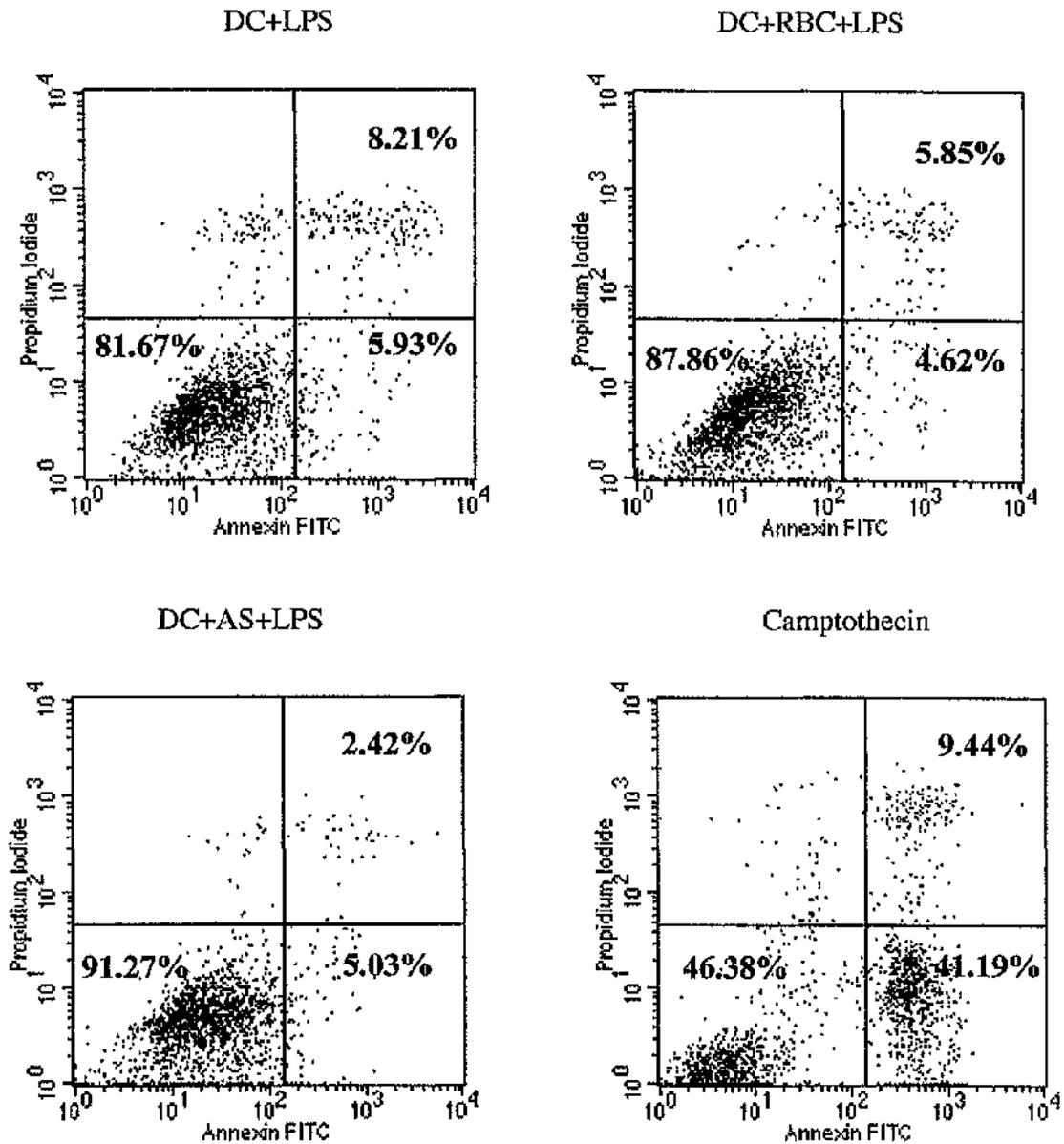


**Figure 3.6** MHC class II, CD40, CD80 and CD86 on bone marrow-derived DCs expressed either as the mean fluorescence intensity (a,c,e and g) or as percentage positive cells (b,d,f and h) following LPS stimulation. DCs were cultured for 24h with non-infected (RBC) or *P. chabaudi* AS infected erythrocytes (AS) as described in figure 3.4, then treated with 1 $\mu$ g/ml of LPS for a further 18h. Control DCs remained unstimulated (DC) or treated with 1  $\mu$ g/ml of LPS (DC+LPS). Results are shown as the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group (a, c, e and g) or as the mean percentage positive cells  $\pm$  standard errors of triplicate cultures per group (b, d, f and h). \*  $p \leq 0.05$  significant difference between DC+AS+LPS and DC+RBC+LPS.  $\sim p \leq 0.05$ , groups significantly different from DC.



**Figure 3.7** Cytokine production by DCs following LPS stimulation after 24h pre-incubation with infected and non-infected erythrocytes. Concentration of IL-12p40 (a) and IL10 (b) secreted by DCs alone (DC), DCs stimulated with 1µg/ml of LPS (LPS), DCs pre-incubated with non-infected erythrocytes (RBC+LPS) or *P. chabaudi* AS infected erythrocytes (AS+LPS) and challenged with LPS.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  infected erythrocytes or with an equal number of non-infected erythrocytes. After 24h of co-culture, DCs were stimulated with 1µg/ml LPS for a further 18h and supernatants harvested for cytokine analysis. DCs treated with LPS alone and those grown in medium only served as positive and negative controls respectively. Error bars

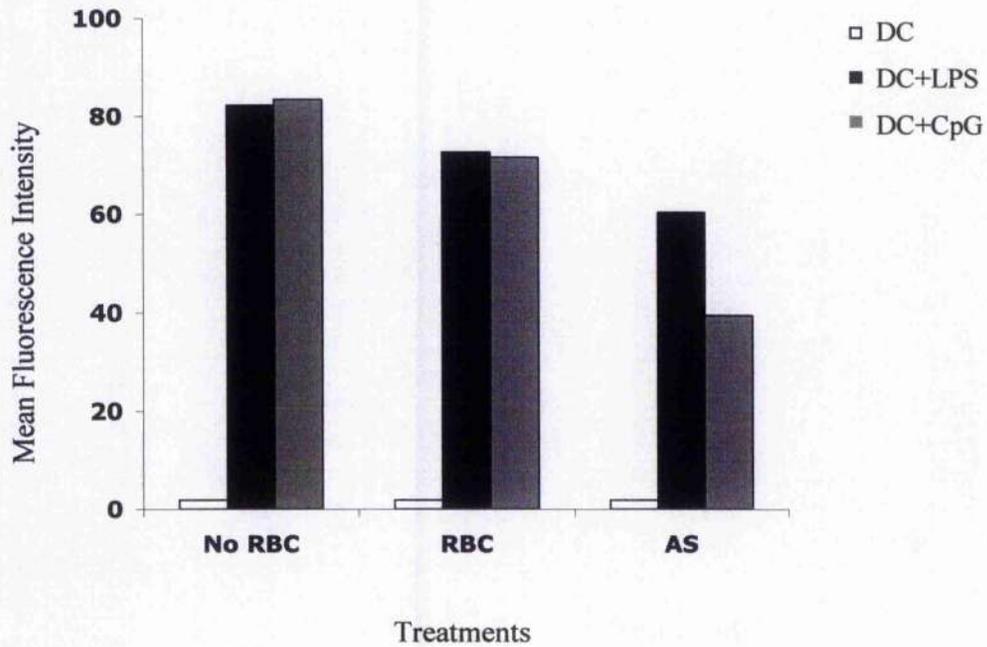
indicate standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between AS+LPS and RBC+LPS.



**Figure 3.8** DC viability following LPS treatment after pre-incubation with *P. chabaudi* AS infected or non-infected erythrocytes.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  infected erythrocytes (DC+AS) or with an equal number of non-infected erythrocytes (DC+RBC) for 24h then treated with  $1 \mu\text{g/ml}$  of LPS. After 18h of culture, DCs were harvested and

the percentage of apoptotic cells within each culture condition was measured using the Annexin V/ PI staining assay. DCs stimulated with 1 µg/ml of LPS only (DC+LPS) were used as controls. Jurkat T cells treated with camptothecin served as positive controls. Flow cytometric data is shown. Results are representative of three separate cultures.

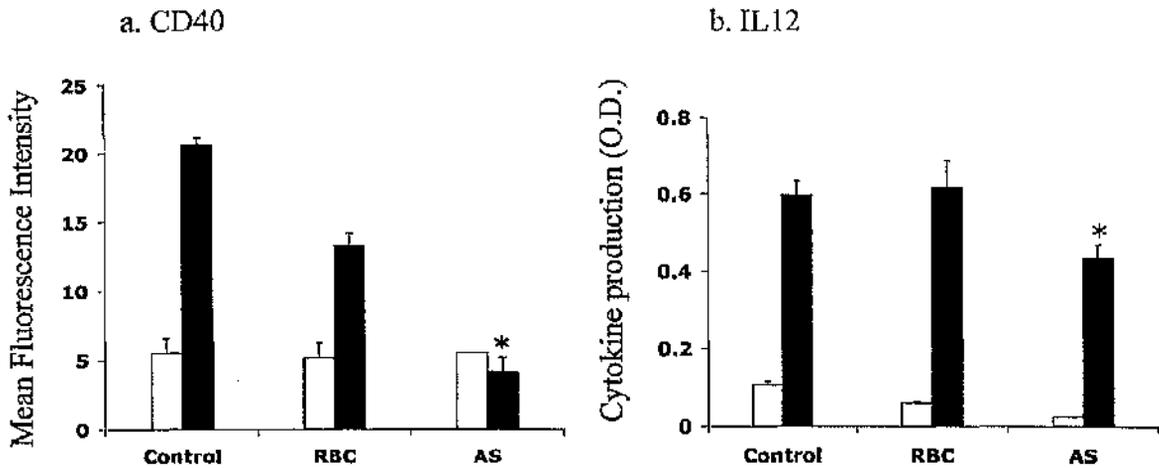
In all the groups tested, cells were primarily Annexin V-FITC and PI negative, indicative of viable cells not undergoing apoptosis. A small percentage of cells were Annexin V-FITC positive and PI negative, indicating cells undergoing apoptosis. A minor population of cells were observed to be Annexin V-FITC and PI positive, indicating that they were in end stage apoptosis or already dead (Vermes *et al.*, 1995).



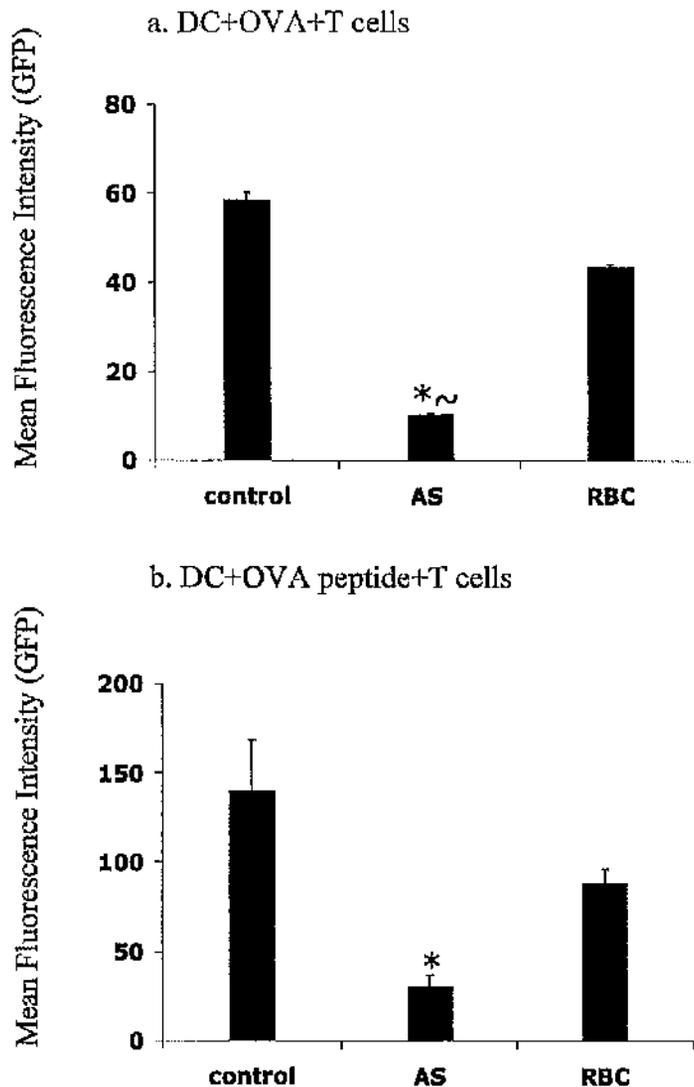
**Figure 3.9** Surface expression of CD40 on bone marrow-derived DCs after 18h stimulation with either LPS or CpG *in vitro*.  $2 \times 10^6$  DCs were incubated with medium alone (no RBC) or in the presence of non-infected (RBC) or *P.chabaudi* AS infected erythrocytes (AS) at a ratio of 1:100. After 24h of culture, DCs were treated with either  $1 \mu\text{g/ml}$  of LPS or  $3 \mu\text{M}$  of CpG for a further 18h. Control DCs remained unstimulated. Results are shown as the mean fluorescence intensity of duplicate cultures per group.

Since CD40-CD40L interaction between DCs and T cells *in vivo* is known to play a crucial role in the production of bioactive IL-12 and upregulation of adhesion and costimulatory molecules (Cella *et al.*, 1996; Schulz *et al.*, 2000), bone marrow-derived DCs were stimulated with CD40L transfected fibroblasts (Figure 3.10). The results show that DCs treated with RBCs significantly upregulated CD40 expression in response to CD40L and produced high levels of IL-12 p40. CD40 ligation however did not rescue the reduced maturation of DCs treated with pRBCs although, as previously observed with the LPS treatment, these cells were still able to produce IL-12 p40 but to a lesser extent than the control groups.

Antigen presentation by DCs plays a crucial role in initiating T cell priming (Banchereau *et al.*, 2000). I therefore investigated the ability of DCs to present heterologous antigens to T cells after treatment with infected erythrocytes. Both OVA protein and OVA peptide (aa.323-339) were used as the antigen. In order to detect antigen presentation independent of costimulation, I took advantage of the OVA-specific T cell hybridoma (DO11-GFP) that expresses green fluorescent protein (GFP) upon TcR ligation (Underhill *et al.*, 1999). The results show that DCs pre-incubated with RBCs prior to OVA loading showed the same antigen presenting ability as cells that were grown in medium only. However, the ability of DCs to activate the OVA-specific T cell hybridoma was significantly reduced when they were pre-incubated with pRBCs before antigen loading (Fig. 3.11). Furthermore, the degree of suppression was significantly greater when OVA protein was used compared to OVA peptide, suggesting that the antigen processing machinery might be affected, although further experiments will be required to confirm this hypothesis.



**Figure 3.10** *P. chabaudi* AS infected erythrocytes inhibit the LPS-induced maturation of DCs independently of CD40 ligation.  $1 \times 10^6$  DCs were cultured with  $1 \times 10^8$  infected erythrocytes (AS) before stimulation with CD40L-expressing fibroblasts (filled bars) or control fibroblasts (open bars) at a 1:1 ratio of fibroblasts:DCs. Untreated DCs (Con) and non-infected erythrocytes treated DCs (RBC) were used as controls. DCs were incubated with fibroblasts for a further 18 h before analysing CD40 expression (a) and cytokine production (b). CD40 expression on DCs is shown as the mean fluorescent intensity as determined by flow cytometric analysis of gated CD11c<sup>+</sup> cells. IL-12 production is shown as the mean Optical Density (OD) read at 450 nm. Results show the mean value  $\pm$  standard error of triplicate cultures per group. \*,  $p \leq 0.05$  (AS vs RBC).



**Figure 3.11** Antigen presentation by DCs treated with *P. chabaudi* AS infected erythrocytes.  $5 \times 10^5$  DCs were cultured with  $5 \times 10^7$  infected erythrocytes (AS). Untreated DCs (control) and non-infected erythrocyte-treated DCs (RBC) were used as controls. After 24 h of culture, the cells were antigen pulsed with (a) 5 mg/ml of OVA or (b) with an equimolar amount of OVA peptide (40  $\mu$ g/ml) for 6 h. The level of antigen presentation was determined by incubating DCs with the OVA-specific DO11.10-GFP hybridoma at 1:1 DC : T cell ratio and T cell activation assessed 24 h later by flow cytometry. Results are expressed as the mean fluorescence intensity of GFP expression by  $CD4^+$  T cells  $\pm$  standard error. \*  $p \leq 0.05$  significant difference between AS and RBC.

~ significant difference between *P. chabaudi* AS-treated DCs pulsed with OVA (a) and *P. chabaudi* AS-treated DCs pulsed with peptide (b).

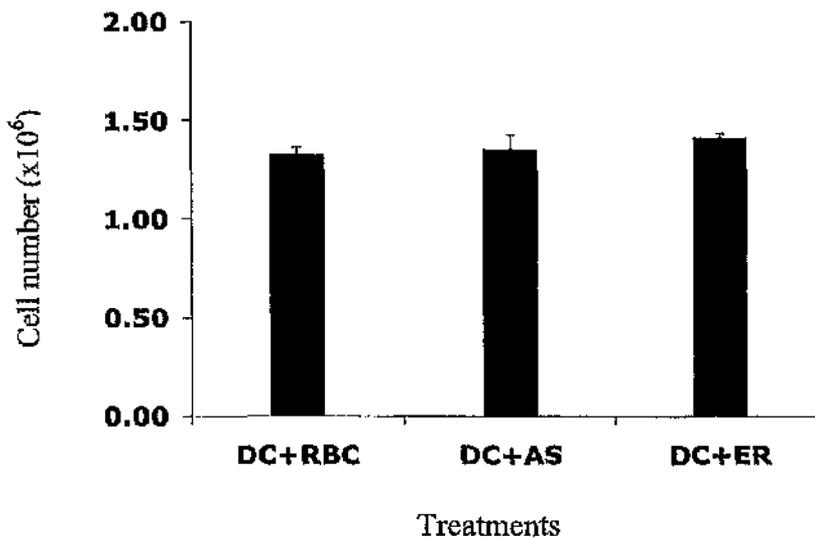
#### 3.2.4 Comparing the effects of *P. chabaudi* AS and *P. chabaudi* ER on DC function *in vitro*.

As previously described in section 1.1.4.1, *P. chabaudi* AS causes an acute primary parasitaemia from which most mice recover. Conversely, *P. chabaudi* ER is a much more virulent strain and it is fatal after the peak of parasitaemia (Beale *et al.*, 1978). Having these two parasite strains available, I decided to compare their effects on DC function with the view of *P. chabaudi* ER having more profound effects on DCs due to its virulence.

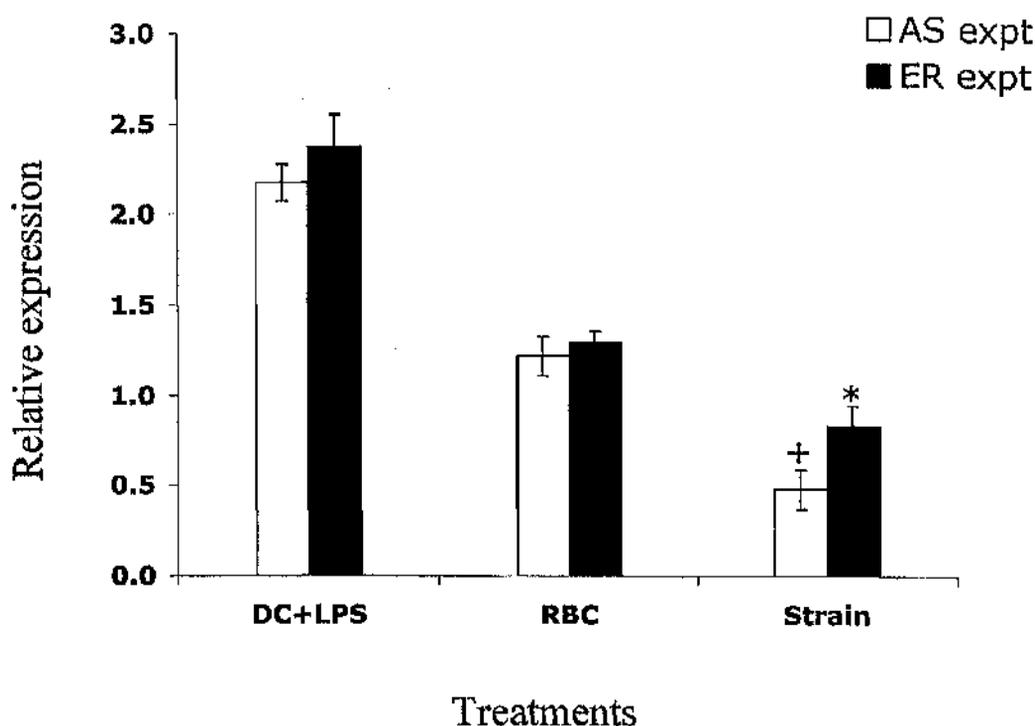
I have previously shown that *P. chabaudi* AS did not affect DC viability (Fig. 3.3). I therefore examined whether the same could be demonstrated using *P. chabaudi* ER. For this purpose DCs were incubated with either *P. chabaudi* AS- or *P. chabaudi* ER-infected erythrocytes or with uninfected controls and their viability was determined 24h later by trypan blue exclusion. The results show that the same number of viable DCs was recovered after each treatment (Fig. 3.12).

Since *P. chabaudi* ER infected erythrocytes did not affect DC viability, I wanted to analyse whether this parasite strain suppressed the LPS-induced maturation and antigen presenting abilities of DCs to a greater extent compared to the effects observed when using *P. chabaudi* AS infected erythrocytes. The results show that the ability of (ER)-pRBC- treated DCs to respond to LPS stimulation *in vitro* was significantly affected as demonstrated by the reduced levels of MHC class II. However, the suppression of MHC class II expression was not significantly greater than the suppression induced by (AS)-pRBCs (Fig. 3.13). The same result was obtained when comparing the antigen presenting ability of DCs treated with (ER)- and (AS)-pRBCs (Fig. 3.14). Both parasite strains inhibited the ability of DCs to present OVA to the OVA-specific T cell hybridoma,

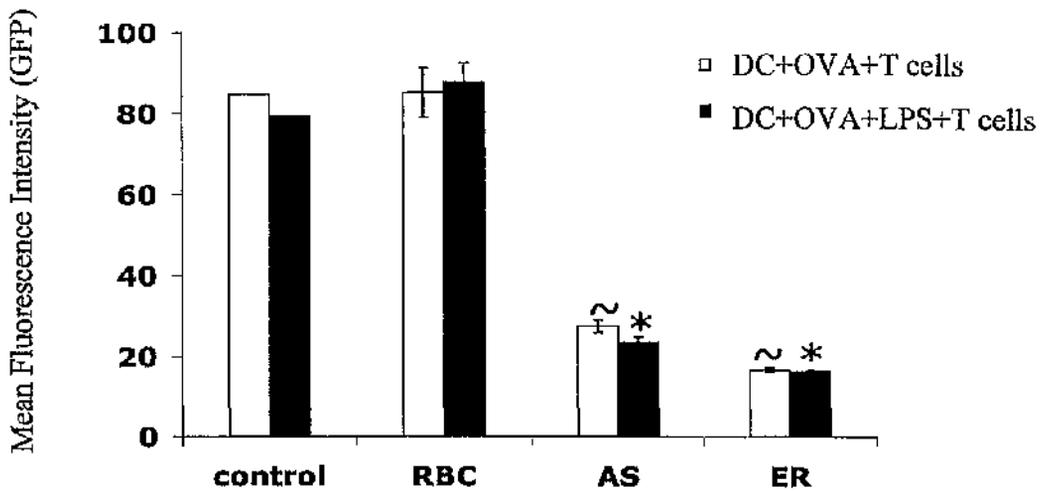
however the effect observed following treatment of DCs with the different parasite strains did not differ significantly. Therefore parasite virulence did not seem to exert more profound effects on some of the key functions of DCs compared to the less virulent strain. Further experiments are necessary to elucidate the role of parasite virulence on DC function. For the purpose of the present study it was sufficient to know that the effects exerted by *P. chabaudi* ER infected erythrocytes were not dramatically different from those induced by *P. chabaudi* AS thus the former could be used in future experiments if required.



**Figure 3.12** DC viability after incubation with different *P. chabaudi* strains.  $2 \times 10^6$  bone marrow-derived DCs were cultured in the presence of non- infected (DC+RBC), *P. chabaudi* AS (DC+AS) or *P. chabaudi* ER (DC+ER) infected erythrocytes at a ratio of 1:100. After 24h incubation, DC viability was assessed by trypan blue exclusion. Results show the mean value of viable cells  $\pm$  standard error of triplicate cultures per group.



**Figure 3.13** Surface MHC class II expression on bone marrow-derived DCs following LPS stimulation after 24h pre-incubation with different *P. chabaudi* strains.  $2 \times 10^6$  DCs were treated with either  $2 \times 10^8$  *P. chabaudi* AS (AS expt, empty bars) or *P. chabaudi* ER infected erythrocytes (ER expt, filled bars). After 24h of incubation, DCs were stimulated with  $1 \mu\text{g/ml}$  of LPS and the expression of MHC class II examined 18h later by flow cytometric analysis of gated  $\text{CD11c}^+$  cells. DCs treated with LPS alone (DC+LPS) and those pre-incubated with non-infected erythrocytes (RBC) prior to LPS challenge served as controls. Results are shown as the relative expression of MHC class II calculated as the mean fluorescence intensity of DCs after the different treatments divided by the mean fluorescence intensity of DCs, which did not receive any activating stimulus  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between *P. chabaudi* ER-treated DCs and the corresponding control RBC. +  $p \leq 0.05$  significant difference between *P. chabaudi* AS-treated DCs and the corresponding RBC control.



**Figure 3.14** Antigen presentation by DCs treated with *P. chabaudi* AS or *P. chabaudi* ER infected erythrocytes.  $5 \times 10^5$  DCs were cultured with  $5 \times 10^7$  infected erythrocytes (AS or ER). Non-infected erythrocyte-treated DCs (RBC) were used as controls. After 24 h of culture, the cells were antigen loaded with 5 mg/ml of OVA (empty bars) and the level of antigen presentation was determined as described in figure 3.10. One set of cells was treated with  $1 \mu\text{g/ml}$  of LPS immediately after addition of T cells (filled bars). \*  $p \leq 0.05$  significant difference between *P. chabaudi* AS- and *P. chabaudi* ER-treated DCs in the LPS treated group compared to the corresponding RBC-treated DCs. ~  $p \leq 0.05$  significant difference between *P. chabaudi* AS- and *P. chabaudi* ER-treated DCs compared to RBC-treated DCs in the LPS negative group.

### 3.3 Discussion

DCs are central to the initiation and regulation of the adaptive immune response during infection (Adams *et al.*, 2005). They are specialised for the uptake, transport, processing and presentation of antigens to T cells (Adams *et al.*, 2005). DCs reside as immature cells with high phagocytic capacity in all peripheral tissues. Encounter with pathogens triggers DC maturation, characterised by phenotypic and functional changes that culminate in their complete transition from antigen-capturing cells to efficient antigen presenting cells. This maturation process is accompanied by cytoskeletal reorganization, reduced phagocytic activity, acquisition of cellular motility, migration to lymphoid tissues, enhanced T cell activation potential and the development of characteristic cytoplasmic extensions usually referred to as 'dendrites' (Quah and O'Neill, 2005). Furthermore, mature DCs secrete cytokines that determine the type of ensuing immune response and upregulate the expression of adhesion and costimulatory molecules, which are involved in bidirectional signalling between DCs and T cells, modulating both T cell activation and DC function (Quah and O'Neill, 2005).

The experiments described in this chapter were aimed at investigating how some of the above functions of DCs are affected during blood-stage malaria infections. I initially examined the ability of pRBCs to induce DC activation by analysing the expression of MHC class II and costimulatory molecules CD40, CD80 and CD86 on their surface. The results showed that pRBCs did not activate DC directly, which was also confirmed by the lack of production of IL-12 and IL-10 cytokines. This finding appeared to contrast previous reports where *P.chabaudi* AS schizonts were found to cause direct activation of DCs *in vitro* (Seixas *et al.*, 2001). Similarly, DCs isolated from *P. yoelii* infected mice during peak parasitaemia were also found to be activated and could efficiently process and present antigen to naïve T cells (Luyendyk *et al.*, 2002; Perry *et al.*, 2005; Perry *et al.*, 2004). Since in this study DCs were exposed to trophozoite-infected erythrocytes, collectively these reports indicate that not only different parasites (*P.yoelii* vs *P.chabaudi*) but also different stages of the same parasite (trophozoites vs schizonts) may exert different effects on DC function.

The concept of chronologically different stages of the parasite life cycle interfering with one another in the context of an on going infection is not new to this field. Ocana-Morgner *et al.* (Ocana-Morgner *et al.*, 2003) have shown that *P. yoelii* blood stage infection inhibited the establishment of a protective cytotoxic T cell response against the initial liver stage, leaving the host susceptible for the next infection. It is therefore possible that specific blood stages might suppress DC function to protect the more vulnerable stages (schizonts and merozoites) in order to allow progression of the disease. Supporting this hypothesis is the finding that human and rodent malaria parasites were found to release pro-inflammatory exoantigens following schizont rupture, which stimulated macrophages to secrete TNF- $\alpha$  *in vitro* (Bate *et al.*, 1988; Kwiatkowski *et al.*, 1989; Taverne *et al.*, 1990b). In the same study, Ocana-Morgner and colleagues also showed that infected erythrocytes did not activate DCs directly *in vitro* (Ocana-Morgner *et al.*, 2003), confirming observations reported in the present study but contrasting previous reports by other authors (Luyendyk *et al.*, 2002; Perry *et al.*, 2005; Perry *et al.*, 2004). However, it is not clear what blood-stage of the parasite was used in the experiments carried out by Ocana-Morgner *et al.* On the other hand a possible reason for these discrepancies might rely on the degree of parasitaemia achieved during infection. In fact, although all of the studies above used the non-lethal parasite line 17X, experiments were carried out in different strains of mice. Ocana-Morgner *et al.* (Ocana-Morgner *et al.*, 2003) used BALB/c mice, which develop a high parasitaemia (49%) when infected with *P. yoelii* 17X whereas other authors used B10.D2 mice, which instead develop a relatively low parasitaemia (13%) (Luyendyk *et al.*, 2002; Perry *et al.*, 2005; Perry *et al.*, 2004), therefore genetic differences may account for the results obtained.

So far I have shown that pRBCs did not induce DC activation. The maturation process is associated with many coordinated events such as production of cytokines, up-regulation of co-stimulatory molecules, and increased ability to activate T cells (Banchereau *et al.*, 2000). I therefore examined the ability of DCs to mature in response to LPS treatment *in vitro* after incubation with pRBCs. The results demonstrate that DCs treated with pRBCs prior to LPS challenge were severely impaired in their ability to upregulate the expression of costimulatory molecules MHC class II, CD40 and CD86. In addition, the

viability of these cells was maintained in both pRBC-treated and control cultures (as examined by Annexin/PI staining). These findings supported previous studies with *P. falciparum* infected erythrocytes and human monocyte-derived DCs (Urban *et al.*, 1999) as well as *in vitro* and *in vivo* studies of *P. yoelii* with murine DCs (Ocana-Morgner *et al.*, 2003). Nevertheless, in the present study pRBC-treated DCs were still able to produce appreciable levels of IL-12p40 in response to LPS although the amount produced was significantly lower than the control cultures. Furthermore, pRBC-treated DCs did not produce significant levels of IL-10 cytokine. The latter result contrasts Ocana-Morgner *et al.* (Ocana-Morgner *et al.*, 2003) and Perry *et al.* (Perry *et al.*, 2005) data where inhibition of IL-12 production was observed in favour of production of high levels of IL-10. Once again I reasoned that the differences observed may be attributed to the different parasites used and that, although in Ocana-Morgner's studies (Ocana-Morgner *et al.*, 2003) *P. yoelii* was found to suppress the LPS-induced maturation of DCs *in vitro* as observed with *P. chabaudi* AS in the present study, the causal effect of the parasites might be by different mechanisms.

As explained previously in section 1.2.3, DCs can sense microbes directly by recognizing molecular patterns within microbial carbohydrates, lipids and nucleic acids using highly conserved pattern-recognition receptors (Janeway and Medzhitov, 2002; Reis e Sousa, 2001). Such receptors include Toll-like receptors (Thoma-Uszynski *et al.*, 2001; Underhill and Ozinsky, 2002). TLR4 has been shown to mediate signalling through interaction with LPS from Gram-negative bacteria (Akira *et al.*, 2001). Unmethylated CpG DNA motifs, highly represented in bacterial DNA, are instead TLR9 ligands (Akira *et al.*, 2001). The distribution of TLRs on DC subsets favours specialisation of each cell type to respond to different pathogens. In particular, human myeloid DCs express all TLRs with the exception of TLR9, which is selectively expressed by plasmacytoid DCs (Colonna *et al.*, 2002). Accordingly, plasmacytoid DCs respond to CpG DNA but not to LPS whereas myeloid DCs respond to LPS but not to CpG DNA (Colonna *et al.*, 2002). By contrast, TLR9 is shared by both myeloid and plasmacytoid DCs in the mouse and they are both activated by CpG DNA (Sparwasser *et al.*, 1998). To analyse whether pRBCs affected TLR9 signalling specifically, pRBC-treated DCs were challenged with

CpG and the levels of surface CD40 expression analysed 18h later by flow cytometry. The results clearly show that even when stimulated with a TLR9 ligand, pRBC-treated DCs were not able to mature efficiently as observed by their reduced levels of CD40 expression compared to control cells. This result demonstrates that pRBCs affects the maturation of DCs independently of the TLR ligand used, inducing a generalised suppression of DC activation. This observation may explain why malaria infected individuals have suppressed immune responses to a number of different pathogens (Greenwood *et al.*, 1972; McGregor, 1962; Williamson and Greenwood, 1978) and why they are more susceptible to secondary infections such as *Salmonella* septicemia (Mabey *et al.*, 1987), tuberculosis (Enwere *et al.*, 1999), herpes zoster (Cook, 1985) and hepatitis B (Thursz *et al.*, 1995).

It has been suggested recently that during malaria infection *in vivo*, DCs are activated during early infection and then show TLR tolerance later in infection, becoming unresponsive to LPS stimulation (Perry *et al.*, 2005). However, this does not seem to be the case with *P. chabaudi* AS since, as explained earlier, there is no evidence of direct maturation of DCs by pRBC in the present study. A possible explanation for the transient increased expression of activation markers observed on DCs *ex vivo* might be the high concentration of pro-inflammatory cytokines caused by the early stage of infection (Langhorne *et al.*, 2004). In support of this, a recent report described that DCs activated through inflammatory cytokines without pathogenic stimulation upregulated markers of activation but were unable to drive CD4<sup>+</sup> T cell differentiation (Sporri and Reis e Sousa, 2005).

The ability of DCs to interact with CD40L on T cells *in vivo* has also been proposed to explain the differences between *in vivo* and *in vitro* studies (Perry *et al.*, 2004). Interestingly in the present study, DC maturation could not be rescued when pRBC-treated DCs were treated with CD40L transfected fibroblasts, suggesting that the suppressive effects exerted by *P. chabaudi* AS-infected erythrocytes may be more profound than those induced by *P. yoelii* infection. The signalling events involved in DC maturation following LPS and CD40L stimulation have been previously described in

Figures 1.3 and 1.4 respectively and undoubtedly represent an oversimplification of the complex series of events that may occur realistically. Inhibition of DC maturation following both LPS and CD40L stimulation suggests that pRBCs might alter the interaction of specific signalling proteins shared by both pathways. For instance, TRAF6 is one of the signalling molecules recruited following both LPS and CD40L stimulation. pRBCs may therefore block DC activation by interfering with the recruitment of TRAF6 or with its interaction with other signalling molecules. Indeed, at the present time, this possibility remains purely speculative and further studies are required to elucidate the maturation signalling events that may be disrupted in DCs following pRBC treatment.

To investigate whether the observed effect on DC function could produce a defect in antigen presentation, I analysed the ability of pRBC-treated DCs to induce proliferation of a T cell hybridoma specific for OVA. These cells only require the presentation of antigen by MHC for activation and are thus insensitive to the ability of APC to provide costimulatory activity (Underhill *et al.*, 1999). The results show that the antigen presenting ability of DCs treated with pRBCs prior to antigen loading was significantly reduced, a result, which concurred with the reduced expression of MHC class II previously found.

Collectively the results presented in this chapter demonstrate that pRBCs exert profound effects on the ability of DCs to carry out key functions that are necessary for the development of an appropriate immune response. It is now widely accepted that encounter with parasites can strongly modify DC function and lead to altered T cell responses both *in vitro* and *in vivo* (Sher *et al.*, 2003). This can be achieved by altering the maturation state of DCs leading to alternatively functional T cells (such as regulatory cells) or a failure of T cell activation leading to T cell anergy, as extensively described in section 1.2.5. Parasites may induce these altered T cell responses as a mechanism to avoid functional T cell responses directed against them, thus enhancing their chances of survival. Besides directly interacting with DCs, parasites may alter the development of protective immune responses by releasing parasite products, which may modulate DC function, or by inducing DCs to secrete immunoregulatory factors such as prostaglandins

(Harizi and Gualde, 2002) and TGF- $\beta$  (Li *et al.*, 2005), which are known to suppress DC function and therefore the induction of appropriate T cell responses. Since DCs play a pivotal role in the induction of primary immune responses, it is reasonable to assume that altered or suppressed T cell responses may result as a consequence of impairment of DC function.

In subsequent chapters I directly analyse all of the above possibilities by investigating the phenotype of the T cell response induced by pRBC-treated DCs, the involvement of selected parasite components, soluble parasite factors and immunoregulatory molecules potentially secreted by DCs following interaction with pRBC, in the observed suppression of DC function. A better insight of the mechanisms employed by the malaria parasite to interfere with DC biology may help to open new avenues for the understanding of immunosuppression observed during malaria infection and develop new treatment strategies.

**4. *P. chabaudi* AS –treated DC fail to induce functional T cell responses *in vitro*.**

## 4.1 Introduction

The ability to prime naïve T cells is a unique and critical function of DCs both *in vitro* and *in vivo* (Adams *et al.*, 2005). Effective priming of naïve T cells results in their clonal expansion and differentiation into cytokine secreting effector cells and memory cells (Adams *et al.*, 2005). As described in section 1.2.6, immune responses to different types of pathogens are associated with different types of effector responses directed by polarised Th1 and Th2 cell subsets. Furthermore, DCs may induce the development of 'alternatively' functional T cells, which are commonly referred to as 'regulatory T cells'. These latter cells include Tr1 cells, which secrete high levels of IL-10, and Th3 cells, which secrete TGF- $\beta$  (Jonuleit and Schmitt, 2003) (see section 1.2.5.2).

Besides inducing functional Th1/Th2 cell responses and T regulatory cells, interaction of DCs with T cells may result in failure of T cell activation leading either to T cell anergy or T cell apoptosis. T cell anergy is defined as a state in which the cell is viable but fails to display certain functional responses (proliferation and IL-2 production) upon exposure to its specific antigen and results from partial T cell activation due to a lack of adequate co-stimulation (Alegre *et al.*, 2001; Lechler *et al.*, 2001) (refer to sections 1.2.5.1 and 1.2.5.3 for more details). Deletion or apoptosis of antigen-specific T cells instead may be induced following engagement of death receptors, such as Fas (Hildeman *et al.*, 2002) or may be controlled by members of the Bcl-2 family of proteins (Li *et al.*, 2004a) (refer to section 1.2.5.4 for more details). The induction of regulatory T cells, T cell anergy and T cell apoptosis represent important mechanisms for the maintenance of central and peripheral tolerance. Nevertheless, there is now evidence indicating that certain pathogens adopt these mechanisms to avoid the establishment of effector T cell responses directed against them (extensively discussed in sections 1.2.6-1.2.10).

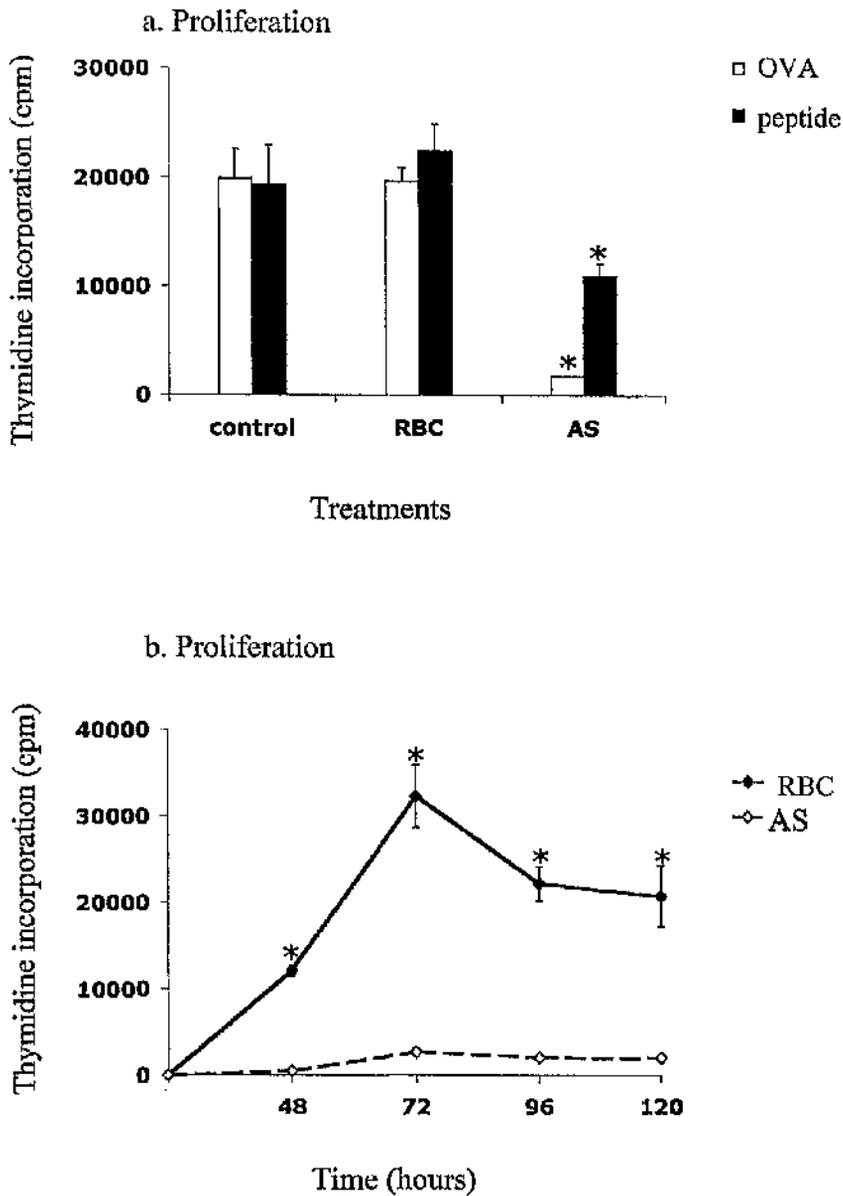
In chapter 3, I analysed the effects that *P. chabaudi* AS-infected erythrocytes have on DCs in terms of antigen presentation, expression of costimulatory molecules and cytokine production, important requirements for activation and differentiation of naïve T cells. I showed that all of the above functions were downregulated following exposure of DCs to

infected erythrocytes, suggesting that the ability of these cells to prime naïve T cells into effector T cells might be affected. In this chapter I addressed this issue directly by analysing the phenotype of the T cell response induced by *P. chabaudi* AS-infected erythrocytes.

## 4.2 Results

### 4.2.1 The ability of DCs to induce the proliferation of naïve TcR transgenic T cells *in vitro* is affected by *P. chabaudi* AS infected erythrocytes

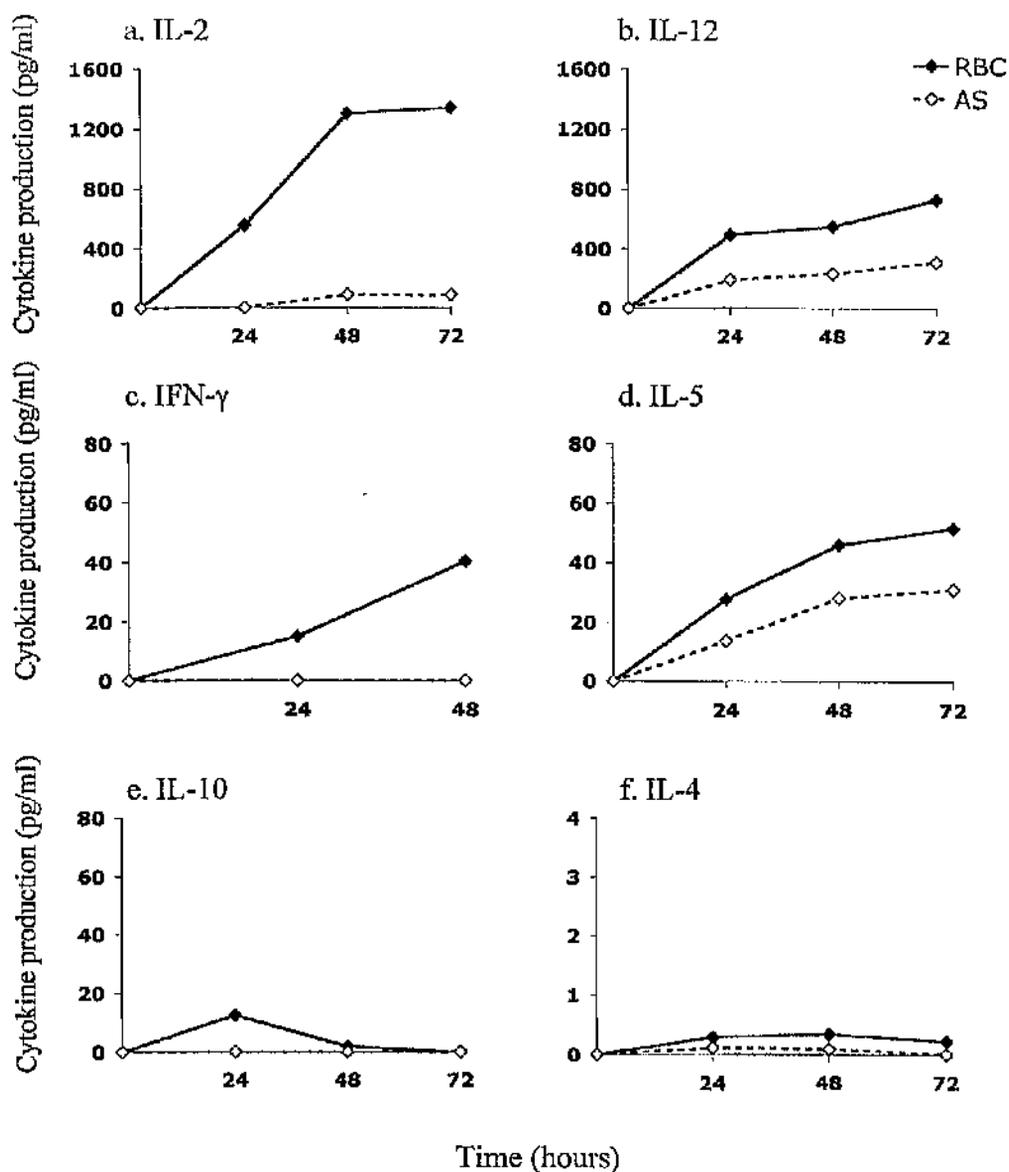
As mentioned earlier, DCs play a pivotal role in the induction of primary immune responses by efficiently activating naïve T cells (Adams *et al.*, 2005). To evaluate whether pRBC-treated DCs retained this ability, I assessed the capacity of these cells to activate naïve, OVA-specific TcR transgenic T cells. DCs were treated with RBCs or pRBCs for 24 h and then pulsed with either OVA protein or OVA peptide for 6 h. Untreated DCs were used as controls. OVA-specific transgenic T cells were added to the culture and the proliferative response assessed after 72h of culture. DCs cultured in the absence of RBCs, and DCs pre-treated with RBCs before OVA loading, had the same ability to induce *in vitro* proliferation of naïve OVA-specific T cells (Figure 4.1a). However, this capacity was significantly reduced when DCs were pre-incubated with pRBCs before antigen loading. In addition, the defect observed was greater when the cells were pulsed with OVA protein than OVA peptide, suggesting that pRBCs may interfere with both the antigen processing and presentation pathways (Figure 4.1a). Furthermore, to exclude the possibility that treatment of DCs with pRBCs could alter the dynamics of the T cell proliferative response, I harvested the T cells at 48, 72, 96 and 120 h of culture (Figure 4.1b). The results clearly show that the ability of pRBC-treated DCs to induce T cell proliferation was significantly downregulated compared to the control group throughout the observation period.



**Figure 4.1** *P. chabaudi* AS infected erythrocytes inhibit the ability of DCs to induce naïve T cell proliferation.  $2 \times 10^5$  DCs were treated with  $2 \times 10^7$  *P. chabaudi* AS infected (AS) or non-infected erythrocytes (RBC) for 24 h. (a - b). DCs were then pulsed with 5 mg/ml of OVA (a-b) or with  $40 \mu\text{g/ml}$  of OVA peptide for 6 h (a). OVA-specific transgenic T cells were isolated from lymph nodes of DO11.10 SCID mice and added to the culture to achieve 1:1 DC: T cell ratio. [ $^3\text{H}$ ] thymidine was added for the last 18h of a 72h culture (a) or of a 48, 72, 96 and 120h cultures (b). Results show mean proliferation of triplicate cultures  $\pm$  standard error. \*,  $p \leq 0.05$  (AS vs RBC).

#### 4.2.2 Cytokine production by TcR transgenic T cells *in vitro* is affected by *P. chabaudi* AS infected erythrocytes

DCs treated with *Plasmodium*-infected erythrocytes or taken from infected mice have been shown to present peptide derived from parasite proteins to CD4<sup>+</sup> T cells (Bruna-Romero and Rodriguez, 2001; Perry *et al.*, 2004). Studies with bone marrow-derived DCs (Seixas *et al.*, 2001) and with splenic DCs (Luyendyk *et al.*, 2002) also showed that *P. chabaudi*- or *P. yoelii*- infected erythrocytes can induce increased expression of MHC class II and costimulatory molecules by DCs as well as production of IL-12, which are necessary for activation of naïve T cells. Furthermore, splenic DCs isolated from *P. yoelii*- infected mice have been shown to activate naïve CD4<sup>+</sup> T cells to produce IL-2 and can support production of IFN- $\gamma$  and TNF- $\alpha$  (Perry *et al.*, 2004). Collectively, these observations suggest that the initial interaction of DCs with infected erythrocytes would result in a Th1 CD4<sup>+</sup> T cell response, as observed during an early acute infection *in vivo* (Langhorne *et al.*, 2002). For this reason, the major cytokines involved in Th1 and Th2 responses were investigated. DCs were treated with RBCs or pRBCs for 24 h and then loaded with OVA protein for 6 h. OVA-specific transgenic T cells were added to the culture and the concentration of IL-2, IL-12, IFN- $\gamma$ , IL-5, IL-10 and IL-4 were measured in supernatants harvested after 24, 48 and 72 h of culture (Figure 4.2a - f). The results revealed that both Th1 and Th2 cytokine production was downregulated in the pRBC-treated groups compared to controls.



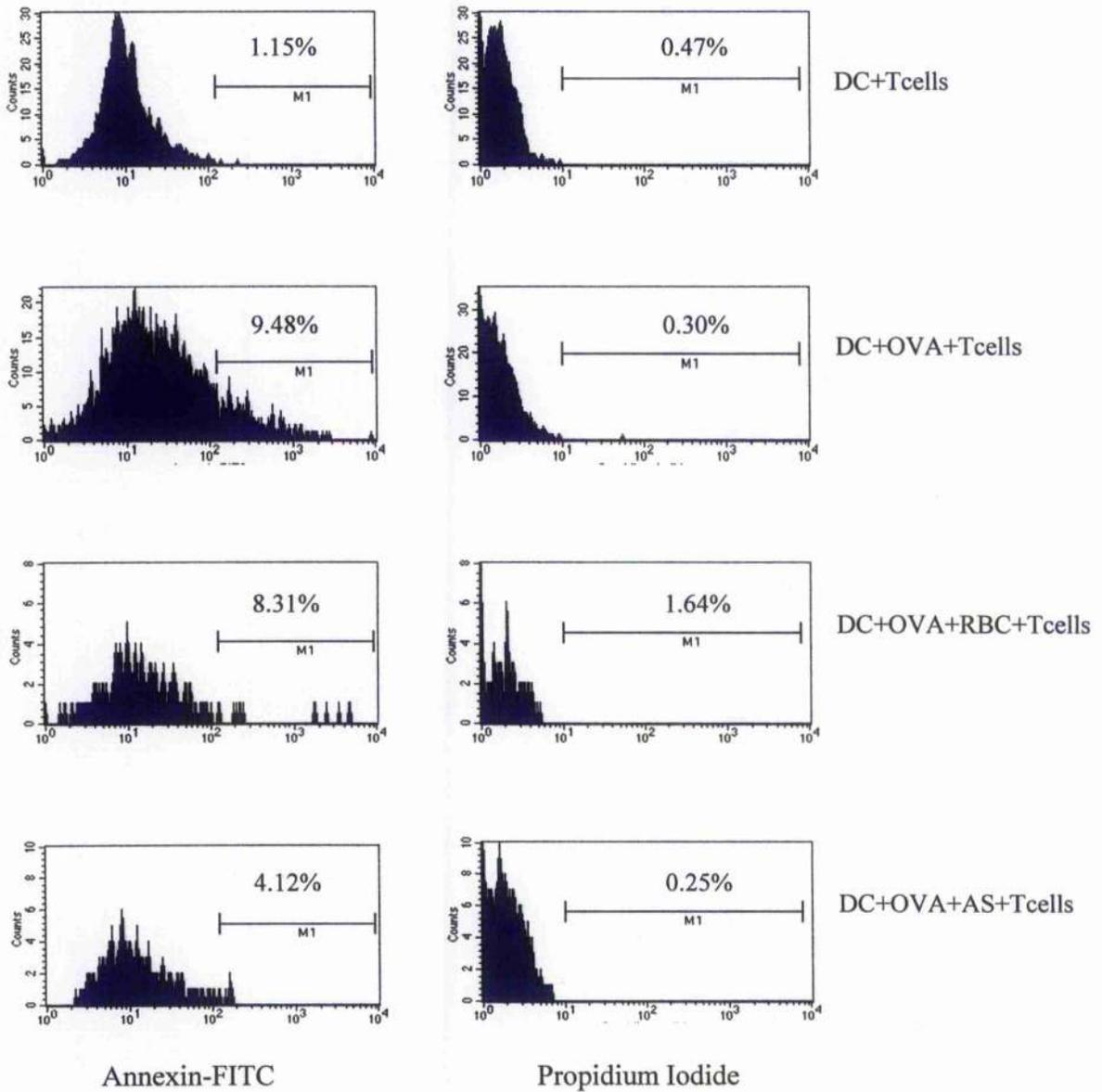
**Figure 4.2** Cytokine production by T cells is downregulated following incubation with *P. chabaudi* AS-treated DCs. The concentration of (a) IL-2, (b) IL-12, (c) IFN- $\gamma$ , (d) IL-5, (e) IL-10 and (f) IL-4 secreted by OVA-specific T cells after incubation with DCs treated with non-infected (filled diamonds) or *P. chabaudi* AS-infected erythrocytes (empty diamonds) were measured in supernatants harvested after 24, 48 and 72 h of culture. Experimental cultures were set up as described in figure 4.1. The data show preliminary results obtained from one experiment only. Results show the mean concentration of duplicate cultures.

#### 4.2.3 TcR transgenic T cell viability is not affected by *P. chabaudi* AS-treated DCs

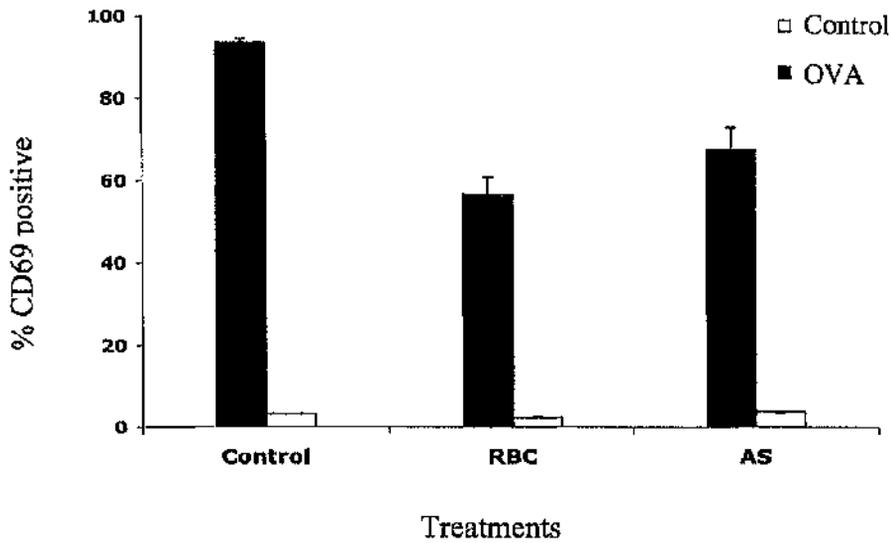
As mentioned earlier, apoptosis of antigen-specific T cells may provide a potential mechanism used by pathogens to evade host protective immune responses directed against them. In fact, apoptotic deletion of T cells has been suggested to be one of the mechanisms responsible for the suppression of T cell responses observed during malaria infection (Wipasa *et al.*, 2001). To investigate the possibility that the lack of T cell proliferation and cytokine production previously observed was due to T cell death, an Annexin V/ Propidium Iodide assay was carried out. Experimental cultures were set up as described in 4.2.2. T cells were harvested after 24h and the percentage of apoptotic cells within each culture condition was determined by flow cytometry. The results showed that the previously observed reduction of T cell proliferation and cytokine production could not be explained by T cell death as similar levels of necrotic and apoptotic T cells were detected in each culture condition (figure 4.3).

#### 4.2.4 The expression of CD69 on TcR transgenic T cells *in vitro* is not affected by *P. chabaudi* AS infected erythrocytes

Having established that pRBC-treated DCs were not able to induce OVA-specific T cell proliferation *in vitro* but did not induce antigen-specific T cell apoptosis, I examined whether these DCs were still capable of providing T cell activating signals. One of the earliest cell surface antigens expressed by T cells following activation is CD69, which is detectable within one hour of ligation of the T cell receptor/CD3 complex (Ziegler *et al.*, 1994). Experimental cultures were set up as described in 4.2.2. T cells were harvested after 24h of culture and the percentage of antigen-specific T cells expressing CD69 determined by flow cytometry. Surprisingly, the results revealed that there was not a significant difference in the percentage of OVA-specific T cells expressing CD69 between RBC and pRBC-treated groups (Figure 4.4), suggesting that although the T cells became equally activated, pRBC-treated DCs failed to induce efficient T cell proliferation.



**Figure 4.3** T cell viability is not affected following incubation with *P. chabaudi* AS-treated DCs. Experimental cultures were set up as described in figure 4.1. After 72h of culture, T cells were harvested and the percentage of apoptotic cells within each culture condition was measured using the Annexin V/ PI staining assay. Flow cytometric data is shown. Histograms are gated on CD4<sup>+</sup> T cells. Results are representative of three separate cultures.



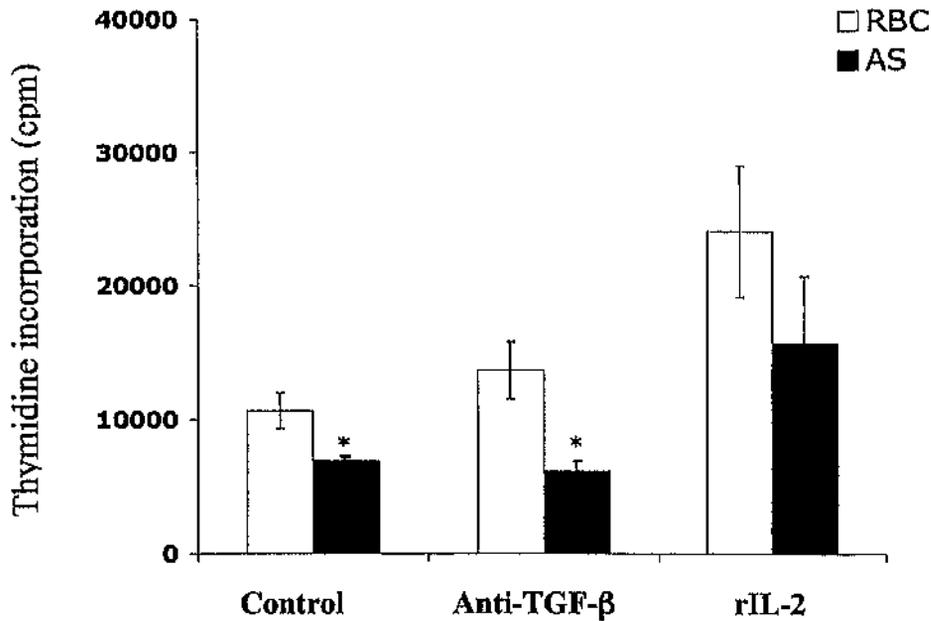
**Figure 4.4** *P. chabaudi* AS-treated DCs do not affect T cell activation. DC-T cell co-cultures were set up as described in figure 4.1. CD69 expression was assessed on DO11.10 T cells harvest from SCID mice by flow cytometric analysis after 24h of DC-T cell co-culture. Results are expressed as the percentage of antigen-specific cells expressing CD69 in cultures stimulated OVA pulsed DC (OVA; filled bars) or DC only (Control; empty bars). Results show the mean of triplicate cultures  $\pm$  standard error.

#### 4.2.5 *P. chabaudi* AS-treated DCs induce the development of anergic T cells *in vitro*.

To further characterise the phenotype of the T cell response induced by pRBC-treated DCs, I tested whether the observed suppression of the OVA-specific T cell proliferation could be rescued by addition of exogenous IL-2, a hallmark of anergic cells (DeSilva *et al.*, 1991). Furthermore, as TGF- $\beta$  is now known to be a potent regulatory cytokine exerting suppressive effects on both DCs and T cells (Li *et al.*, 2005), I also investigated whether it played a role in the observed downregulation of the OVA-specific T cell response. As explained in sections 1.2.6-1.2.10, pathogens may drive the development of inappropriate (Th1 vs Th2) or ineffective (anergic T cells or regulatory T cells) T cell responses that cannot support the development of a protective immune response. They may achieved this by directly inducing DCs to produce TGF- $\beta$ , which will in turn suppress the development of protective T cell responses; alternatively they may alter the activation state of DCs leading them to favour the development of Th3 cells, which secrete TGF- $\beta$  (Jonuleit and Schmitt, 2003) (refer to section 1.2.7 for more details). Moreover, it was recently shown that soluble components of *P. falciparum*, *P. berghei* and *P. yoelii* infected erythrocytes activated both native (platelet-derived) and recombinant latent TGF- $\beta$  directly, suggesting that malaria parasites may use this cytokine to avoid protective immune responses being directed against their elimination (Omer *et al.*, 2003).

Experimental cultures were set up as described in 4.2.2. Exogenous rIL-2 and anti-TGF- $\beta$  neutralizing antibodies were added at the beginning of the proliferation assay and T cells harvested after 72h of culture. The results showed that DCs pre-treated with RBCs before OVA loading induced normal proliferation of naïve OVA-specific T cells. However, this capacity was significantly reduced when DCs were pre-incubated with pRBCs prior to antigen loading therefore confirming previous observations (Figure 4.5). Treatment with anti-TGF- $\beta$  antibody did not rescue the T cell proliferative response, therefore excluding the possibility that this cytokine might be involved in the observed suppression of the OVA-specific T cell proliferation. The proliferation of T cells was instead increased to levels equal to those reached by control cultures when exogenous

rIL-2 was added to the culture. This result suggested that pRBC-treated DCs induced the development of T cells with an anergic phenotype.



**Figure 4.5** *P. chabaudi* AS-treated DC induce anergic T cell responses.  $2 \times 10^5$  DCs were treated with  $2 \times 10^7$  *P. chabaudi* AS infected (AS) or non-infected erythrocytes (RBC) for 24 h. 10ng/ml of rIL-2 or 5 $\mu$ g/ml of anti-TGF- $\beta$  were added at the beginning of the proliferation assay. DCs were pulsed with 5 mg/ml of OVA. OVA-specific transgenic T cells were isolated from lymph nodes of DO11.10 SCID mice and added to the culture to achieve 1:1 DC: T cell ratio. [ $^3$ H] thymidine was added for the last 18h of a 72h culture. Results show mean proliferation of triplicate cultures  $\pm$  standard error. \*,  $p \leq 0.05$  (AS vs RBC).

### 4.3 Discussion

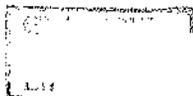
As extensively described in section 1.1.5, studies using murine models of malaria have shown that T cells play a pivotal role in protective immunity to malaria. This knowledge was substantiated by early experiments showing that parasitaemia could not be controlled in T cell-deficient animals (Jayawardena *et al.*, 1977; Weinbaum *et al.*, 1976). Nevertheless, T cell responses were shown to be suppressed during malaria infection in both humans and experimental animal models. Suppression of T cell responses to both parasite and heterologous antigens was observed in *P. falciparum* infected individuals. Patients with acute *P. falciparum* malaria were found to have a parasite-specific T cell proliferative defect that persisted for more than four weeks following treatment (Ho *et al.*, 1986). This observation suggested that blood-stage malaria infections might suppress responses important for immunity to malaria to allow parasite survival as well as raising the possibility that patients infected with *P. falciparum* might not respond as well to a malaria vaccine as would uninfected individuals. Depressed responsiveness to heterologous antigens was originally reported in 1962 by measuring the antibody responses of infected and uninfected children to vaccination with tetanus toxoid (McGregor, 1962). Later studies confirmed this observation as well as reporting reduced antibody responses to the O antigen of *Salmonella typhimurium* in children with acute malaria (Greenwood *et al.*, 1972) (see section 1.1.7 for more details on malaria-induced immunosuppression).

Many different mechanisms have been suggested to explain the suppression of T cell proliferative responses during malaria infection. Apoptosis of T cells (Wipasa *et al.*, 2001), parasite inhibition of macrophage activation and antigen processing (Arese and Schwarzer, 1997; Schwarzer *et al.*, 1998; Schwarzer *et al.*, 2003; Schwarzer *et al.*, 1992; Scorza *et al.*, 1999), inhibition of DC maturation or alteration of DC function (Urban *et al.*, 1999; Urban and Roberts, 2002, 2003; Urban *et al.*, 2001b) and involvement of T regulatory cells (Hisaeda *et al.*, 2004) are among some possible explanations.

In this chapter I investigated the phenotype of the T cell response induced by pRBC-treated DCs. I showed that the interaction of pRBC-treated DCs with T cells resulted in almost complete loss of T cell proliferation and cytokine production. More importantly, this defect was not due to the failure of T cells to recognise antigen as they increased the expression of the early activation marker CD69 to the same extent as control cultures. These observations suggested that pRBC-treated DCs were able to induce T cell activation, however the signalling events leading to T cell proliferation were somehow impaired, resulting in failure to induce effector Th1/Th2 cell responses. Moreover, the observed inhibition of T cell proliferation did not seem to be mediated by IL-10 as this cytokine was not present in the culture, excluding the potential induction of IL-10-secreting T regulatory cells by pRBC-treated DCs. Furthermore, the involvement of TGF- $\beta$ , potentially produced either by the DCs themselves following interaction with pRBCs or following the development of Th3 regulatory T cells, was excluded by adding a neutralising antibody to the culture, as it did not rescue the suppressed T cell proliferative response. Instead, the proliferation of T cells returned to levels equal to those reached by control cultures when exogenous rIL-2 was added to the culture system, indicating that pRBC-treated DCs promoted the development of T cells with anergic properties. As mentioned earlier, anergic cells result from partial T cell activation due to a lack of adequate co-stimulation (Alegre *et al.*, 2001; Lechler *et al.*, 2001) In this regard, the data presented here are consistent with previous observations described in chapter 3, where pRBCs were shown to affect DC maturation by down-regulating the expression of costimulatory molecules CD40 and CD86.

In conclusion I have demonstrated that *P. chabaudi* AS-infected erythrocytes affected the ability of DCs to induce an efficient T cell response, leading to the development of an anergic rather than an effector T cell phenotype. This observation agrees with the suppression of immunity observed in malaria-infected patients. The inability to mount an appropriate T cell response may explain the reason why many infected individuals suffer increased secondary infections such as Salmonella and meningitis (Williamson and Greenwood, 1978) as well as not being able to control viral infections such as EBV (Whittle *et al.*, 1984). A better insight of the mechanisms employed by the malaria

parasite to interfere with DC biology could therefore help to open new avenues for the understanding of immunosuppression observed during malaria infection and develop new treatment strategies.



**Investigating the effects of *Plasmodium chabaudi chabaudi* AS-infected  
erythrocytes on Dendritic Cell function *in vitro***

**Volume II**

**Caterina Di Lorenzo BSc (Hons)**

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

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Glasgow

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**5. Investigating the mechanisms by which *P. chabaudi* AS-  
infected erythrocytes induce suppression of  
DC function *in vitro*.**

## 5.1 Introduction

In the past few years there has been growing interest in studying DC function in the context of infections as these cells have a crucial role in the activation of naïve T cells and thereby, in the induction of adaptive immunity. It is becoming increasingly clear that many pathogens have evolved immune evasion strategies that may be targeted at the induction of the immune response by interfering with DC biology (Rescigno and Borrow, 2001; Sher *et al.*, 2003).

It has been reported that soluble molecules from the eggs of the helminth parasite *S. mansoni* (SEA) suppressed the LPS-induced activation of immature murine DCs, including MHC class II, costimulatory molecules CD80 and CD86 as well as IL-12 production (Kane *et al.*, 2004). *T. gondii* was also found to suppress the ability of immature DC to participate in innate immunity and to induce adaptive immune responses by rendering DCs resistant to subsequent activation by TLR ligands or CD40 ligation (McKee *et al.*, 2004). The functional consequences of *T. gondii*-mediated suppression of DC activation were in fact manifested in a relative inability of infected immature DC to activate naïve CD4<sup>+</sup> T cells, or to secrete cytokines, such IL-12 and TNF- $\alpha$ , that play important roles in innate and/or adaptive immunity (McKee *et al.*, 2004). Furthermore, it has been demonstrated that infection of mature human monocyte-derived DCs by cytomegalovirus resulted in a reduced ability to stimulate T cells in an allogeneic mixed leukocyte reaction *via* a novel mechanism, the release of soluble CD83 (Senechal *et al.*, 2004). As mentioned in previous chapters, *P. falciparum* asexual erythrocytic stages were shown to impair the ability of human DCs to undergo maturation on exposure to LPS *in vitro* and it has been suggested that this effect was mediated through the binding of CD36 to a parasite-derived molecule (PfEMP1) on the infected erythrocyte surface (Urban *et al.*, 1999; Urban *et al.*, 2001b).

Understanding the actual mechanisms used by parasites to suppress DC function may aid in the development of appropriate and effective vaccines. For instance, the effects of immunosuppressive agents potentially used by pathogens to downregulate DC function

could be reversed following their identification and knowledge of their mechanism of action, leading to enhancement of DC function and improvement of DC-based therapeutic treatments. In fact, with their powerful antigen presenting ability, DCs have the potential to overcome parasite-induced suppression of immune responses and instead induce effective anti-parasite immunity. Thus, DC-based therapies may provide a useful approach to induce potent cell-mediated responses against parasite. In order to optimise this approach, a thorough understanding of how parasites interact with DCs and the mechanisms they adopt to induce DC suppression would be extremely beneficial for future vaccine design.

In this chapter I investigated the role of selected parasite components in the parasite-mediated inhibition of DC function. Both haemozoin, the end-product of the haemoglobin catabolism by intraerythrocytic malaria parasite, and soluble parasite factors secreted by pRBCs (which have not been identified yet) were found to contribute significantly to the immunosuppressive effects induced in DCs, demonstrating that products of the intra-erythrocytic stages of malaria parasites can directly alter murine DC function *in vitro*.

## 5.2 Results

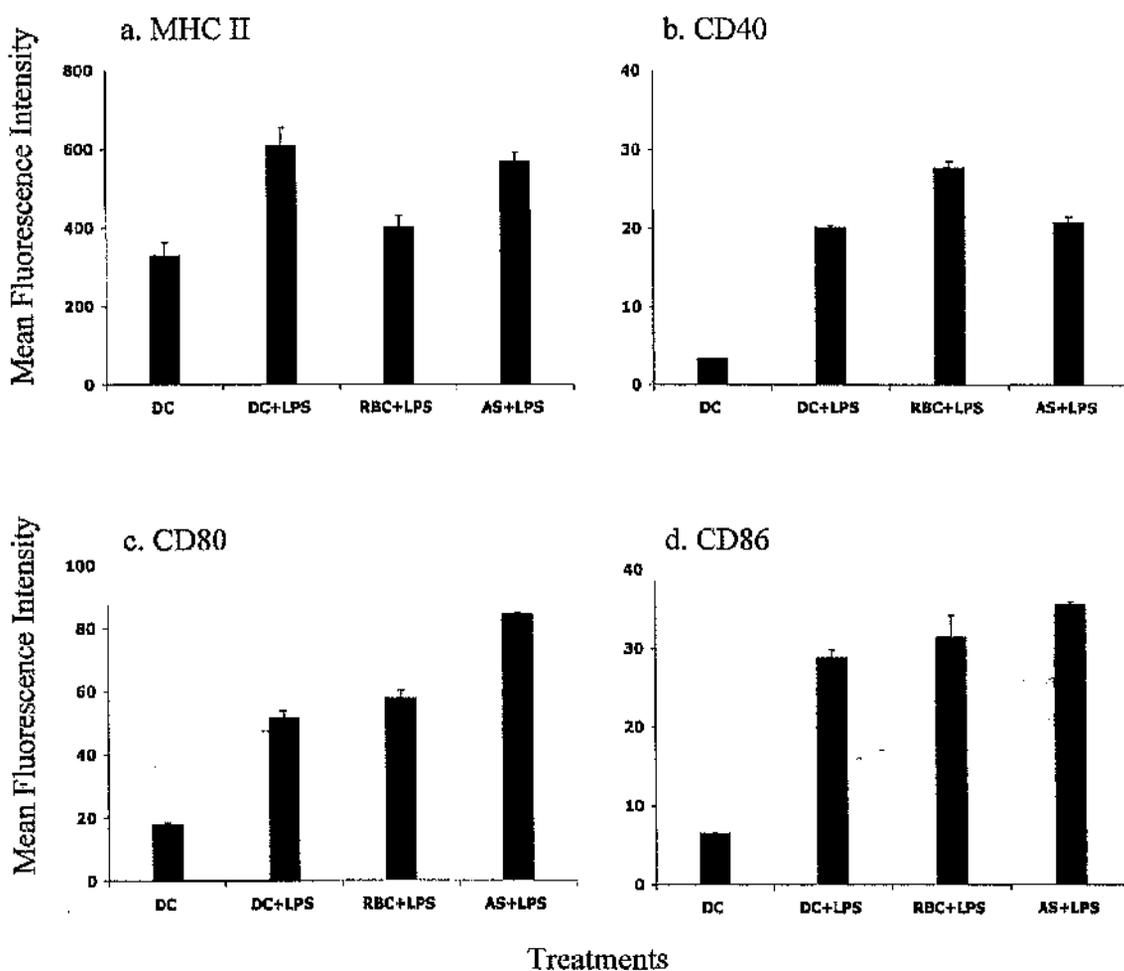
5.2.1 DCs incubated simultaneously with *P. chabaudi* AS and LPS retained their ability to mature *in vitro*.

Having established in previous chapters that pRBCs affected key DC functions, I focused on dissecting the possible mechanisms used by the parasites to exert their effects. To address this issue I initially examined whether pRBCs induced immediate suppression of DC function by simultaneously incubating pRBCs and LPS with bone marrow-derived DCs. The expression of MHC class II and the costimulatory molecules CD40, CD80 and CD86 by DCs was examined 24h later by flow cytometry. The results showed that DCs expressed low levels of surface MHC class II, CD40, CD80 and CD86 when cultured in growth medium alone, thus confirming their immature state in culture (Fig. 5.1). Stimulation with LPS promoted a significant increase in the expression of all of the costimulatory molecules tested. Furthermore, DCs incubated simultaneously with pRBCs and LPS retained their ability to mature *in vitro* as they showed similar expression levels of MHC class II, CD40, CD80 and CD86 to DCs treated with RBCs and LPS or LPS alone. This finding suggested that pRBCs required a longer pre-incubation period with DCs to affect the LPS-induced maturation of DCs *in vitro*.

5.2.2 *P. chabaudi* AS-infected erythrocytes required 6h of incubation with DCs to exert their suppressive effects.

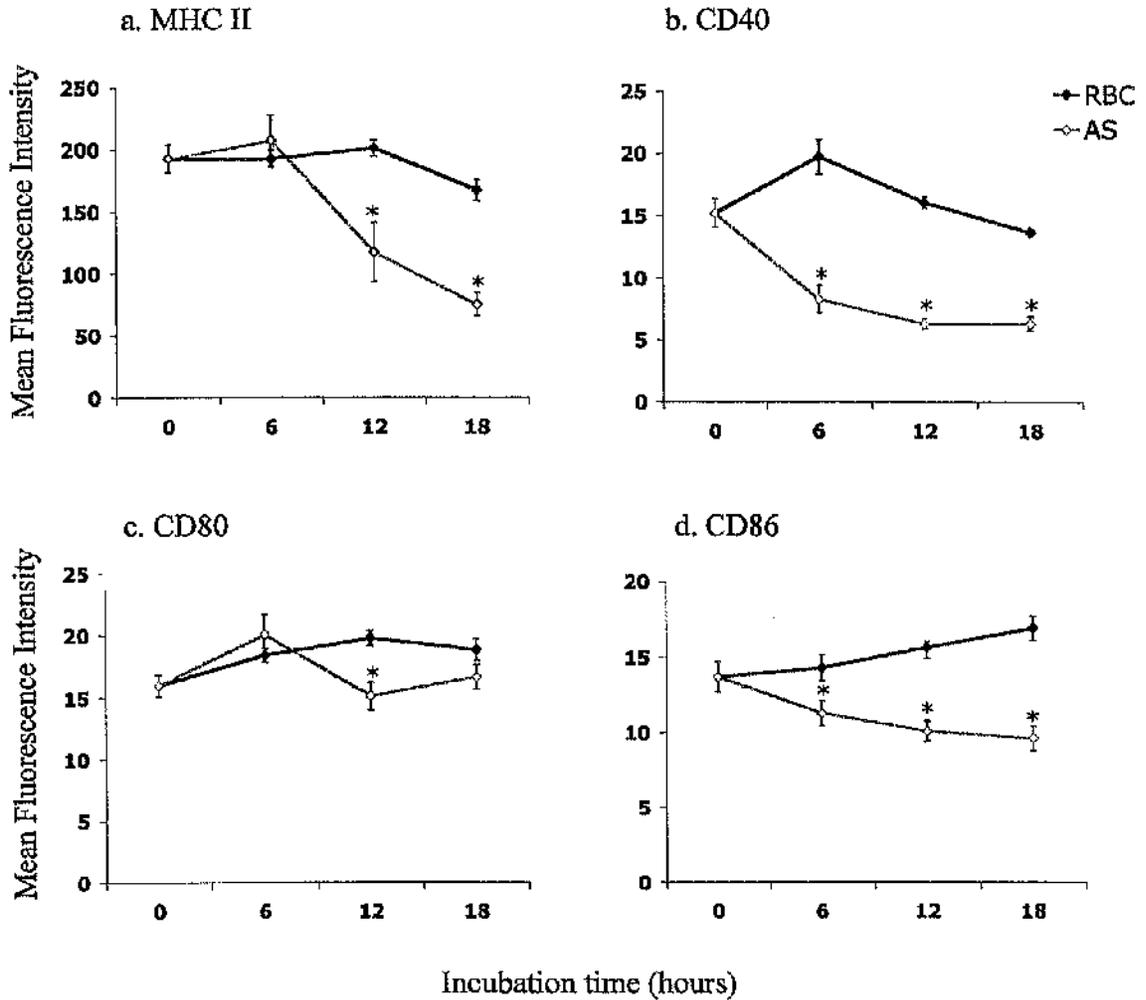
In order to investigate when DC-pRBC interactions led to suppression of DC function, DCs were incubated with RBCs or pRBCs for 6, 12 or 18h. Each experimental culture was then stimulated with LPS for a further 18h and the expression levels of MHC class II and costimulatory molecules CD40, CD80 and CD86 analysed 24h later by flow cytometry. The results showed that the ability of DCs to respond to LPS treatment *in vitro* was significantly affected when DCs were pre-incubated with pRBCs for 6h before LPS challenge as observed by the reduced levels of CD40 and CD86 expression (Fig.

5.2). The upregulation of MHC class II and CD80 in response to LPS stimulation was instead inhibited after 12h of incubation with pRBCs suggesting that the expression of costimulatory molecules CD40 and CD86 might be more sensitive to the suppressive effects induced by pRBCs. Furthermore, although the expression of CD80 was downregulated after 12h of incubation with pRBCs, it was not significantly affected when DCs were pre-incubated with pRBCs for 18h prior to LPS challenge. This finding suggested that the effects induced by pRBCs on the expression level of CD80 were transient.



**Figure 5.1** pRBCs did not immediately suppress the LPS-induced maturation of DCs *in vitro*.  $2 \times 10^6$  DCs were cultured simultaneously with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes and  $1 \mu\text{g/ml}$  of LPS (AS+LPS) or with non-infected erythrocytes and LPS

(RBC+LPS) for 24h. Unstimulated DCs (DC) or DCs stimulated with 1  $\mu\text{g/ml}$  of LPS (DC+LPS) served as negative and positive controls respectively. Surface expression of MHC class II (a), CD40 (b), CD80 (c) and CD86 (d) on DCs was determined by flow cytometric analysis of gated CD11c<sup>+</sup> cells. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group.

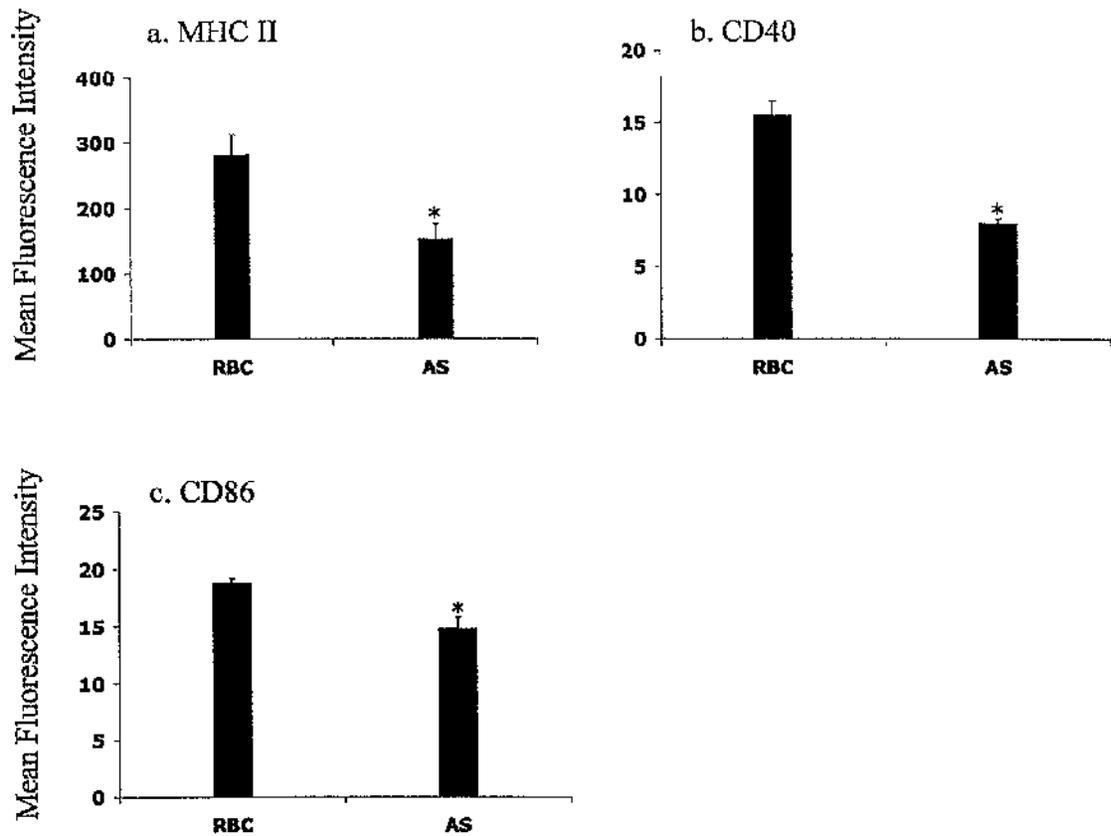


**Figure 5.2** pRBCs required 6h incubation with DCs to downregulate the expression of costimulatory molecules on DCs in response to LPS treatment *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes (AS) or with non-infected erythrocytes (RBC) for 6, 12 and 18h. Each culture was then stimulated with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Surface expression of MHC class II (a), CD40 (b), CD80 (c) and

CD86 (d) on DCs was determined by flow cytometric analysis of gated CD11c<sup>+</sup> cells. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS at the indicated time point.

### 5.2.3 Erythrocytes infected with *P. chabaudi* AS-ring stage suppressed the LPS-induced maturation of DCs *in vitro*.

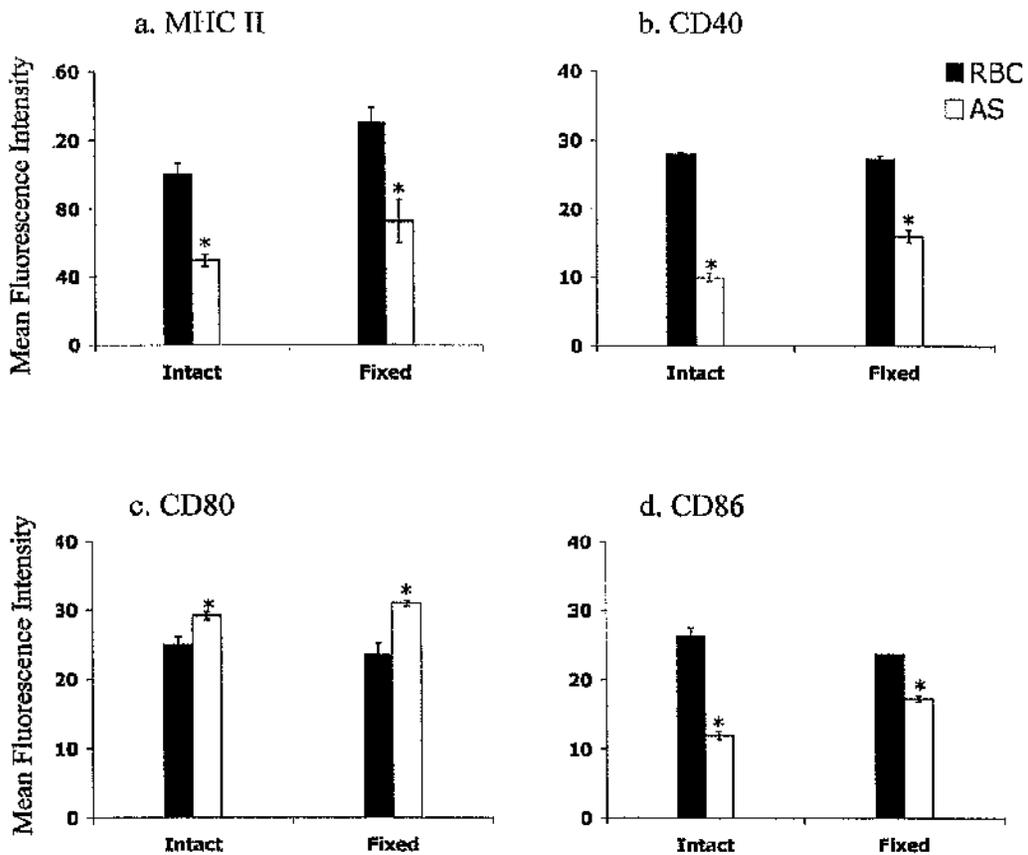
As previously mentioned in section 1.4, trophozoite-infected RBCs were used in all the experiments described in this thesis. However, it has been reported that *P. chabaudi* AS schizonts activated DCs *in vitro* (Seixas *et al.*, 2001), suggesting that different blood stages might exert different effects on DC function. I therefore investigated the effects that the early ring stage of the parasite's intra-erythrocytic cycle had on the LPS-induced maturation of DCs *in vitro*. DCs were incubated with RBCs or pRBCs. After 24h of co-culture, DCs were stimulated with LPS and the expression of MHC class II, CD40, CD80 and CD86 examined 18h later by flow cytometry (Fig. 5.3). DCs incubated with RBCs matured in response to LPS treatment as observed by the elevated levels of all of the costimulatory molecules analysed. However, when DCs were incubated with pRBCs prior to LPS challenge, the expression levels of MHC class II, CD40 and CD86 were significantly downregulated. This finding suggested that the early stages of the parasite's intra-erythrocytic life cycle (ring and trophozoites) led to suppression of DC maturation in response to LPS treatment *in vitro* whereas, according to previous reports (Seixas *et al.*, 2001), more mature stages (schizonts) led to DC activation. It is therefore possible that specific blood stages might suppress DC function to protect the more vulnerable stages (schizont and merozoites) in order to allow progression of the disease, as previously discussed in chapter 3. The latter hypothesis can only be tested *in vivo*, as it is not clear whether trophozoite-infected erythrocytes have the ability to mature to the schizont stage *in vitro* under cell culture condition (refer to 5.2.9 for details).



**Figure 5.3** pRBCs harbouring early ring stages inhibited the LPS-induced maturation of DCs *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes (AS) or with non-infected erythrocytes (RBC). After 24h of co-culture, DCs were challenged with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Surface expression of MHC class II (a), CD40 (b), and CD86 (c) on DCs was determined by flow cytometric analysis of gated CD11c<sup>+</sup> cells. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS.

#### 5.2.4 Fixed *P. chabaudi*-infected erythrocytes suppressed the LPS-induced maturation of DCs.

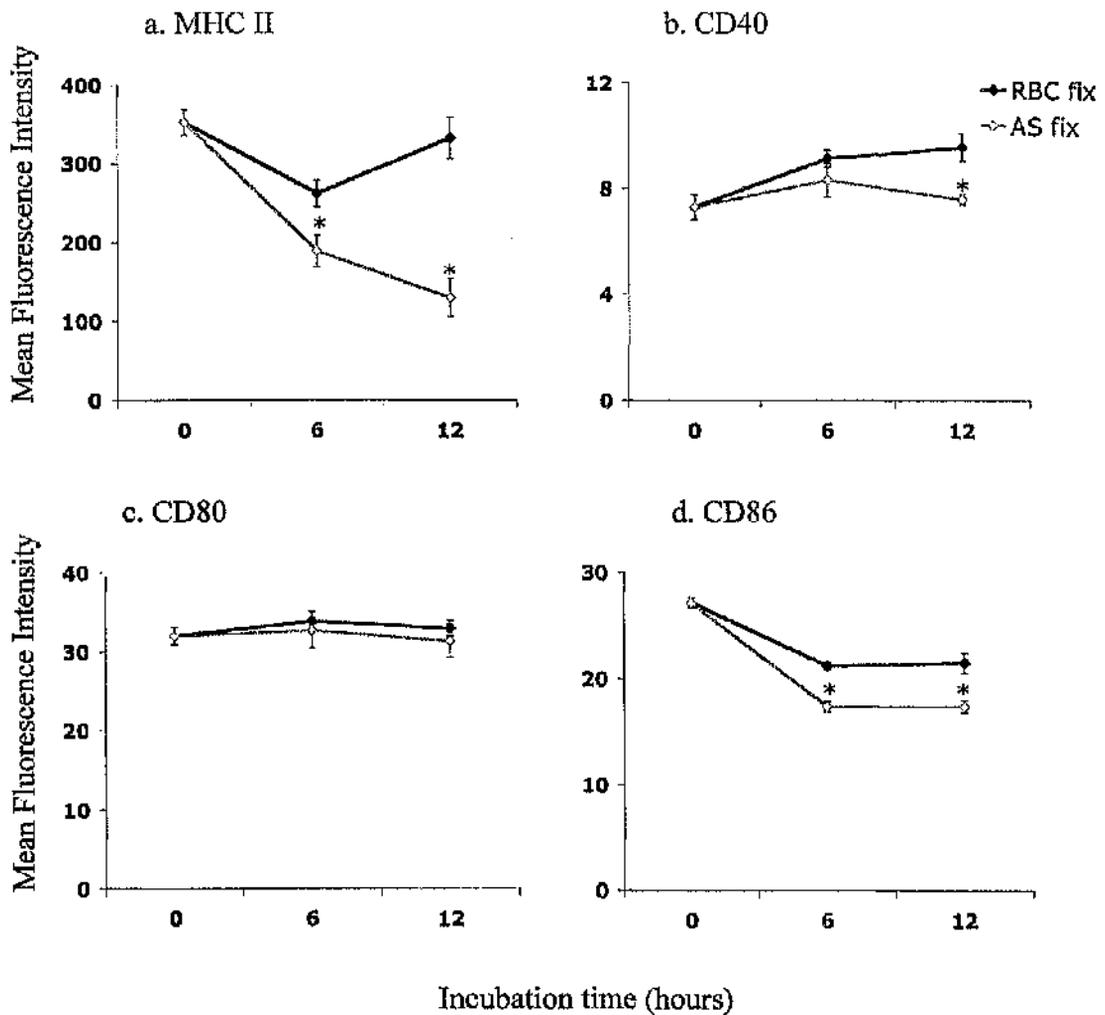
To analyse whether parasite viability or development was necessary to modulate DC function, pRBCs (and RBC controls) were fixed with paraformaldehyde, which cross-links proteins, normally through free amino groups and preserve cell structure. DCs were incubated with fixed RBCs or fixed pRBCs for 24h and then stimulated with LPS for 18h. The expression of MHC class II and co-stimulatory molecules CD40, CD80 and CD86 were examined 18h later by flow cytometry. Non-fixed RBCs and pRBCs served as controls. DCs treated with RBCs or fixed RBCs prior to LPS challenged matured efficiently as observed by the high levels of all of the costimulatory molecules tested (Fig 5.4). As previously observed in chapter 3, treatment of DCs with viable pRBCs significantly downregulated the expression levels of MHC class II, CD40 and CD86 following LPS stimulation. The same result was observed when fixed-pRBCs were used indicating that parasite viability was not a necessary requirement to induce suppression of DC function *in vitro*. Furthermore, the data also indicate that the suppression of the LPS-induced maturation observed when using viable pRBCs was not due to a more mature erythrocytic stage of the parasite, which might have developed following adaptation to cell culture conditions; pRBC fixation would in fact arrest parasite growth ensuring that only trophozoite-stages are present in the culture. Therefore the above results indicate that the observed suppression of the LPS-induced maturation of DCs is specifically mediated by the trophozoite stage.



**Figure 5.4** Fixed pRBCs inhibited the LPS-induced maturation of DCs *in vitro*.  $2 \times 10^6$  DCs were treated for 24 h with non-infected (RBC; filled bars) or *P. chabaudi* AS-infected erythrocytes (AS; open bars) that were non-fixed (intact) or fixed (fixed) prior to stimulation with LPS (1  $\mu\text{g}/\text{ml}$ ) for 18 h. DC activation was characterised by examination of (a) MHC class II, (b) CD40, (c) CD80 and (d) CD86 expression on the DC surface by flow cytometric analysis of gated CD11c<sup>+</sup> cells. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS.

### 5.2.5 Fixed-pRBCs required 6h of incubation with DCs to exert their suppressive effects.

To further confirm that trophozoite-infected erythrocytes were responsible for the modulation of DC functions previously analysed in chapter 3, I initially investigated whether the dynamics of the LPS-induced suppression of DCs induced by fixed-pRBCs were similar to those observed when using viable parasites. DCs were incubated with fixed-RBCs or fixed-pRBCs for 6 or 12h. Each experimental culture was then stimulated with LPS and the expression levels of MHC class II and costimulatory molecules CD40, CD80 and CD86 analysed 18h later by flow cytometry. The ability of DCs to respond to LPS treatment *in vitro* was significantly affected when DCs were pre-incubated with fixed-pRBCs. Particularly MHC class II and CD86 expression were affected after only 6h of incubation of DCs with fixed-pRBCs (Fig. 5.5). The upregulation of CD40 in response to LPS stimulation was instead inhibited after 12h of incubation with pRBCs. Contrary to observations made with viable pRBCs, the expression of CD80 was not significantly affected when DCs were incubated with fixed-pRBCs for 12h prior to LPS stimulation. Overall, these findings suggested that the dynamics of suppression induced by fixed-pRBCs were very similar to that induced by non-fixed pRBCs, confirming that viable pRBCs were not necessary to induce suppression of DC function *in vitro* and that trophozoite-stages are responsible for the observed effects.



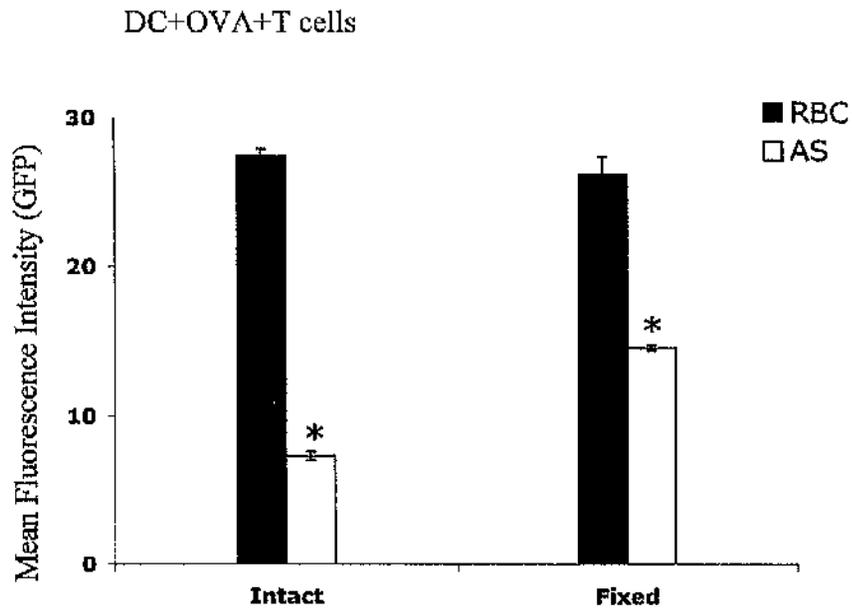
**Figure 5.5** Fixed pRBCs required 6h incubation with DCs to downregulate the expression of costimulatory molecules on DCs in response to LPS treatment *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  fixed *P. chabaudi* AS-infected erythrocytes (AS fix) or with fixed non-infected erythrocytes (RBC fix) for 6 and 12h. Each culture was then stimulated with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Surface expression of MHC class II (a), CD40 (b), CD80 (c) and CD86 (d) on DCs was determined by flow cytometry. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS at the indicated time point.

### 5.2.6 Fixed-pRBCs inhibit the antigen presenting ability of DCs *in vitro*.

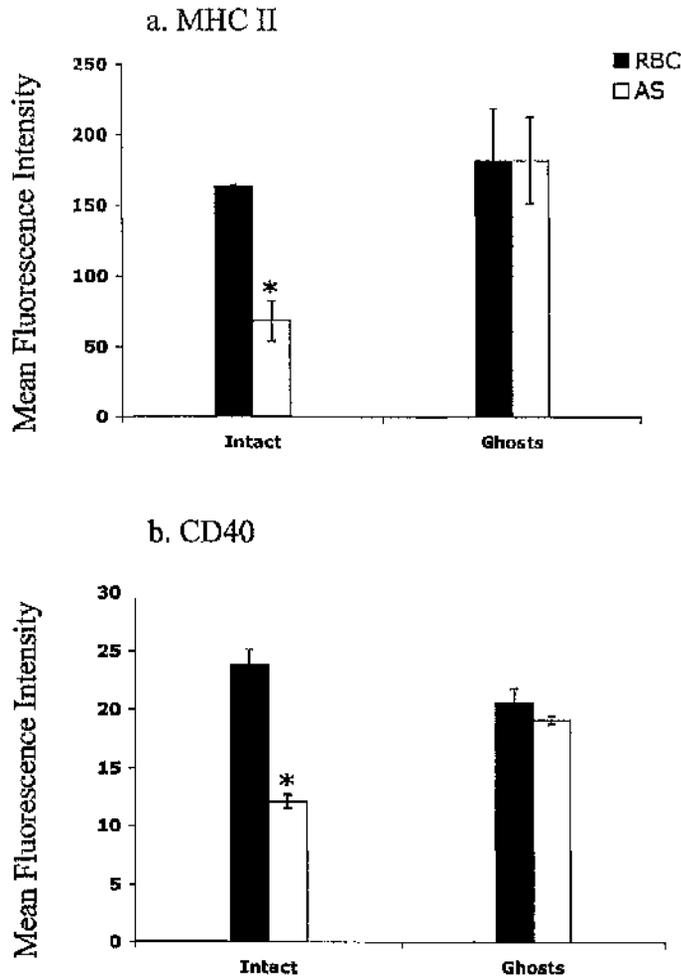
To further ensure that fixed-pRBCs suppressed DC functions in a manner similar to that observed when viable pRBCs were used, the antigen presenting ability of fixed-pRBC-treated DCs was analysed by using DO11.10 OVA-specific T cell hybridoma cells that express green fluorescent protein (GFP) upon TcR ligation (Underhill *et al.*, 1999) as previously described in section 3.2.3. DCs were incubated with fixed-RBCs or fixed-pRBCs for 24h and then pulsed with OVA protein for 6h. Non-fixed RBCs and pRBCs were used as controls. OVA-specific T cells were added to each culture and GFP expression, indicative of antigen presentation independent of co-stimulation, examined 24h later by flow cytometry. DCs incubated with RBCs or fixed-RBCs prior to antigen loading efficiently presented OVA as observed by high levels of GFP expression by T cells. However, the ability of DCs to activate the OVA-specific T cell hybridoma was significantly reduced when they were incubated with pRBCs or fixed-pRBCs (Fig. 5.6), indicating that viable and fixed-pRBCs might use a common suppressive mechanism.

### 5.2.7 pRBC-ghosts did not suppress the LPS-induced maturation of DCs *in vitro*.

In order to understand the mechanisms involved in the parasite-mediated modulation of DC function, I initially analysed the effect that parasite proteins, expressed on the erythrocyte membrane, had on DC function. DCs were exposed to plasma membranes (ghosts) isolated from RBCs or pRBCs. After 24 h of culture, DCs were challenged with LPS and the expression of costimulatory molecules examined 18 h later by flow cytometry. Intact RBCs and pRBCs were used as controls (Fig. 5.7). DCs upregulated the expression of costimulatory molecules MHC class II and CD40 when they were incubated with RBCs or RBC-ghosts prior to LPS challenge. pRBC-treated DCs were not able to efficiently respond to LPS treatment as the levels of MHC class II and CD40 were significantly downregulated, confirming previous results. However, DCs exposed to pRBC-ghosts retained their ability to respond to LPS stimulation as the levels of MHC class II and CD40 were not significantly affected. This finding suggested that parasite molecule expressed on the erythrocyte membrane did not modulate DC activation.



**Figure 5.6** Fixed *Plasmodium chabaudi* AS-infected erythrocytes inhibited antigen presentation by DCs.  $5 \times 10^5$  DCs were treated with non-infected (RBC; filled bars), or *P. chabaudi* AS-infected erythrocytes (AS; open bars) that were non-fixed (intact) or fixed (fixed). After 24 h of culture, the cells were pulsed with 5 mg/ml of OVA for 6 h. The level of antigen presentation was determined by incubating DCs with the OVA-specific DO11.10-GFP hybridoma at 1:1 DC : T cell ratio and T cell activation assessed 24 h later by flow cytometry. Results are expressed as the mean fluorescence intensity of GFP expression by  $CD4^+$  T cells  $\pm$  standard error. \*,  $p \leq 0.05$  significant difference between RBC and AS.

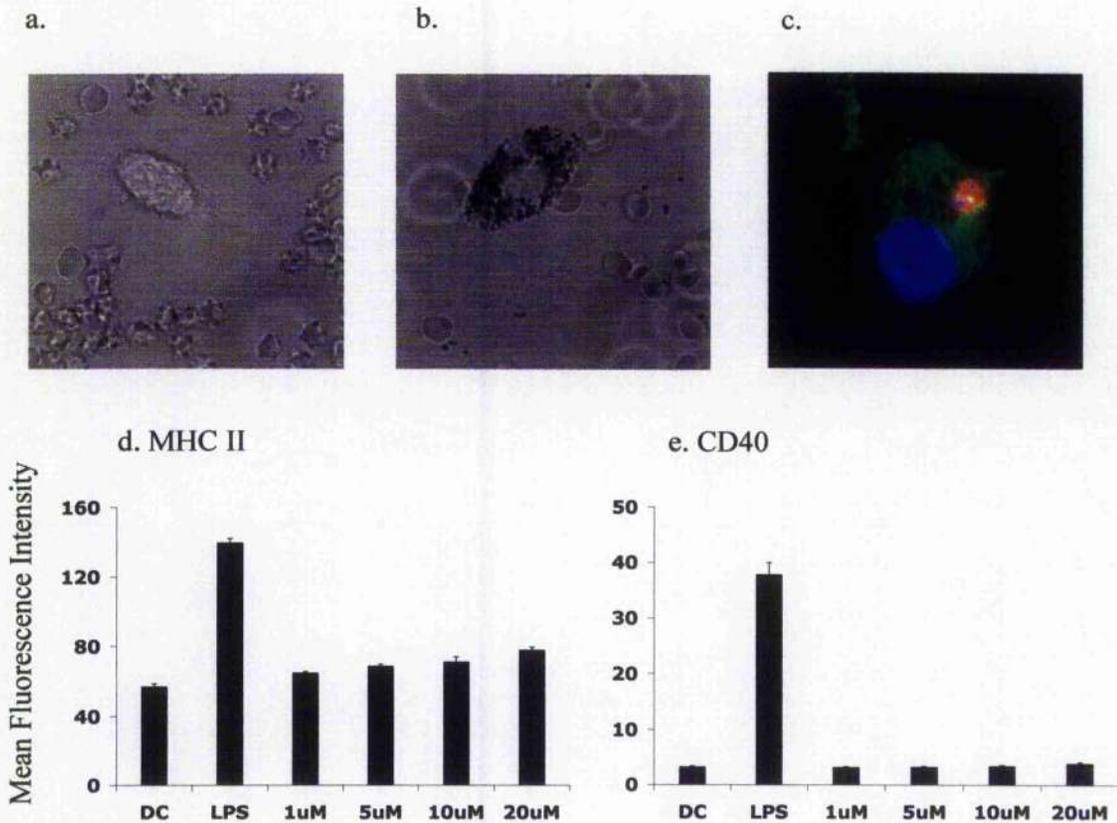


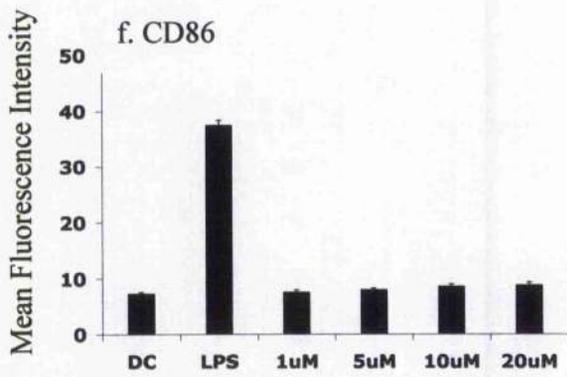
**Figure 5.7** pRBC-ghosts did not affect the ability of DCs to mature in response to LPS treatment *in vitro*.  $5 \times 10^5$  DCs were incubated with intact erythrocytes (intact) or RBC ghosts (ghosts) from non-infected (RBC; filled bars) or *P. chabaudi* AS-infected erythrocytes (AS; open bars), for 24 h prior to stimulation with LPS (1  $\mu\text{g}/\text{ml}$ ) for 18 h. DC activation was characterised by analysis of (a) MHC class II and (b) CD40 expression on the DC surface by flow cytometry. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS.

5.2.8 Activation of DCs in response to LPS treatment *in vitro* was suppressed by haemozoin.

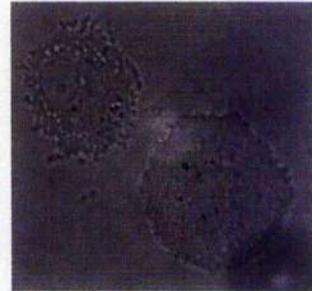
Having established that the LPS-induced maturation of DCs *in vitro* could be modulated by pRBCs, but was not due to the effects of parasite proteins expressed on the erythrocyte cell membrane, I focused my attention on haemozoin (HZ), a byproduct of haemoglobin digestion. One of the reasons that led me to investigate the role of HZ in the parasite-induced modulation of DC function was the altered morphology of DCs following a 24h incubation with pRBCs (Fig. 5.8a-b). DCs were dispensed on polylysine-coated coverslips and left to adhere overnight before addition of RBCs or pRBCs. After 24h of co-culture, coverslips were transferred onto glass slides and examined by bright-field microscopy. DCs showed their characteristic cell morphology with many long processes when cultured with RBCs. In contrast, incubation with pRBCs caused a dramatic change in cell morphology with "black pigment" clearly visible inside DCs. This may be due to the uptake of pRBCs and HZ formation. To verify whether DCs could internalise intact pRBCs, DCs and pRBCs co-cultures were set up as described above and incubated for 3h. DCs were stained with Cholera toxin, which stain lipid rafts and provide a clear cell membrane staining, facilitating DC visualisation by fluorescence microscopy (Nichols, 2002; Ribi *et al.*, 1988). pRBCs were instead stained with Biotin-conjugated rat anti-mouse TER-119 monoclonal antibody, which reacts with a 52-kDa molecule associated with glycophorin A on cells of the erythroid lineage (Kina *et al.*, 2000). Coverslips were transferred onto glass slides using mounting medium containing DAPI to reveal cell nuclei (Fig. 5.8c). Examination of prepared slides by fluorescence microscopy revealed the presence of pRBC inside DC as shown by visible blue stains inside the RBC, indicative of parasite nuclei. To investigate the effects of HZ on DC function, I initially analysed its ability to activate DCs directly *in vitro*. DCs were treated with 1, 5, 10 or 20  $\mu$ M of HZ and the expression of MHC class II and costimulatory molecules CD40 and CD86 examined 24h later. DCs grown in medium only and those treated with LPS served as negative and positive control respectively. DCs expressed very low levels of surface MHC class II, CD40 and CD86 when cultured in growth medium alone, confirming their immature state in culture (Fig. 5.8d-f). Stimulation with LPS promoted a significant

increase in the expression level of all of the costimulatory molecules tested. However, DCs incubated for 24h with HZ did not increase expression of MHC class II, CD40 and CD86, indicating that HZ did not induce DC activation at any of the concentrations tested. To ensure that DCs internalised HZ efficiently, DC were observed by bright-field microscopy (Fig. 5.8g-j). Deposits of HZ were clearly visible, as black “spots” inside DCs. Furthermore the magnitude of HZ deposition inside DCs seemed to be proportional to the concentration of HZ added to the cultures. I then examined whether HZ-treated DCs were still able to respond to LPS treatment *in vitro* (Fig. 5.8k-m). Experimental cultures were set up as described above and stimulated with LPS for 18h. The results clearly showed that as the concentration of HZ increased, the LPS-induced maturation of DCs was downregulated, as observed by the reduced levels of MHC class II, CD40 and CD86. Collectively these findings suggest that HZ is a key factor involved in the suppression of murine DC function *in vitro*.

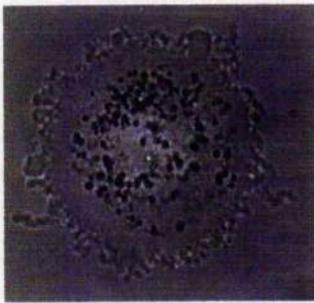




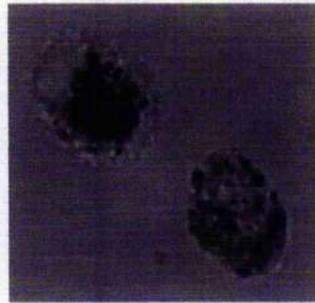
g. 1 $\mu$ M HZ



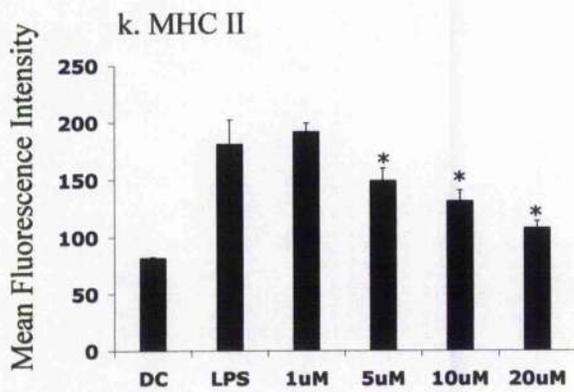
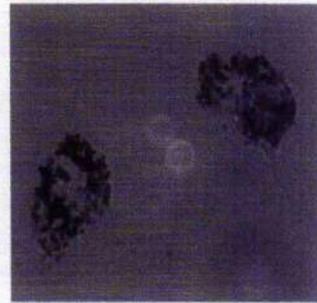
h. 5  $\mu$ M HZ



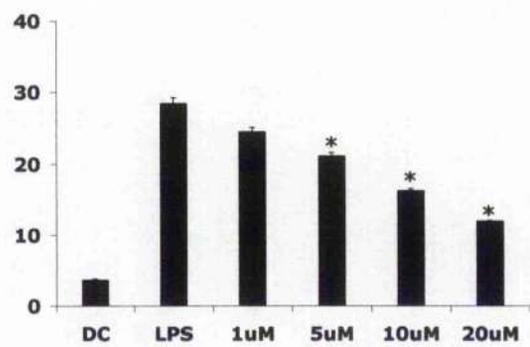
i. 10  $\mu$ M HZ

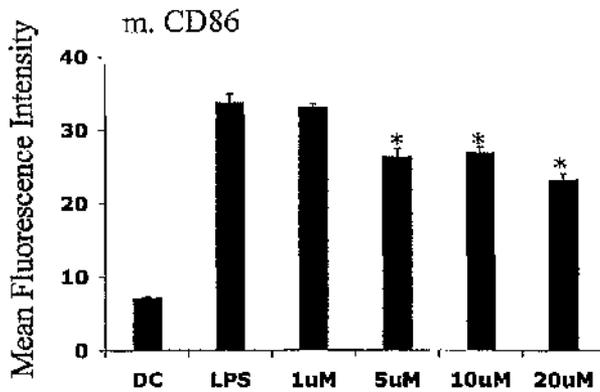


j. 20  $\mu$ M HZ



l. CD40





**Figure 5.8** Haemozoin (HZ) affected the ability of DCs to respond to LPS treatment *in vitro*. (a-b)  $1 \times 10^5$  DCs were incubated with *P. chabaudi* AS-infected or non-infected erythrocytes. After 24h of co-culture, cells were examined by bright-field microscopy using a 63x oil immersion lens. (c) Experimental cultures were prepared as described above. After 3h of co-culture, DCs were stained with  $0.5 \mu\text{g/ml}$  of CTxB-Alexa Fluor 488 (green) and then fixed with 0.5% of paraformaldehyde. Infected erythrocytes were stained with Biotinylated-TER-119 antibody, which reacts with a 52-kDa molecule associated with glycoprotein A on cells of the erythroid lineage, and detected using streptavidin-FITC (red). Cell nuclei were revealed using Vectashield with DAPI (blue). Images were obtained using an Axiovert S-100 Zeiss microscope fitted with a 63x oil immersion lens. (d-f)  $2 \times 10^6$  DCs were cultured with  $1 \mu\text{M}$ ,  $5 \mu\text{M}$ ,  $10 \mu\text{M}$  and  $20 \mu\text{M}$  of HZ. After 24 h, the level of expression of (d) MHC class II, (e) CD40 and (f) CD86 was determined by flow cytometry. (g-j)  $2 \times 10^6$  DCs were cultured with (g)  $1 \mu\text{M}$ , (h)  $5 \mu\text{M}$ , (i)  $10 \mu\text{M}$  and (j)  $20 \mu\text{M}$  of HZ. After 24h of culture, cells were examined by bright-field microscopy using a 63x oil immersion lens. (k-m) After 24h of culture with HZ,  $1 \mu\text{g/ml}$  of LPS was added to DCs and the levels of (k) MHC class II, (l) CD40 and (m) CD86 analysed 18 h later by flow cytometry. All results are shown as the mean fluorescence intensity of triplicate cultures  $\pm$  standard error. \*,  $p \leq 0.05$  ( $\mu\text{M}$  HZ vs LPS).

### 5.2.9 A soluble factor(s) released by pRBCs modulated the LPS-induced maturation of DCs *in vitro*.

Having established that HZ played an important role in modulating DC function, I decided to investigate whether the parasite used additional mechanisms to efficiently exert its suppressive effects on DC function. I addressed this issue by using a transwell culture system where pRBCs and DCs were separated by a 0.4µm pore size membrane. This allowed me to investigate whether DCs had to interact directly with pRBCs to be modulated. DCs were cultured in the lower compartment of a transwell culture plate. pRBCs were added to the upper compartment and incubated for 24h. DCs were then challenged with LPS and the expression of MHC class II and CD40 examined 24h later by flow cytometry. Supernatants were also collected for cytokine analysis. RBCs and pRBCs cultured with DCs directly were used as controls.

DCs cultured directly with RBCs or separated by a membrane efficiently responded to LPS challenged as observed by the high levels of expression of both MHC class II and CD40 (Fig. 5.9a-b). Consistent with previous results, treatment of DCs with pRBCs significantly affected their ability to mature in response to LPS stimulation as the expression levels of MHC class II and CD40 were significantly downregulated. In addition, IL-12 production by DCs treated with pRBCs was significantly suppressed and small amounts of IL-10 were detected (Fig 5.9c-d). The same results were found when a membrane separated DCs and pRBCs, suggesting that a soluble factor(s) secreted by pRBCs might also be involved in the modulation of DC function *in vitro*. To ensure that this was the case, further experiments were designed to rule out the possibility that HZ was causing the observed effects. In fact, although the size of *P. chabaudi* AS HZ has not been reported, studies using different species of *Plasmodium* revealed that the average size of HZ crystals ranged between 50 and 600nm (Noland *et al.*, 2003). Having used a 0.4µm pore size membrane to separate DCs from pRBCs, the possibility still remained that during the 24h incubation period some pRBCs could have ruptured as a result of the culture conditions, allowing HZ to pass through the membrane. Alternatively, the parasites could have undergone schizogony during the incubation time, releasing HZ into

the culture. Conflicting ideas exist regarding the ability of the parasites to undergo the full erythrocytic cycle under cell culture conditions (i.e. 37°C, 5%CO<sub>2</sub>). In fact *Plasmodium* species are generally cultured in plates placed in a candle jar that provides an atmosphere of 3% CO<sub>2</sub>-17% O<sub>2</sub> or in vials that allow for continuous flow of medium into culture vessels with an atmosphere of 7% CO<sub>2</sub>-1% O<sub>2</sub>-92% N<sub>2</sub> (Schuster, 2002). However, it is possible that some parasites might adapt to the new growth conditions and be able to undergo schizogony (fig.5.9e-f). For these reasons, a series of experiments involving high-speed centrifugations were designed to ensure that supernatants harvested from pRBCs after 24h incubation under cell culture conditions were HZ-free.

RBCs and pRBCs were cultured for 24h at 37°C and 5%CO<sub>2</sub>. Cells were centrifuged for 10 min at 450g (later referred to as 'low-speed centrifugation'). Supernatants were collected and either added to DCs or centrifuged further at 32000g (later referred to as 'high-speed centrifugation') for 20 min before addition to DCs. To further ensure that uncontaminated supernatants were used in the experiments, an additional set of supernatants were prepared and subjected to high-speed centrifugation followed by filter sterilisation through a 0.2µm pore size membrane filter before addition to DCs. A pellet was recovered following the high-speed centrifugation and was also incubated with DCs to analyse whether it would suppress responses to LPS. After 24h incubation, DCs were challenged with LPS and the levels of CD40 expression examined 18h later by flow cytometry (Fig 5.9g).

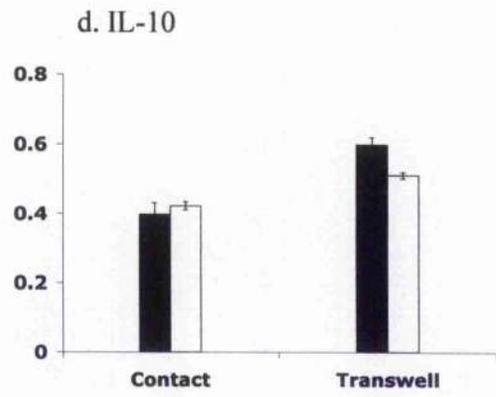
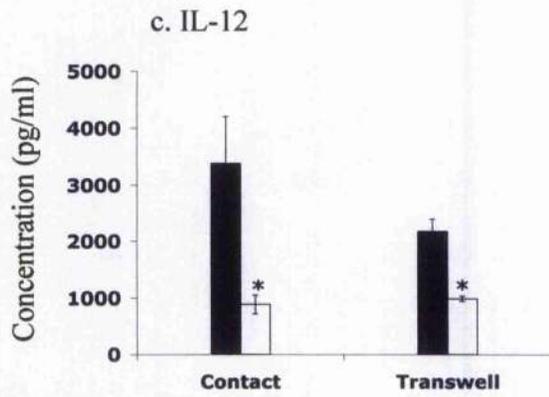
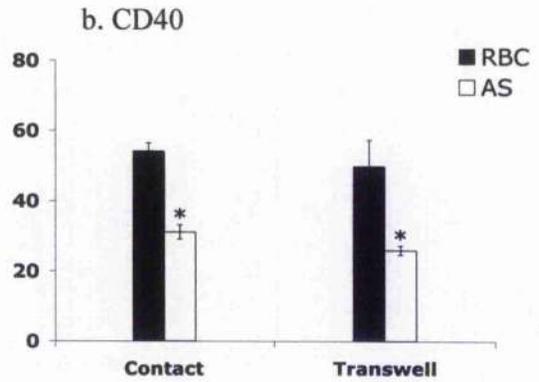
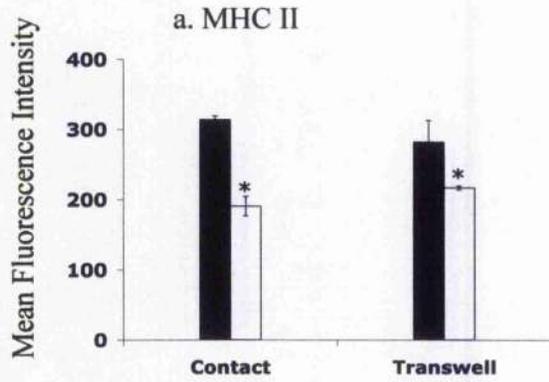
DCs incubated with RBC supernatants derived from both the low- and the high-speed centrifugations matured efficiently in response to LPS stimulation as observed by the high levels of CD40 expression. However, DCs treated with pRBC supernatants obtained following both low- and high-speed centrifugations showed significantly lower levels of CD40 expression indicating that DCs were not able to respond to LPS treatment *in vitro*. Visualisation of DCs by bright field microscopy revealed that pRBC supernatants derived from the low-speed centrifugation contained parasite components as "black depositions" were clearly visible inside DCs and these deposits could therefore be responsible for the observed effect (Fig. 5.9h). However, pRBC supernatants derived from the high-speed

centrifugation lacked parasite contamination as DCs clearly retained their characteristic cell morphology and lacked black depositions in the cytoplasm (Fig. 5.9i). This finding reinforced the idea that a soluble factor(s) might be responsible for the observed suppression of DC maturation.

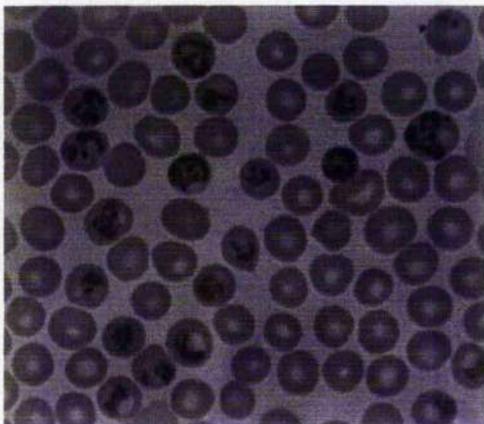
The pellet recovered from the high-speed centrifugation did not downregulate the expression of CD40 (Fig 5.9g), indicating that DCs were able to mature efficiently in response to LPS stimulation *in vitro*. This finding suggested either that the pellet did not contain HZ or the amount of HZ released by pRBCs was not significantly high to induce inhibition of DC function, the latter probably due to inappropriate culture conditions for parasite growth. The notion of having a pellet, which did not contain or had very low levels of HZ was further reinforced by the solubility of the pellet in the culture medium where HZ is instead insoluble.

To further characterise the nature of the soluble factor(s) released by pRBCs, I heat inactivated the supernatants obtained following the high-speed centrifugation before they were added to DCs. RBC and pRBC supernatants were prepared as described above and heated to 100°C for 20min then centrifuged for 10 min at 450g and passed through a 0.2 µm pore size membrane filter before addition to DCs. After 24h incubation DCs were challenged with LPS and the expression of CD40 examined 18h later (Fig. 5.9j).

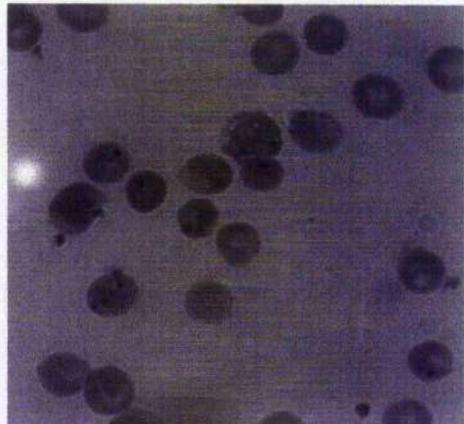
Confirming previous findings, DCs incubated with the pRBC supernatant preparations derived from both the low- and high-speed centrifugations were unable to mature efficiently following LPS stimulation as observed by the downregulation of CD40 expression. However, when the supernatants were heat inactivated, DCs retained their ability to respond to LPS treatment, as the level of CD40 expression by DCs was not significantly different from the RBC control. This finding suggested that soluble parasite factors must be in their native form to cause suppression of DC function *in vitro*.

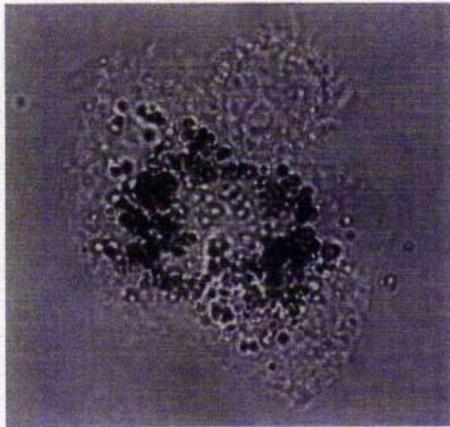
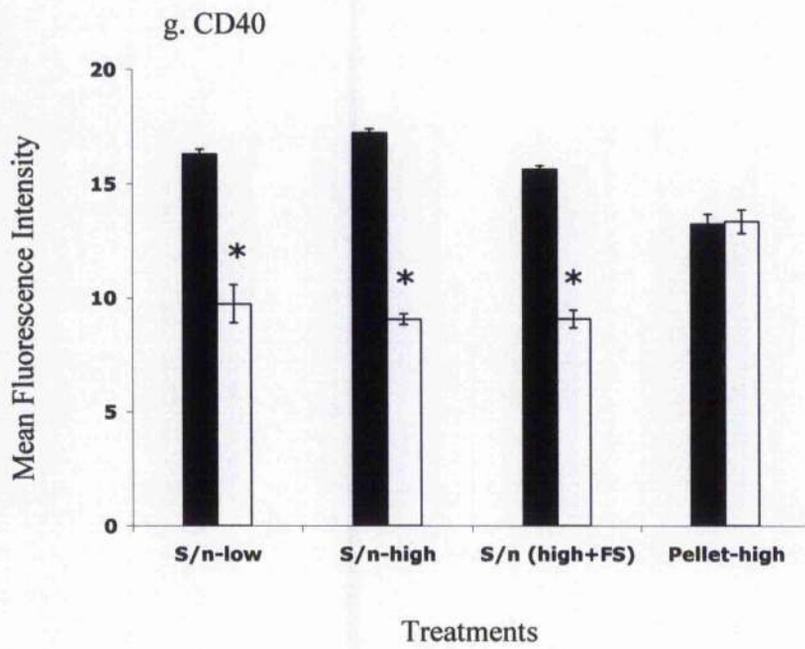


e. Trophozoite-infected erythrocytes

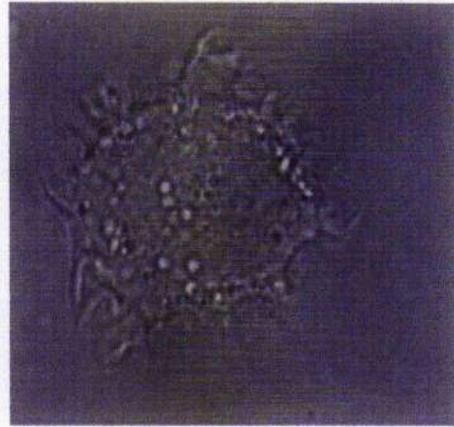


f. Trophozoite-infected erythrocytes after 24h incubation

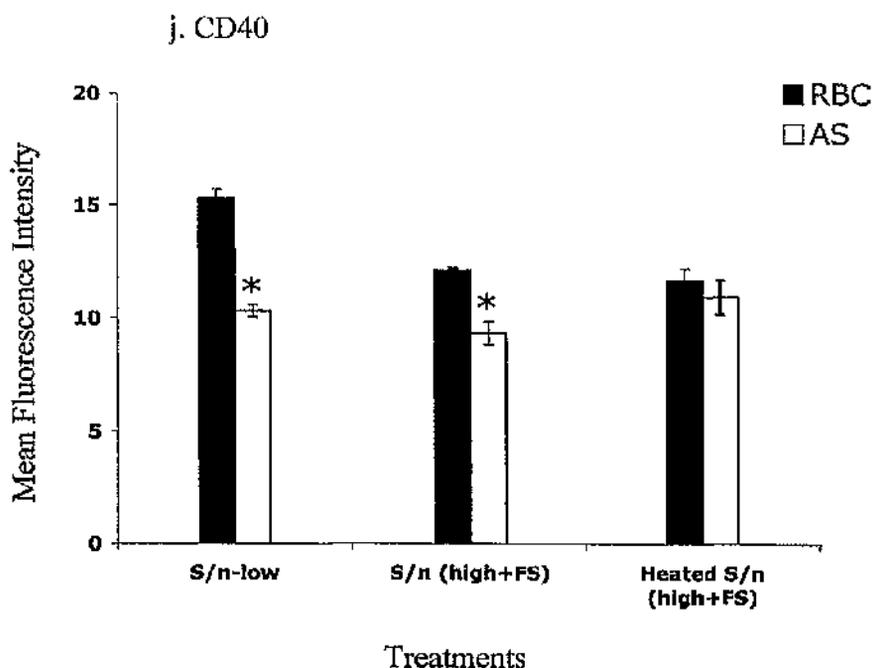




h. DC incubated for 24h with pRBC-supernatant derived from the low-speed centrifugation (450g)



i. DC incubated for 24h with pRBC-supernatant derived from the high-speed centrifugation (32000g)



**Figure 5.9** pRBCs released a soluble factor that affected the ability of DCs to respond to LPS treatment *in vitro*. (a-b) DCs were treated for 24 h with non-infected (RBC; filled bars) or *P. chabaudi* AS-infected erythrocytes (AS; open bars), either directly (contact) or separated by a 0.4  $\mu\text{m}$  pore size membrane (Transwell) prior to stimulation with LPS (1  $\mu\text{g}/\text{ml}$ ) for 18 h. DC activation was characterised by examination of (a) MHC class II and (b) CD40 expression on the DC surface by flow cytometric analysis of gated CD11c<sup>+</sup> cells. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS. (c-d) Concentration of IL-12p40 (c) and IL10 (d) secreted by DCs that were treated for 24 h with non-infected (RBC; filled bars) or *P. chabaudi* AS-infected erythrocytes (AS; open bars) either directly (contact) or separated by a 0.4  $\mu\text{m}$  pore size membrane (Transwell) prior to stimulation with LPS (1  $\mu\text{g}/\text{ml}$ ) for 18 h. After 24h of culture, supernatants were harvested for cytokine analysis. Error bars indicate standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS. (e-f) Giemsa's stained thin blood smears taken either directly from a *P. chabaudi* AS infected BALB/c mouse (e) or after 24h incubation of pRBCs under cell culture conditions (37°C, 5%CO<sub>2</sub>). Cells

were photographed using an Axiovert S-100 Zeiss microscope using a 63x oil immersion lens. (g) DCs were treated for 24 h with supernatants derived from non-infected (RBC; filled bars) or *P. chabaudi* AS-infected erythrocytes (AS; open bars) prior to stimulation with LPS (1 µg/ml) for 18h. Supernatants were obtained after incubating RBCs or pRBCs for 24h and subjecting them to either a 450g centrifugation (S/n-low) or a 32000g centrifugation (S/n-high). An additional set of cells was centrifuged at 32000g followed by filter sterilisation through a 0.2 µm pore size membrane (S/n (high+ FS)). DC activation was characterised by examination of CD40 expression on the DC surface by flow cytometric analysis of gated CD11c<sup>+</sup> cells. Results show the mean fluorescence intensity ± standard errors of triplicate cultures per group. \* p≤0.05 significant difference between RBC and AS. (h-i) DCs were incubated with the supernatant of *P. chabaudi* AS infected erythrocytes derived either from a 450g centrifugation (h) or a 32000g centrifugation (i). After 24h, cells were examined by bright-field microscopy using a 63x oil immersion lens. (j) DCs were incubated with *P. chabaudi* AS supernatants obtained following a 450g or a 32000g centrifugation as described in (g). An additional set of supernatants obtained following a 32000g centrifugation, was heated to 100°C and filter sterilised through a 0.2 µm pore size membrane prior to addition to DCs (heated S/n (high+FS)). Results show the mean fluorescence intensity ± standard errors of triplicate cultures per group. \* p≤0.05 significant difference between RBC and AS.

### 5.3 Discussion

In previous chapters I showed that malaria infected erythrocytes did not directly activate bone marrow-derived DCs *in vitro*; rather, they inhibited the LPS induced upregulation of Class II MHC and costimulatory molecules (CD40 and CD86) on DCs (chapter 3) and significantly reduced the ability of these cells to induce naïve T cell proliferation (chapter 4).

In search of a mechanistic explanation for these observations, I initially examined whether pRBCs induced immediate suppression of DC function. To address this issue DCs were incubated simultaneously with pRBCs and LPS and the expression of MHC class II and the the costimulatory molecules CD40, CD80 and CD86 examined 24h later by flow cytometry. The results showed that pRBC-treated DCs were able to mature in response to LPS treatment, as the levels of all of the costimulatory molecules tested were significantly upregulated. This finding suggested that pRBCs required a longer incubation period with DCs to exert their inhibitory effect. The above observation led me to investigate the time that pRBCs required to interact with DCs in order to modulate their function. DCs were incubated with pRBCs for 6, 12 and 18h. Each experimental culture was then challenged with LPS and the expression of MHC class II and the costimulatory molecules CD40, CD80 and CD86 examined 24h later by flow cytometry. The results showed that pRBCs had to interact with DCs for at least 6h before inhibiting their ability to respond to LPS stimulation *in vitro*, as observed by the reduced expression of CD40 and CD86. The level of MHC class II was instead suppressed following a 12h incubation period with pRBCs, perhaps indicating that CD40 and CD86 are more sensitive to the suppressive effects induced by pRBCs. Alternatively, they might be selectively downregulated by the parasites before significantly affecting MHC class II expression to ensure that appropriate co-stimulation is not provided to T cells, thus impeding the development of appropriate T cell responses directed against them. Interestingly, the level of CD80 expression was also significantly downregulated following a 12h incubation of DCs with pRBCs. Nevertheless, the latter appeared to be a transient suppression, as the expression of CD80 returned to normal levels when analysed

after an 18h incubation period. Whether these differences in kinetics reflect a specific mechanism used by pRBCs to suppress DC function or merely represent differences in their overall sensitivity to the parasite remains unclear. Nevertheless, these data suggest that in order to induce their suppressive effects, pRBCs have to interact with DCs for at least 6h. The erythrocytic cycle of *P. chabaudi* requires 24h to complete within its host, with the parasites developing into a more mature stage every 6h. Therefore, the observed suppression of DC function might be the result of the interaction of a more mature parasite form with DCs. Alternatively, following phagocytosis, trophozoite-infected erythrocytes might remain viable within the DCs and actively suppress their function. I therefore decided to examine whether the suppression of DC function was specifically induced by the trophozoite stage and whether their viability was a necessary requirement.

To address the above questions, pRBCs were fixed and incubated with DCs prior to LPS challenge. Fixed pRBCs were found to significantly affect the ability of DCs to mature in response to LPS stimulation. Furthermore, they also required at least 6h of incubation with DCs to exert their inhibitory effect and significantly reduced the antigen presenting ability of DCs. Therefore fixed-pRBCs showed a similar pattern of modulation of DC function observed when using non-fixed pRBCs. The lack of a requirement for viable parasites confirmed that trophozoite-infected erythrocytes rather than a more mature erythrocytic stage (schizont) were responsible for the observed suppression of DC maturation. It also suggested that an isolatable parasite product was mediating this effect. To address this issue, I initially focused on analysing the effect that parasite proteins expressed on the erythrocyte membrane have on DC function.

It is known that the erythrocytic development of the parasites is coupled with changes in the host cells, including the host cell plasma membranes (Wunderlich *et al.*, 1987). It is also well established that parasites express 'neo-proteins' on the host cell surface some of which are reported to induce protective immunity (Aikawa and Miller, 1983; Schmidt-Ullrich *et al.*, 1983; Sherman, 1985). Moreover, it has been suggested that the ability of *P. falciparum* to inhibit DC maturation is mediated through the binding of CD36 to a parasite-derived molecule (PfEMP1) on the infected erythrocyte surface (Urban *et al.*,

1999; Urban *et al.*, 2001b). Erythrocytes infected with *P. chabaudi* AS were also shown to be able to adhere to specific cell types and sequester by interacting with molecules such as CD36 (Mota *et al.*, 2000). To assess the involvement of parasite proteins present on the erythrocyte membrane in the observed suppression of DC activation, I exposed DCs to plasma membrane ghosts derived from infected or non-infected erythrocytes, prior to LPS challenge. Ghosts isolated from infected erythrocytes did not alter the ability of DCs to respond to LPS treatment *in vitro*, suggesting that parasite antigens expressed on the erythrocyte plasma membranes did not induce the suppression previously described following contact with DCs *in vitro*. In support of this finding is the observation that immunisation of mice with pRBC ghosts induced protection from parasite challenge (Wunderlich *et al.*, 1988), suggesting that ghosts, unlike intact parasites, are not inherently immunosuppressive.

As mentioned earlier, trophozoite-infected erythrocytes were used in all the experiments described in this thesis. In order to develop to this stage, parasites have to polymerise the haeme, a toxic byproduct of haemoglobin digestion, into the non-toxic form known as haemozoin (HZ), which accumulates into the parasite's digestive vacuole (Francis *et al.*, 1997; Olliaro and Goldberg, 1995; Slater *et al.*, 1991). Since fixed pRBCs had an effect on the LPS-induced maturation of DCs and pRBC-ghosts did not, HZ seemed to be a good candidate to investigate the mechanism involved in the parasite-induced modulation of DC function. I therefore incubated DCs with HZ, purified from supernatants obtained from cultures of *P. falciparum* gametocytes. The results showed that purified HZ did not activate DCs directly, however it significantly inhibited the LPS-induced maturation of DCs in a dose dependent manner. These observations are consistent with previous reports suggesting that HZ is able to impair the differentiation and functional capacity of human monocytes and murine macrophages through the production of IL-10 and/or induction of peroxisome proliferators-activated receptor- $\gamma$  (Deshpande *et al.*, 2004; Morakote and Justus, 1988; Schwarzer *et al.*, 1998; Scorza *et al.*, 1999; Skorokhod *et al.*, 2004). However, the precise mechanisms accounting for the suppressive effects of HZ remain to be elucidated, although in an early study, HZ was reported to increase degradation of protein kinase C, which plays a crucial role in signal transduction and expression of

membrane antigens (Schwarzer *et al.*, 1993). In addition, an other report by the same authors suggested that accumulation of HZ within phagolysosomes indirectly caused a defect in antigen processing through the generation of potent bioactive lipid derivatives (Schwarzer *et al.*, 2001). Both of the above studies were carried out using human monocytes. Furthermore, a more recent report showed that HZ inhibited the maturation of human monocyte-derived DCs (Skorokhod *et al.*, 2004). Nevertheless, the involvement of HZ in suppression of DC function remains controversial, as other investigators suggest a pro-inflammatory role of HZ, possibly *via* TLR-9 activation (Coban *et al.*, 2005; Coban *et al.*, 2002; Jaramillo *et al.*, 2005; Jaramillo *et al.*, 2003; Jaramillo *et al.*, 2004; Pichyangkul *et al.*, 1994; Pichyangkul *et al.*, 2004). One possible explanation for this disparity in results may simply relate to differences in the HZ preparations. For instance, Paolo Arese and colleagues prevented the complete removal of adherent membranes from the HZ preparation as no phagocytosis of the pigment was observed in this case (Schwarzer *et al.*, 1992), an approach also used in the present study. Conversely, other investigators had additional incubation steps with proteinases and lipases in their preparations (Coban *et al.*, 2002; Jaramillo *et al.*, 2003).

While carrying out the experiments described in this chapter, I realised that the observed suppression of DC function was consistently greater when live infected erythrocytes were used. This observation suggested that other parasite products could also be involved and might therefore have cumulative effects on the suppression of DC function. In order to address this issue, I initially examined whether interaction of DCs with pRBCs was necessary to modulate DC function. DCs and pRBCs were separated by a 0.4 $\mu$ m pore size membrane and cultured for 24h prior to LPS stimulation. The ability of DCs to mature in response to LPS treatment *in vitro* was significantly downregulated suggesting that soluble parasite products could be present in the cultures and might mediate the observed effect. However, It has been reported that the size of HZ crystals belonging to different species of *Plasmodium* ranged between 50 and 600nm (Noland *et al.*, 2003). Although the ability of the parasites to undergo the full erythrocytic cycle under cell culture conditions has not been reported yet, the possibility still remained that the observed effect was due to HZ passing through the 0.4 $\mu$ m pore size membrane used to

separate the cells. For this reason, a series of experiments involving low- and high-speed centrifugations were designed to ensure that pRBC supernatants were free from HZ. The LPS-induced maturation of DCs was found to be significantly affected when DCs were incubated for 24h with the supernatants derived from both the low- and high-speed centrifugations. Particularly, it was clear that pRBC supernatants derived from the high-speed centrifugation lacked HZ as visualisation of DCs by bright field microscopy showed absence of black pigment within DCs and the cells retained their characteristic morphology. This finding reinforced the idea that a soluble factor(s) might be responsible for the suppression observed. Furthermore, the fact that the early ring stages were found to suppress the LPS-induced maturation of DCs further supported the involvement of soluble parasite factors in the observed modulation of DC function. To further characterise the nature of this factor, pRBC supernatants obtained following the high-speed centrifugation were heat-inactivated prior to addition to DCs. After 24h incubation DCs were challenged with LPS and the level of CD40 expression on DCs analysed 18h later by flow cytometry. The results obtained from this experiment showed that DCs retained their ability to mature in response to LPS treatment *in vitro* when treated with heat-inactivated supernatants, suggesting that a soluble factor(s) potentially secreted by the parasites has to be in its native form to induce suppression of DC activation. Treatment of the pRBC-derived supernatant with proteinases and lipases would aid in the identification of the nature of this potential immunosuppressive factor, an experiment, which due to time constraints could not be carried out for the present study. Interestingly, early reports have shown that blood-stage exoantigens from *P. yoelii* and *P. falciparum* were pro-inflammatory and induced the release of TNF- $\alpha$  from macrophages (Bate *et al.*, 1992b; Taverne *et al.*, 1990a; Taverne *et al.*, 1990b). The capacity of the exoantigens to stimulate macrophages to secrete TNF- $\alpha$  did not require the presence of a protein or a carbohydrate but was associated with a lipid, whose activity was shown to be abolished by treatment with phospholipase C (Bate *et al.*, 1992a; Bate *et al.*, 1992b).

More recent studies using *P. yoelii* infections showed that a soluble factor(s) specifically inhibited IL-2 production by responding CD4<sup>+</sup> T cells (Luyendyk *et al.*, 2002). The authors used a transwell culture system where T cells purified from OVA TcR transgenic

mice were cultured with spleen cells from uninfected mice in the inner well of the two-chamber system. Spleen cells from infected mice were instead placed in the outer well. The soluble factor was suggested to be produced by CD11b<sup>+</sup> cells purified from spleen cells isolated from infected mice. The effect that pRBC alone had on the IL-2 production by T cells was not directly examined in the above study.

Another study by Ocana-Morgner *et al.* (Ocana-Morgner *et al.*, 2003) found that interaction of blood-stage parasites with DCs induced the secretion of soluble factors that inhibited the activation of CD8<sup>+</sup> T cells *in vitro*. The authors suggested that these factors were secreted by DCs as the inhibitory activity was not found in the incubation medium of pRBC alone thus confirming the results previously found by Luyendyk *et al.* (Luyendyk *et al.*, 2002). However, Ocana-Morgner *et al.* carried out their study by transferring incubation media onto fresh DCs, rather than using a transwell system and they did not take into account the fact that other parasite products (such as HZ) could be present in the culture media. Nevertheless it is still possible that the differences observed between the above and the present studies were due to the different parasite species used in the experiments (*P. yoelii* vs *P. chabaudi*).

In the present chapter I have demonstrated that haemozoin, rather than infected erythrocyte membranes, is responsible for the observed suppression of DC activation *in vitro*. Furthermore, soluble factors secreted by pRBCs also seem to contribute significantly to the immunosuppressive effects induced in DCs. Although parasites may affect DC function by actively secreting suppressive factors, they may also induce DCs to secrete or express factors that could in turn modulate their activation state, resulting in an overall inability of these cells to induce protective responses against the parasites. In the next chapter I analyse this possibility directly by investigating the involvement of some of the most common immunoregulatory agents, which could be potentially induced by DCs following interaction with pRBCs, in the observed suppression of DC activation. Identification, followed by a deep understanding of the mechanisms of action of these suppressive agents, may be beneficial for future therapeutic treatments and vaccine designs where replacement of DC suppression in favour of DC activation would be

particularly advantageous. Furthermore, the identification of specific immunosuppressive factors released by DCs may also help in settings where immunosuppression would be clinically beneficial, such as in the case of autoimmune diseases.

**6. Investigating the role of selected immunoregulatory molecules on the LPS-induced activation of DCs *in vitro***

## 6.1 Introduction

As previously described in section 1.2.6, pathogens generally cause priming of immature DCs to become mature effector DCs that drive appropriate Th1 or Th2 responses. However, there is now evidence describing the ability of certain pathogens to prime for an alternative subset of DCs, characterised by having altered activation states (Auffermann-Gretzinger *et al.*, 2001; Dolganiuc *et al.*, 2003; Geijtenbeek *et al.*, 2003; Karp *et al.*, 1996; McGuirk *et al.*, 2000; Nigou *et al.*, 2001; Urban *et al.*, 1999; van der Kleij *et al.*, 2002). These 'alternatively activated DCs' may not be able to induce appropriate effector T cell responses directed against various pathogens. Instead they may drive the generation of anergic or regulatory T cells, or even induce T cell apoptosis, providing a mechanism used by certain pathogens to avoid host immune responses directed against their elimination. Examples of pathogens promoting the generation of this type of DCs were extensively discussed in sections 1.2.7.1 and 1.2.7.2. *P. falcatiparum* has been identified as being one of the pathogens belonging to this category as it has been shown to suppress the maturation of DCs and therefore their ability to stimulate naïve T cells (Urban *et al.*, 1999).

Besides directly modulating DC activation, pathogens may induce host cells to secrete or express factors that will in turn affect the activation state of DCs. Production of PGE<sub>2</sub>, NO and TGF-β, as well as PPAR-γ activation and IDO expression by DCs, all represent possible immunoregulatory mechanisms that pathogens may potentially use to suppress DC function (Gosset *et al.*, 2001; Grohmann *et al.*, 2000; Harizi *et al.*, 2003; Holt *et al.*, 1993; Li *et al.*, 2005). All of the above molecules and their potential involvement in modulating DC functions have been previously discussed in section 1.2.8. Although they could all have a direct effect on T cells, in this chapter I focus on their effects on the LPS-induced maturation of DCs as a preliminary study. However, as it was not possible to use positive controls in my experiments and as I have not replicated the above findings in the system used in the present study, some caution should be exercised when interpreting the results presented in this chapter. Furthermore, due to time constraints, all the inhibitors or antagonists were used at a single concentration previously shown to block respective

activities by other investigators. To ensure the validity of the results presented herein, optimal dilutions for each of the molecules tested should be determined in the future.

Understanding the involvement of the above mediators in the regulation of immune responses in human diseases could have important consequences in the improvement of therapeutic treatments. Agonists of PPAR- $\gamma$  are already being used for the treatment of type II (non-insulin dependent) diabetes and are also considered as potential therapeutic agents for cancer treatment (Dubuquoy *et al.*, 2002; Murphy and Holder, 2000). Moreover it has been shown that administration of rosiglitazone (a PPAR- $\gamma$  ligand) reduces the severity of inflammation in patients with mild to moderate cases of ulcerative colitis (Kornbluth, 2001; Lewis *et al.*, 2001). Similarly, encouraging results from phase II clinical trials were obtained by inducing IDO expression in autoimmune rheumatoid arthritis (Kremer *et al.*, 2003), implying that it might be possible to induce IDO expression in settings where immunosuppression would be clinically beneficial. Therefore, the experiments presented in this chapter were designed with the view that a better understanding of the involvement of these mediators following pRBC-DC interaction could be useful for therapeutic approaches and help to improve the effectiveness of potential malaria vaccines.

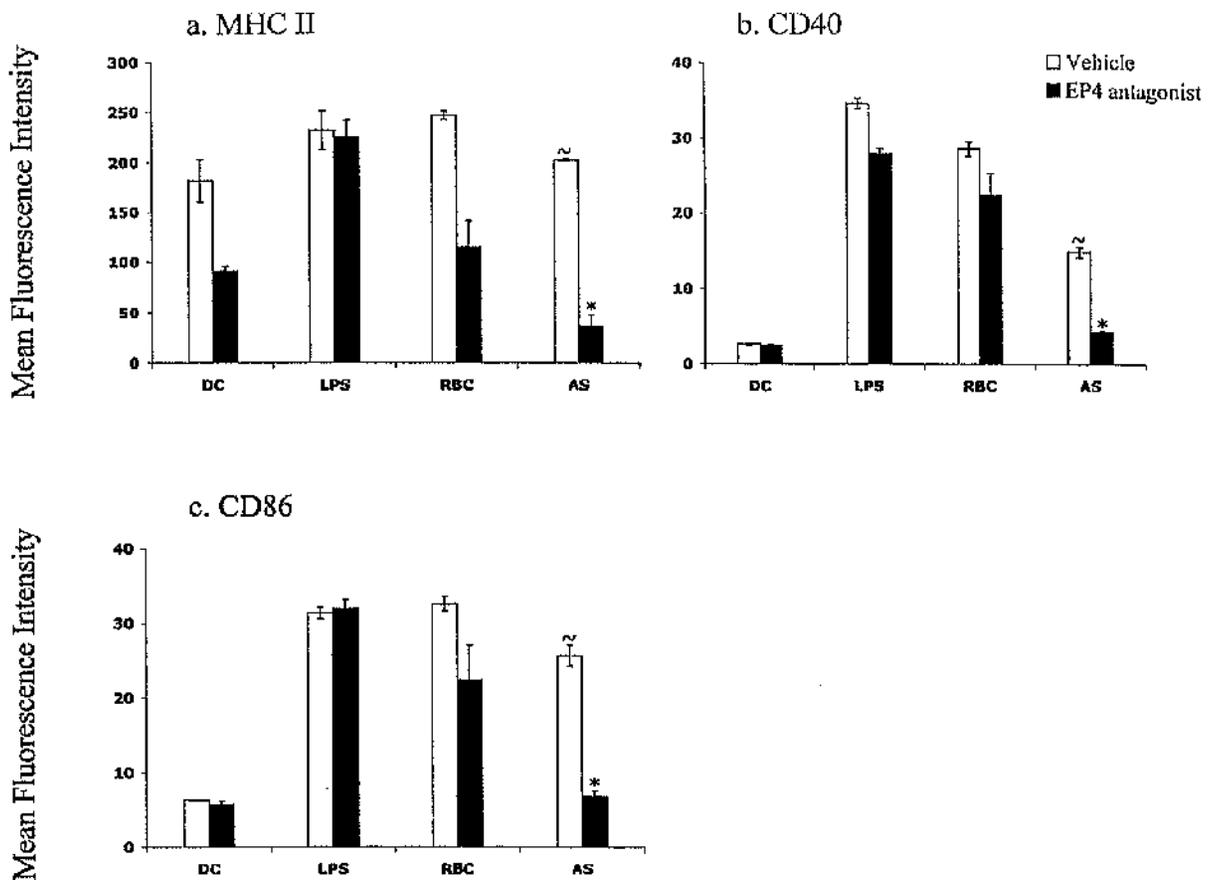
## 6.2 Results

### 6.2.1 PGE<sub>2</sub>-EP<sub>4</sub> signalling is not involved in the suppression of the LPS-induced maturation of *P. chabaudi* AS-treated DCs *in vitro*.

The role of host eicosanoids in modulating immunity is well documented and it is now widely accepted that DCs produce lipid mediators, such as PGE<sub>2</sub>, which are well known to be involved in immune suppression (Harizi and Gualde, 2004). Furthermore, DCs themselves have been shown not to be refractory to the effects of PGE<sub>2</sub>. In fact, exogenously added or endogenously released PGE<sub>2</sub> was reported to act on DCs by inducing the production of endogenous IL-10, which suppresses IL-12 production and alters antigen presentation by inhibiting MHC class II protein expression (Harizi *et al.*, 2002). Moreover, many parasites, including *P. falciparum* (Kilunga Kubata *et al.*, 1998), have been reported to produce prostaglandins and prostaglandin-like molecules (Noverr *et al.*, 2003), representing a possible mechanism used by parasites to alter DC function. This knowledge prompted me to investigate whether the observed suppressive effects induced by pRBCs on DC function were partly mediated by the action of PGE<sub>2</sub> that could be potentially produced either by DCs following interaction with pRBCs or by the parasite itself.

PGE<sub>2</sub> exerts its action by binding to four different types of G-protein-coupled receptors known as EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> (Narumiya *et al.*, 1999). Although mouse bone marrow-derived DCs express all four EP receptor subtypes, their production of cytokines and expression of MHC class II molecules were shown to be modulated through EP<sub>2</sub>- and EP<sub>4</sub>- dependent mechanisms (Harizi *et al.*, 2003). These data were proved by the use of EP<sub>2</sub> and EP<sub>4</sub> agonists, which significantly promoted the production of endogenous IL-10 from bone marrow-derived DCs and decreased their expression of MHC class II molecules (Harizi *et al.*, 2003) As an EP<sub>4</sub>-selective antagonist was readily available, I decided to carry out a preliminary experiment to investigate whether PGE<sub>2</sub>-EP<sub>4</sub> signalling was involved in the suppression of the LPS-induced maturation of pRBC-treated DCs.

DCs were cultured with RBCs or pRBCs in the presence or absence of the EP<sub>4</sub> antagonist for 24h and then stimulated with LPS *in vitro*. The expression of MHC class II and the co-stimulatory molecules CD40 and CD86 were examined 18h later by flow cytometry. The results showed that DCs cultured in growth medium in the presence or absence of the EP<sub>4</sub> antagonist expressed low levels of surface MHC class II, CD40 and CD86, confirming their immature state in culture (figure 6.1). When stimulated with LPS, DCs cultured alone or with RBCs upregulated the expression level of all of the costimulatory molecules tested regardless of the presence of the EP<sub>4</sub> antagonist. As shown previously, DCs cultured with pRBCs had a significantly reduced ability to respond to LPS treatment *in vitro*. Furthermore, the presence of the EP<sub>4</sub> antagonist in the culture did not rescue the ability of DCs to mature in response to LPS, suggesting that the PGE<sub>2</sub>-EP<sub>4</sub> signalling pathway did not play a role in the modulation of DC function during incubation with pRBCs. Nevertheless, as mentioned above, PGE<sub>2</sub> might exert its effects through other receptors thus the involvement of PGE<sub>2</sub> in the observed inhibition of DC maturation cannot be ruled out yet and further experiments will be required to elucidate the role of PGE<sub>2</sub> in the modulation of DC function during interaction with pRBCs.



**Figure 6.1** Effect of PGE<sub>2</sub>-EP<sub>4</sub> signalling on the LPS-induced maturation of *P. chabaudi* AS- treated DCs *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes (AS) or with non-infected erythrocytes (RBC) in the absence (vehicle) or presence of  $1 \mu\text{M}$  ONO-AE3-208 (EP4 antagonist) (Kabashima *et al.*, 2003b). After 24h of co-culture, DCs were challenged with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Control DCs remained unstimulated (DC) or treated with LPS alone (LPS). Surface expression of MHC class II (a), CD40 (b) and CD86 (c) on DCs was determined by flow cytometric analysis of gated CD11c<sup>+</sup> cells. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS treated with the EP4 antagonist. ~  $p \leq 0.05$  significant difference between RBC and AS treated with vehicle only.

### 6.2.2 PPAR- $\gamma$ signalling is not necessary to induce suppression of LPS-induced activation of DCs *in vitro*.

Recent reports have shown that DCs express PPAR- $\gamma$  and that agonist-induced activation of PPAR $\gamma$  can influence DC maturation (Gosset *et al.*, 2001). Furthermore Appel *et al.* (Appel *et al.*, 2005) reported that TLR ligand-induced activation and migration of human monocyte-derived DCs, as well as their ability to initiate lymphocyte proliferation, was impaired upon PPAR- $\gamma$  activation. For these reasons I investigated the involvement of PPAR- $\gamma$  activation in the reduced ability of pRBC-treated DCs to mature in response to LPS challenge.

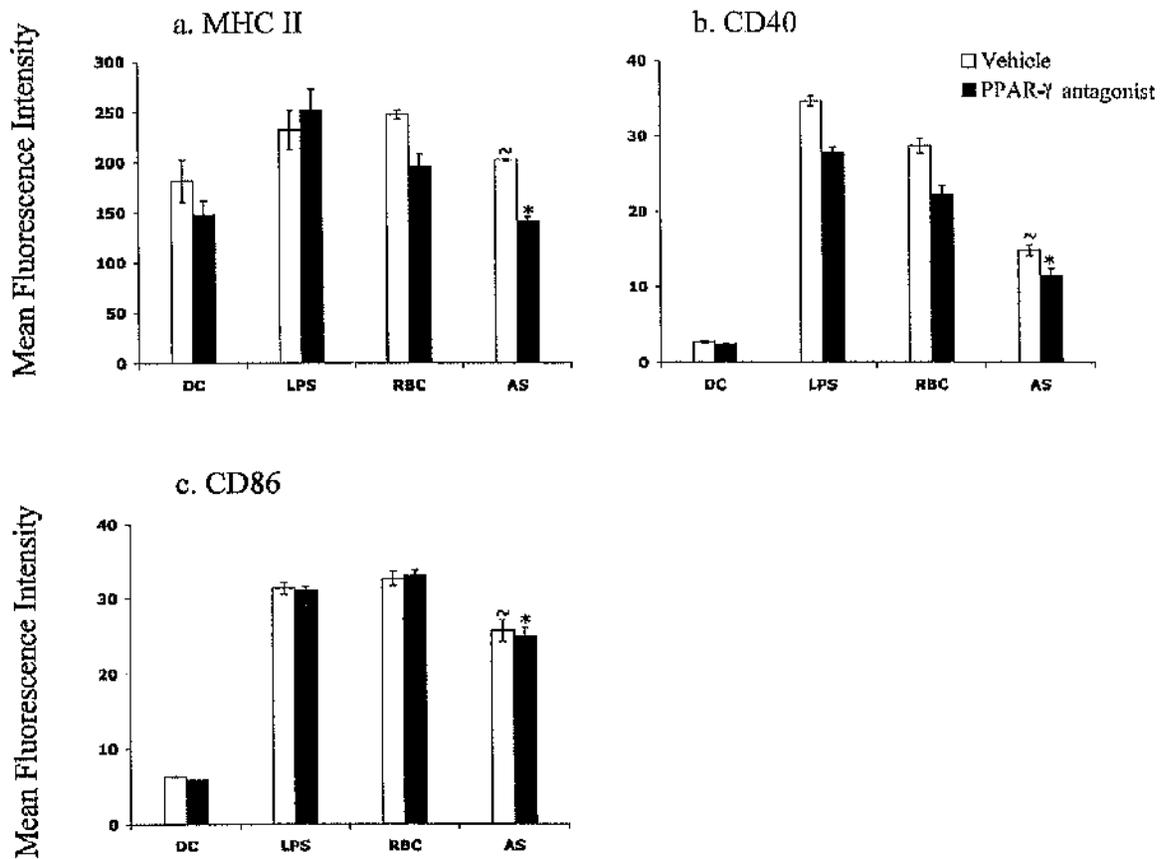
Experimental cultures were set up as described in 6.2.1 and a PPAR- $\gamma$  antagonist was used to block PPAR- $\gamma$  activation. DCs cultured in growth medium in the presence or absence of the PPAR- $\gamma$  antagonist expressed low levels of surface MHC class II, CD40 and CD86 and when challenged with LPS, they upregulated the expression level of all of the costimulatory molecules tested. However DCs cultured with pRBCs were not able to respond to LPS treatment *in vitro* even when PPAR- $\gamma$  activation was blocked (figure 6.2). This result excludes the possibility that PPAR- $\gamma$  ligation is responsible for the observed suppression of DC function during incubation with pRBCs.

### 6.2.3 Nitric oxide is not necessary to induce suppression of LPS-induced activation of DCs *in vitro*.

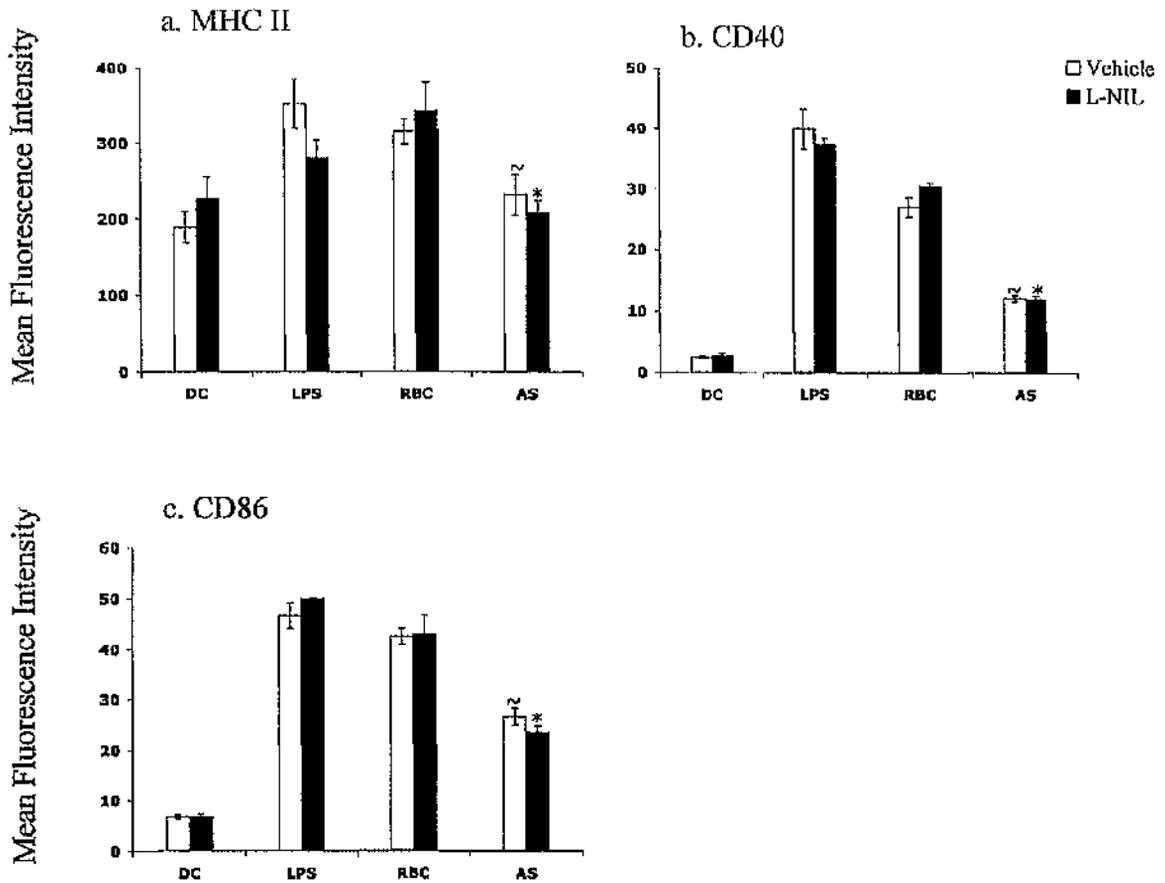
Nitric oxide (NO) and other reactive nitrogen intermediates (RNI) have been reported to suppress T cell proliferative responses during many infections (Candolfi *et al.*, 1994; Gregory *et al.*, 1993; O'Connor *et al.*, 2000; Sternberg and McGuigan, 1992) as well as being involved in the modulation of DC function. NO was shown to selectively downregulate the expression of the costimulatory molecule CD86 on monocyte-derived DCs in response to LPS or soluble CD40 ligand and led to a decreased capacity of DCs to stimulate T cells *in vitro* (Corinti *et al.*, 2003). Furthermore, NO was reported to inhibit the release of IL-10 and IL-12p40 by mature DCs as well as the secretion of chemokines

crucially involved in T cell recruitment (IP-10/CXCL10 and RANTES/CCL5) (Corinti *et al.*, 2003).

Here I examined the role of NO in the observed suppression of pRBC-treated DC function by blocking the action of NO synthase, the enzyme responsible for its generation (Korhonen *et al.*, 2005). Experimental cultures were set up as previously described in 6.2.1. Cells were treated with L-NIL, a potent and selective inhibitor of NO synthase (figure 6.3). DCs cultured in growth medium alone or with RBCs were able to upregulate the expression of MHC class II, CD40 and CD86 following LPS treatment regardless of the presence of L-NIL in culture. However, blocking NO synthase activity did not reverse the maturation defect observed in the pRBC-treated cultures as DCs did not upregulate the levels of the costimulatory molecules tested in response to LPS challenge. This finding suggests that NO does not play a significant role in the pRBC-induced suppression of DC maturation.



**Figure 6.2** Effect of PPAR- $\gamma$  on the LPS-induced maturation of *P. chabaudi* AS- treated DCs *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes (AS) or with non-infected erythrocytes (RBC) in the absence (vehicle) or presence of  $10 \mu\text{M}$  GW9662 (PPAR- $\gamma$  antagonist) (Hammad *et al.*, 2004). After 24h of co-culture, DCs were challenged with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Control DCs remained unstimulated (DC) or treated with LPS alone (LPS). Surface expression of MHC class II (a), CD40 (b) and CD86 (c) on DCs was determined as described in figure 6.1. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS treated with the PPAR- $\gamma$  antagonist. <sup>~</sup>  $p \leq 0.05$  significant difference between RBC and AS treated with vehicle only.

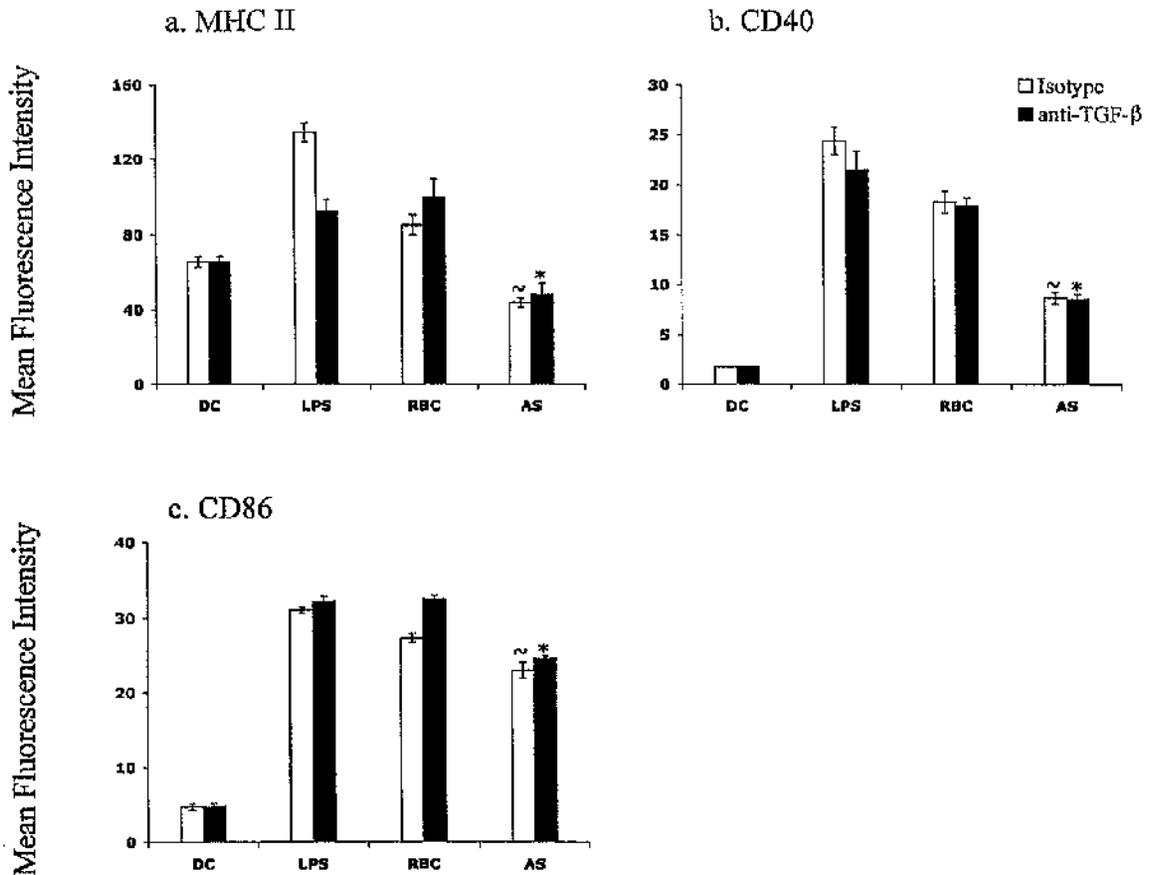


**Figure 6.3** Effect of NO on the LPS-induced maturation of *P. chabaudi* AS- treated DCs *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes (AS) or with non-infected erythrocytes (RBC) in the absence (vehicle) or presence of 1mM L-NIL (L-NIL), a selective inhibitor of NO synthase (Moore *et al.*, 1994). After 24h of co-culture, DCs were challenged with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Control DCs remained unstimulated (DC) or treated with LPS alone (LPS). Surface expression of MHC class II (a), CD40 (b) and CD86 (c) on DCs was determined as described in Figure 6.1. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between RBC and AS treated with L-NIL. ~  $p \leq 0.05$  significant difference between RBC and AS treated with vehicle only.

#### 6.2.4 TGF- $\beta$ is not necessary to induce suppression of LPS-induced activation of DCs *in vitro*.

TGF- $\beta$  has been reported to regulate the maturation of differentiated DCs and DC-mediated T cell responses (Li *et al.*, 2005). The presence of TGF- $\beta$  in LPS-stimulated DC cultures inhibited the expression of MHC class II and costimulatory molecules, which attenuated the antigen presenting function of DCs (Geissmann *et al.*, 1999). In addition, DC maturation and IL-12 production induced by inflammatory cytokines such as IL-1 and TNF- $\alpha$  were also shown to be inhibited by TGF- $\beta$  (Geissmann *et al.*, 1999). It is therefore reasonable to assume that pathogens may induce host cells to secrete active TGF- $\beta$ , which could potentially affect DC function and promote their survival. The significance of TGF- $\beta$  activation for parasite survival has recently been demonstrated for malaria parasites (Omer *et al.*, 2003). Soluble components of *P. falciparum*, *P. berghei* and *P. yoelii* infected erythrocytes were shown to be directly able to activate both native (platelet-derived) and recombinant latent TGF- $\beta$  (Omer *et al.*, 2003).

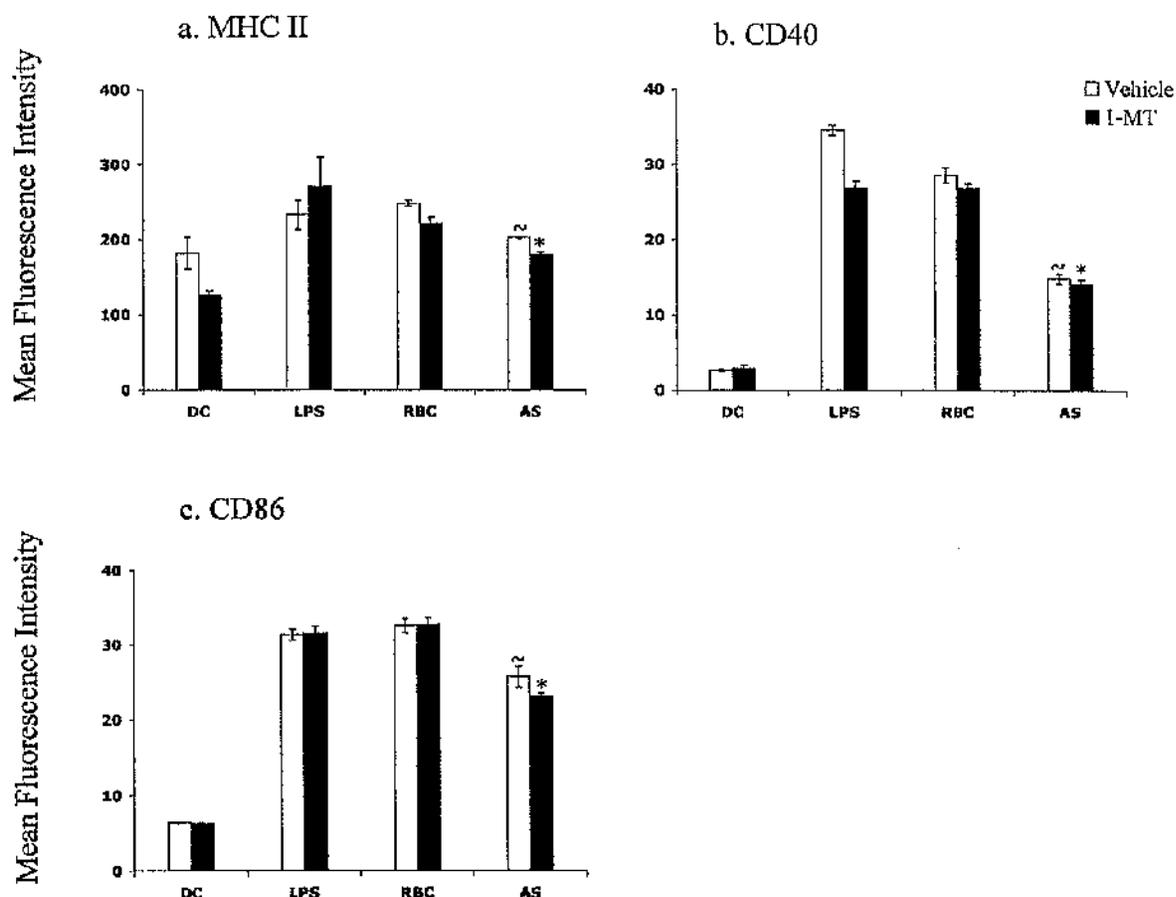
Due to the involvement of TGF- $\beta$  in the modulation of DC function, I investigated its role in the observed suppression of the LPS-induced maturation of pRBC-treated DCs. Experimental cultures were set up as described in 6.2.1 and an anti-TGF- $\beta$  antibody was used to block any suppressive effects that this cytokine may exert on DC function. The results clearly showed that the ability of pRBC-treated DCs to respond to LPS treatment *in vitro* was not rescued by blocking TGF- $\beta$  production as the levels of MHC class II, CD40 and CD86 were significantly lower than control cultures (figure 6.4). This finding suggests that TGF- $\beta$  does not play a significant role in the inability of pRBC-treated DCs to mature in response to LPS challenge *in vitro*.



**Figure 6.4** Effect of TGF- $\beta$  on the LPS-induced maturation of *P. chabaudi* AS- treated DCs *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes (AS) or with non-infected erythrocytes (RBC) in the presence of  $5 \mu\text{g/ml}$  anti-TGF- $\beta$  antibody (anti-TGF- $\beta$ ) or IgG1 isotype control (Isotype). After 24h of co-culture, DCs were challenged with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Control DCs remained unstimulated (DC) or treated with LPS alone (LPS). Surface expression of MHC class II (a), CD40 (b) and CD86 (c) on DCs was determined as described in figure 6.1. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \* $p \leq 0.05$  significant difference between RBC and AS treated with the anti-TGF- $\beta$  antibody. ~  $p \leq 0.05$  significant difference between RBC and AS treated with the isotype control.

### 6.2.5 Indoleamine 2,3-dioxygenase expression is not necessary to induce suppression of LPS-induced activation of DCs *in vitro*.

Recently there has been a growing interest in investigating an immunosuppressive mechanism, involving tryptophan catabolism by DCs that express the enzyme indoleamine 2,3-dioxygenase (IDO). It has been reported that one mechanism by which IDO exerts its immunosuppressive effects is by altering the biology of the IDO-expressing APCs (Hwu *et al.*, 2000; Munn *et al.*, 1999). This notion prompted me to investigate the role of IDO in the inhibition of DC function observed following treatment with pRBCs. A pharmacological inhibitor of IDO (1-MT) (Cady and Sono, 1991) was used to inhibit IDO activity *in vitro*. DCs were cultured with RBCs or pRBCs in the presence or absence of 1-MT for 24h and then stimulated with LPS *in vitro*. The expression of MHC class II and co-stimulatory molecules CD40 and CD86 were examined 18h later by flow cytometry (figure 6.5). As previously shown, DCs cultured with pRBCs were not able to respond to LPS treatment *in vitro* as the levels of MHC class II, CD40 and CD86 were significantly lower than control cultures. Furthermore, the presence of 1-MT in the culture did not reverse the maturation defect observed following LPS stimulation, suggesting that IDO expression in DCs did not play a significant role in the inhibition of DC activation following incubation with pRBCs.



**Figure 6.5** Effect of indoleamine 2,3-dioxygenase on the LPS-induced maturation of *P. chabaudi* AS- treated DCs *in vitro*.  $2 \times 10^6$  DCs were cultured with  $2 \times 10^8$  *P. chabaudi* AS-infected erythrocytes (AS) or with non-infected erythrocytes (RBC) in the absence (vehicle) or presence of 2nM 1-MT (1MT) (Cady and Sono, 1991). After 24h of co-culture, DCs were challenged with  $1 \mu\text{g/ml}$  of LPS for a further 18h. Control DCs remained unstimulated (DC) or treated with LPS alone (LPS). Surface expression of MHC class II (a), CD40 (b) and CD86 (c) on DCs was determined as described in figure 6.1. Results show the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \* $p \leq 0.05$  significant difference between RBC and AS treated with 1-MT.  $\sim p \leq 0.05$  significant difference between RBC and AS treated with vehicle only.

### 6.3 Discussion

In previous chapters I have demonstrated that *P. chabaudi* AS-treated DCs had limited ability to mature in response to LPS treatment *in vitro* (chapter 3) and could not induce an appropriate T cell response, leading to the development of an anergic rather than an effector T cell phenotype (chapter 4). Subsequent studies revealed that haemozoin played an important role in the observed modulation of DC biology (chapter 5). However transwell experiments opened up the possibility that soluble factors, either secreted by the parasites or the DCs themselves, might be involved in altering DC function thus exerting cumulative effects on the observed suppression of DC function (chapter 5). In fact, due to the long co-existence of the malaria parasite with its host, it is very likely that the parasite has developed a number of mechanisms whereby it manipulates the host's immune response to enhance its chances of survival.

In this chapter I investigated whether selected immunoregulatory molecules were involved in the observed suppression of the LPS-induced maturation of pRBC-treated DCs. I focused on the possible effects that PGE<sub>2</sub> (Harizi and Gualde, 2004) NO (Bogdan, 2001; Brunet, 2001) and TGF- $\beta$  (Geissmann *et al.*, 1999) might have on DCs due to their well-known involvement in immune regulation in many different infections. I also investigated the role of PPAR- $\gamma$  (Appel *et al.*, 2005; Gosset *et al.*, 2001) and IDO expression (Hwu *et al.*, 2000; Munn *et al.*, 1999) on the observed suppression of DC function due to their reported roles in immune modulation.

As extensively discussed in section 1.2.8.1, prostaglandins are well known immunoregulatory molecules (Harizi and Gualde, 2004). DCs have been reported to produce prostaglandins, especially PGE<sub>2</sub>, which can act in an autocrine manner and suppress some key functions of the secreting cell itself or in a paracrine manner and modulate the biology of cells present in the local environment (Harris *et al.*, 2002). *P. falciparum* was shown to produce prostaglandins, both endogenously and exogenously (Kilunga Kubata *et al.*, 1998). Prostaglandin production was shown to be highest in the trophozoite and schizont stages in the parasite life cycle (Kilunga Kubata *et al.*, 1998).

This observation raised the possibility that parasite-derived prostaglandins might play a role in the modulation of the host defence mechanism against malaria infection. For instance, an increased level of TNF- $\alpha$  during malaria infection was reported to suppress parasite growth *in vivo* (Clark *et al.*, 1987; Taverne *et al.*, 1994). Lowering the host TNF- $\alpha$  production by parasite-produced PGE<sub>2</sub> would therefore be beneficial to the parasite, since PGE<sub>2</sub> regulates the levels of TNF- $\alpha$  (Kunkel *et al.*, 1988; Renz *et al.*, 1988). It is therefore reasonable to assume that parasites may suppress immune responses directed against them either by producing prostaglandins themselves or by inducing DCs to secrete these lipid mediators, which will in turn suppress their function. For these reasons, I investigated whether PGE<sub>2</sub> was responsible for the reduced ability of pRBC-treated DCs to mature in response to LPS-treatment *in vitro*. Since PGE<sub>2</sub> was shown to modulate DC function *via* the EP<sub>4</sub> receptor subtype (Harizi *et al.*, 2003) and having an EP<sub>4</sub> antagonist readily available in the laboratory, I decided to examine whether the observed suppression of DC function was partly mediated by the action of PGE<sub>2</sub> through interaction with the EP<sub>4</sub> receptor. In this context, the results showed that PGE<sub>2</sub>-EP<sub>4</sub> signalling did not play a significant role in suppressing the LPS-induced activation of DCs following incubation with pRBCs. Nevertheless, the involvement of PGE<sub>2</sub> in parasite-induced suppression of DC function cannot yet be ruled out. In fact, DCs can produce other prostanoids including thromboxanes A<sub>2</sub> (TXA<sub>2</sub>) and PGD<sub>2</sub>, which have been reported to modulate the adaptive immune response through their action on DCs. For instance, PGD<sub>2</sub> was reported to affect the migratory properties of Langerhans cells (Angeli *et al.*, 2001) and the maturation of human DCs (Gosset *et al.*, 2003), as well as suppressing the CD40- and LPS-induced IL-12 secretion by murine splenic DCs (Faveeuw *et al.*, 2003). Moreover, PGE<sub>2</sub>-induced inhibition of DC function was also reported to be mediated by interaction with the EP<sub>2</sub> receptor (Harizi *et al.*, 2003).

A recent study reported the induction of IDO expression in DCs following interaction of PGE<sub>2</sub> with the EP<sub>2</sub> receptor subtype (Braun *et al.*, 2005). IDO-expressing DCs were shown to suppress T cell proliferative response *in vitro* (Terness *et al.*, 2002) and were tolerogenic when injected *in vivo* (Grohmann *et al.*, 2000). Using a pharmacological inhibitor of IDO (1-MT), I investigated whether IDO expression could mediate the

suppression of LPS-induced activation observed in pRBC-treated DCs. The results demonstrated that the maturation defect could not be rescued following treatment of DCs with 1-MT, excluding the involvement of IDO in the parasite-induced modulation of DC function observed in the present study.

Like PGE<sub>2</sub>, NO can exert its effects in an autocrine or paracrine fashion on neighbouring cells (Korhonen *et al.*, 2005). Both exogenous and endogenous NO have been reported to suppress the antigen presenting function of DCs by selectively downregulating the expression of costimulatory molecules in response to LPS or soluble CD40L *in vitro* as well as inhibiting the release of IL-10 and IL-12p40 by mature DCs (Corinti *et al.*, 2003). Therefore parasites may potentially induce the production of NO by DCs to subvert immune responses directed against them. Using a selective inhibitor of NO synthase activity, I tested whether NO played a role in the inability of pRBC-treated DCs to mature in response to LPS challenge. Blocking NO synthase activity did not reverse the maturation defect thus excluding the possibility that NO might be directly involved in the modulation of DC function in the culture system used.

TGF- $\beta$  is a potent regulatory cytokine produced by a wide variety of cells including DCs (Li *et al.*, 2005) and it has been reported to affect DC function. For instance, the presence of TGF- $\beta$  in LPS-stimulated DC cultures was shown to inhibit the expression of MHC class II and costimulatory molecules, which attenuated the antigen presenting function of DCs (Geissmann *et al.*, 1999). In addition, TGF- $\beta$  was also reported to be responsible for the suppression of DC maturation and IL-12 production induced by inflammatory cytokines such as IL-1 and TNF- $\alpha$  (Geissmann *et al.*, 1999). As previously discussed in section 1.2.8.5, there is evidence that many pathogens have evolved mechanisms to induce host cells to secrete active TGF- $\beta$ , which in turn suppresses APC function, enhancing pathogen survival. In particular, soluble components of *P. falciparum*, *P. berghei* and *P. yoelii* infected erythrocytes were shown to be directly able to activate both native (platelet-derived) and recombinant latent TGF- $\beta$  (Omer *et al.*, 2003). However, in the present study TGF- $\beta$  did not seem to be an important factor involved in the suppression of DC maturation observed following pRBC treatment, as the addition of a

TGF- $\beta$ -neutralising antibody in the culture did not rescue the LPS-induced maturation of pRBC-treated DCs.

The involvement of PPAR- $\gamma$  in the modulation of DC function has been previously discussed in section 1.2.8.2. Activation of PPAR- $\gamma$  has been shown to inhibit the maturation of both murine (Faveeuw *et al.*, 2000) and human monocyte-derived DCs (Gosset *et al.*, 2001). A more recent report showed that TLR ligand-induced activation and migration of human monocyte-derived DCs, as well as their ability to initiate lymphocyte proliferation, was impaired upon PPAR- $\gamma$  activation (Appel *et al.*, 2005). Activation of PPAR- $\gamma$  resulted in down-regulation of costimulatory and adhesion molecules by DCs and reduced secretion of cytokines and chemokines involved in T lymphocyte activation and recruitment (Appel *et al.*, 2005).

The involvement of PPAR- $\gamma$  activation during malaria infection has also been reported. PPAR- $\gamma$  was found to enhance the phagocytosis of *P. falciparum* infected erythrocytes by macrophages by upregulating the expression of CD36 (Serghides and Kain, 2001). However, it was shown that *P. falciparum* infected erythrocytes inhibited DC maturation *in vitro* through the binding of CD36 to a parasite-derived molecule (PfEMP1) on the infected erythrocyte surface (Urban *et al.*, 1999; Urban *et al.*, 2001b). Therefore what seems to be initially a protective mechanism induced by PPAR- $\gamma$  activation could actually enhance parasite survival within the host by altering DC activation states. Another study carried out by Paolo Arese and colleagues showed that the parasite pigment haemozoin inhibited the maturation of human monocyte-derived DCs *via* PPAR- $\gamma$  activation (Skorokhod *et al.*, 2004). Haemozoin was also found to be responsible for the suppression of DC function in the present study, as discussed in chapter 5. However, PPAR- $\gamma$  did not seem to mediate this suppression as the use of a PPAR- $\gamma$  antagonist did not rescue the ability of pRBC-treated DCs to respond to LPS treatment *in vitro*.

The results presented in this chapter clearly rule out the participation of some of the most common immunoregulatory molecules studied in the suppression of pRBC-treated DC function, although the role of PGE<sub>2</sub> requires further investigation due to its reported

ability to module DC function *via* interaction with the EP<sub>2</sub> receptor subtype. Additional studies are therefore necessary to gain a better understanding of the mechanisms by which the malaria parasite exerts its immunosuppressive effects on DC function. The events leading to DC hyporesponsiveness following interaction of DCs with infected erythrocytes are undoubtedly complicated given the antigenic and developmental complexity of the malaria parasite and the long co-evolution with its host. Improving the knowledge of these events could have important implications for vaccine design and immunotherapy and might aid in the development of effective DC-based vaccines. Since none of the molecules tested in this chapter had an effect on DC function, gene expression analysis seemed to be an alternative way to gain an overall idea of the molecular events involved during DC-pRBC interaction.

## **7. Gene expression analysis of *P. chabaudi* AS-treated DCs**

## 7.1 Introduction

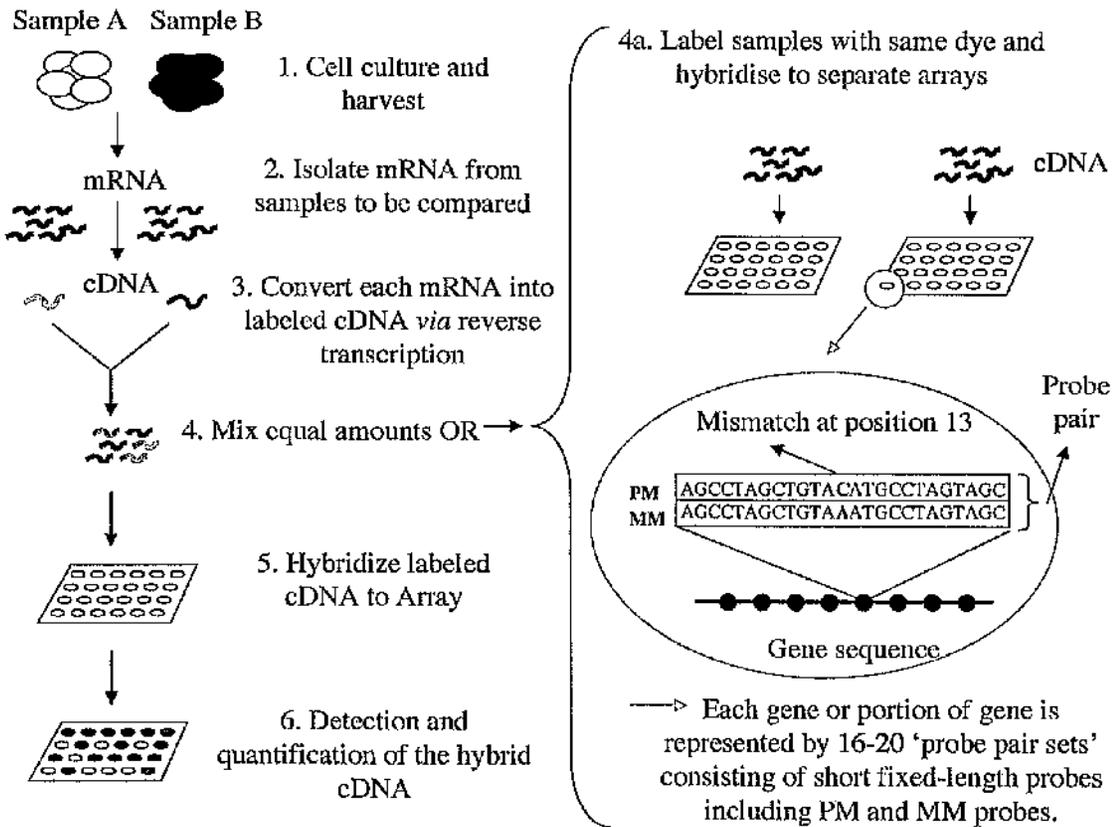
Gene array technology is a powerful method that provides researchers with the opportunity to analyse the expression patterns of thousands of genes simultaneously in a short period of time (Schena *et al.*, 1995). In a clinical context, identification of differentially expressed genes may lead to improved diagnosis and treatment guidance, early disease detection and clinical outcomes prediction. Moreover, relating specific groups of genes with specific biological states is a critical step toward understanding the underlying molecular mechanisms characterising a specific disease and will therefore aid in the identification of novel therapeutic targets (Troyanskaya *et al.*, 2002).

Spotted long cDNA microarray and *in situ* synthesis oligonucleotide arrays (Affymetrix) are currently the most widely used array systems (Li *et al.*, 2002). The basic concept behind the use of DNA arrays for gene expression is quite simple: labeled cDNA 'targets' (derived from the mRNA of an experimental sample) are hybridized to nucleic acid probes attached to the solid support. Multiple samples of interest are labeled either with different dyes and hybridized simultaneously against a single array (spotted cDNA array) or with the same dye and hybridized separately against multiple arrays (*in situ* oligonucleotide arrays) (Huber *et al.*, 2002). In each case, the relative abundance of a gene transcript in these samples is determined by comparing the corresponding fluorescence intensities (Brown and Botstein, 1999) (Diagram 7.1).

The existence of different types of arrays for measuring gene expression makes consistency and reproducibility across technology important issues. Being able to use data interchangeably across techniques would be very beneficial, as this would potentially reduce the need to duplicate experiments. Unfortunately, at the present time, there are issues in both technologies that should be resolved before it is possible to transform measurements from either technology to a general index of gene expression (Kuo *et al.*, 2002; Li *et al.*, 2002).

Spotted two-colour DNA array

Affymetrix Oligonucleotide Array



**Diagram 7.1** Principle of microarray technology. mRNA is isolated from samples of interest and converted into labelled cDNA, which is then hybridized to the array. The DNA sequences spotted on the array are referred to as 'probes', whereas the nucleic acid samples hybridized onto the array (whose identity/abundance is being detected) are generally referred to as 'targets'. When using a spotted-DNA array, labeled targets are mixed and hybridized simultaneously on a single array. If oligonucleotide arrays are used, targets are hybridized onto separate arrays. The Affymetrix oligonucleotide array uses 'probe-pair sets' consisting of a collection of 'probe pairs' (16-20) related to a common gene or fraction of a gene. Probe pairs are short fixed-length probes consisting of a Perfect Match (PM, a 25 base-pairs oligonucleotide complementary to a sequence of interest) and a Mismatch probes (MM, same as PM but with a single base change at position 13, in order to achieve more homogeneous hybridization conditions. Regardless

of the type of array used, the relative abundance of a gene transcript is determined by comparing the corresponding fluorescence intensities. In the spotted two-colour DNA array, the ratio of the red and green fluorescence intensities for each spot is indicative of the relative abundance of the corresponding DNA probe in the two nucleic acid target samples (green, gene is over-expressed in green-labeled sample compared to red-labeled sample; red, gene is over-expressed in red-labeled sample compared to green-labeled sample; yellow, gene is equally expressed in both samples). In the case of oligonucleotide arrays, the expression measures are generally based on differences of PM-MM, with the intention to correct for background and non-specific binding.

### **7.1.1 Spotted cDNA *versus* Oligonucleotide (Affymetrix) arrays**

Sensitivity and specificity are the two major features that researchers are concerned with in performing microarray analysis. The length of the DNA employed as the probe sequence on the arrays is a key factor in determining the specificity and sensitivity of the two commercial arrays mentioned above (Li *et al.*, 2002). In spotted cDNA arrays, the sequence of probes can vary from 500 to 5000 base pairs. One of the problems associated with having such large sequences is that it becomes difficult to control the hybridization efficiency of various cDNA probes. Hybridization conditions are based on the length and composition of nucleic acid fragments (Southern *et al.*, 1999). As A-T pairs are known to be less stable than G-C pairs (Southern *et al.*, 1999), probes with higher G-C content tend to have increased hybridization stability due to the higher proportion of stable base pairs (Herwig *et al.*, 2000).

The issue of non-specific hybridization is also an important limitation of the cDNA-based arrays (Kuo *et al.*, 2002; Li *et al.*, 2002). In addition to probe length affecting hybridization efficiency, probe sequences with sequence homology to other probes may further impinge on this issue (Schadt *et al.*, 2000). Different genes may in fact encode common domains and may have some degree of sequence identity with proteins from other genes (Evertsz *et al.*, 2001; Kane *et al.*, 2000). As a result, gene families pose a potential problem because in many cases these genes have a great degree of sequence

identity and can only be distinguished from each other by the design and use of gene-specific hybridization probes (Li *et al.*, 2002). Spotting more specific cDNA probes of the unique regions from a set of genes and reducing the length of the probe sequences could reduce some of the limitations, which might be encountered when using cDNA arrays (Kuo *et al.*, 2002).

In order to accomplish high sensitivity and specificity, Affymetrix introduced a system that involves the use of a series of specific and non-specific gene probe sets that are intended to result in a more accurate discrimination between true signal and random hybridization. Each gene or portion of a gene in the Affymetrix system is represented by 16-20 oligonucleotides of 25 base-pairs (Kothapalli *et al.*, 2002). The fixed-length probes are designed to be perfectly complementary to a target sequence, minimizing cross-hybridization with highly homologous genes (Kuo *et al.*, 2002; Li *et al.*, 2002). A partner probe that is identical except for a single base mismatch in its center, which theoretically provides maximal disruption of hybridization (Kothapalli *et al.*, 2002), is generated for each probe (see 4a in Figure 1). The difference in hybridization signals between the partners, as well as their intensity ratios, serves as indicators of specific target abundance. These probe pairs, called the Perfect Match probe (PM) and the Mismatch probe (MM), are intended to give more homogeneous hybridization conditions in order to increase the specificity and sensitivity of the assay (Li *et al.*, 2002). Unfortunately, the use of the mismatch probe information can sometimes interfere with fold change calculations of gene expression. For instance, the strong signal associated with some of the MM probes can interfere with the detection of some genes present in the samples (Kothapalli *et al.*, 2002), (Table 7.1 summarises the main differences between the two microarrays described).

**Spotted arrays****Affymetrix arrays**

One probe <i>per</i> gene	16-20 probe-pairs <i>per</i> gene
Probes of varying lengths	Probes are 25 base-pair long
Large probe sequences reduce hybridization efficiency and maximize cross-hybridization with homologous genes	Fixed-length probes improve hybridization efficiency and minimize cross-hybridization with homologous genes
	<u>Problem:</u> Sometimes strong signals associated with MM probe can interfere with detection of some genes
Two target samples <i>per</i> array	One target sample <i>per</i> array

**Table 7.1** Spotted cDNA *versus* Affymetrix oligonucleotide arrays: Summary of differences and limitations.

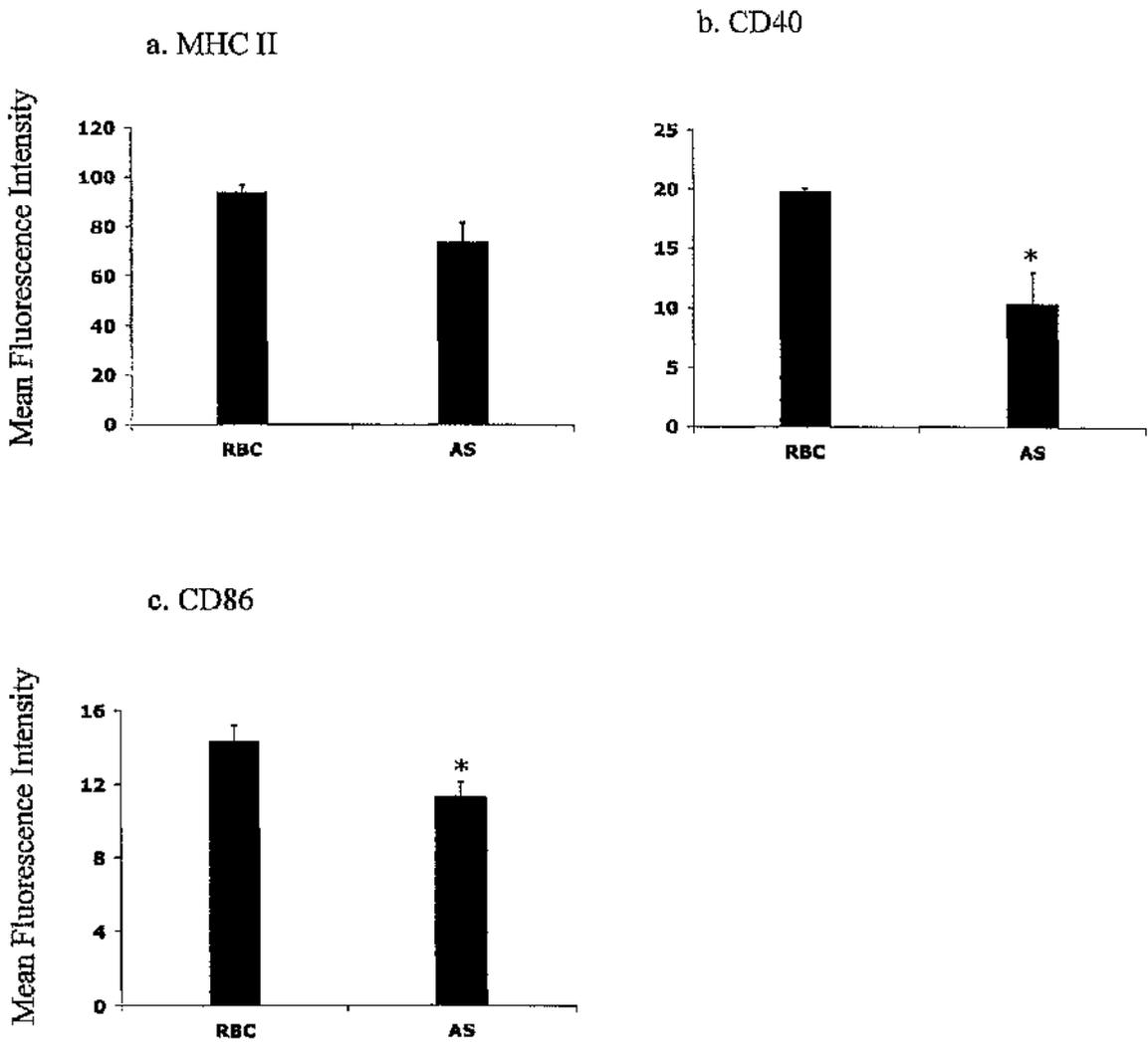
Despite potential disadvantages associated with the MM probes, the Affymetrix oligonucleotide arrays seem to give a more accurate and comprehensive scenario of gene-expression profiles, which in turn ensures with a high probability that further research work will proceed down the correct path (Kuo *et al.*, 2002; Li *et al.*, 2002). For these reasons, the Affymetrix arrays were used in the present study to analyse the gene expression profile of DCs following treatment with *P. chabaudi* AS infected erythrocytes or uninfected controls, to gain an overall idea of the molecular events involved during DC-pRBC interaction. Although gene array technologies allow the simultaneous measurement of the expression level of thousands of genes in a cell population, these experiments may lead to a high level of 'noise' in the resulting data. This latter problem may be worsened if a number of variables are introduced in the experimental design, therefore particular care must be taken when planning array experiments. To keep the level of noise in the resulting data to a minimum, bone marrow-derived DCs were used in the experiments described in this chapter, rather than DCs purified from infected animals. This approach allowed the investigation of changes in DC gene expression, which are

solely due to parasite interaction with the DCs, without being influenced by various other factors, such as the presence of cytokines and other cells in the environment. The present work was therefore designed with the view of gaining a deeper insight of the parasite-induced changes in DCs and provides a way to select specific gene products, which may potentially play an important role in the observed suppression of DC function, thus providing the basis for future investigation.

## 7.2 Results

Previous experiments have shown inhibition of the LPS-induced maturation of DCs beginning to occur by 6h of incubation with pRBCs (chapter 5). Therefore gene expression in these DCs must be either up- or down regulated by this time. Although suppression of DC function was most marked after 24h, I was concerned that changes in gene expression may have come and gone by this point. I therefore extracted RNA for microarray analysis from DCs, which have been incubated with pRBCs for 6h. To ensure that these DCs were functionally impaired, a set of cells was stimulated with LPS for a further 18h and the expression of MHC class II and costimulatory molecules CD40 and CD86 analysed by flow cytometry (Figure 7.1). The results show that CD40 and CD86 expression was significantly reduced in pRBC-treated DCs compared to RBC controls. Instead, MHC class II expression was not affected at this time point. This finding is consistent with previous results shown in chapter 5, indicating that after 6h incubation with pRBCs, DCs begin to show signs of a functional defect.

Details on cell preparations and RNA extraction were described in chapter 2. The Sir Henry Wellcome Functional Genomics Facility at the University of Glasgow performed the microarray experiment and data analysis. Results shown in Table 7.2 and 7.3 were selected based on their potential involvement in the modulation of DC function and were obtained by using the Rank Product (RP) method, where data are expressed as rank lists (Breitling *et al.*, 2004b). Results shown in Table 7.4 and 7.5 were instead obtained by using the iterative Group Analysis (iGA) approach, which identifies functional classes of gene that are significantly changed (Breitling *et al.*, 2004a). The basic principles of the above methods were described in chapter 2.



**Figure 7.1** Surface expression of MHC class II (a), CD40 (b) and CD86 (c) on bone marrow-derived DCs following LPS stimulation *in vitro*. DCs were cultured for 6h with non-infected (RBC) or *P. chabaudi* AS infected erythrocytes (AS) and then treated with 1  $\mu$ g/ml of LPS for a further 18h. Results were obtained by flow cytometric analysis of gated CD11c<sup>+</sup> cells and are shown as the mean fluorescence intensity  $\pm$  standard errors of triplicate cultures per group. \*  $p \leq 0.05$  significant difference between AS and RBC.

**Table 7.2** Upregulated genes expressed by DCs treated with *P. chabaudi* AS-infected erythrocytes for 6h. Results shown were obtained using the Rank Products approach (RP) (Breitling *et al.*, 2004b).

<b>RP</b>	<b>Title</b>	<b>Rank</b>
4.58	COX-2	4
23.05	Thrombospondin-1	17
257.99	12-Lipoxygenase	153
266.79	Haeme Oxygenase-1	157
321.66	Clusterin	182

**Table 7.3** Downregulated genes expressed by DCs treated with *P. chabaudi* AS-infected erythrocytes for 6h. Results shown were obtained using the Rank Products approach (RP) (Breitling *et al.*, 2004b).

<b>RP</b>	<b>Title</b>	<b>Rank</b>
67.90	CD47	39
280.81	BNIP3	143
330.24	COX-1	173
500.66	Bcl-2-like protein 11	261
557.61	Tyrosine-3 Mono-oxygenase	280
563.92	Cytochrome c	286
710.17	Fis 1	352
816.90	Hipk2	404
1065.95	Caspase 3	500
1395.57	Tripartite motif protein 27	665

**Table 7.4** Functional classes of genes significantly upregulated by DCs incubated with *P. chabaudi* AS-infected erythrocytes for 6h, as determined by the iGA approach (Breitling *et al.*, 2004a).

<b>GROUP</b>	<b>p-Value</b>	<b>Title</b>
<b>PR001811 - Small chemokine, IL-8 like</b>	6.1e-03	CXCL1 (GRO/MIP-2/KC)
	1.8e-04	CXCL2 (GRO/MIP-2/KC)
	3.5e-06	CCL4 (MIP-1 $\beta$ )
	4.3e-08	CCL3 (MIP-1 $\alpha$ )
	3.9e-10	gene model 1960, (NCBI)
	2.1e-11	CCL2 (JE)
	1.9e-1	CXCL5 (GCP-2/LIX)
<b>6935 - chemotaxis</b>	3.5e-02	CXCL2 (GRO/MIP-2/KC)
	1.2e-03	CCL4 (MIP-1 $\beta$ )
	3.3e-05	CCL3 (MIP-1 $\alpha$ )
	1.4e-06	CCL2 (JE)
	2.8e-08	IFN- $\alpha$ -inducible protein
	4.1e-10	CXCL5 (GCP-2/LIX)
<b>8009 - chemokine activity</b>	6.8e-03	CXCL1 (GRO/MIP-2/KC)
	2.2e-04	CXCL2 (GRO/MIP-2/KC)
	4.7e-06	CCL4 (MIP-1 $\beta$ )
	6.5e-08	CCL3 (MIP-1 $\alpha$ )
	3.4e-09	CXCL5 (GCP-2/LIX)
<b>Inflammatory_Response_Pathway - GenMAPP</b>	5.6e-03	IL-6
	7.8e-05	IL-1 $\beta$
	7.3e-07	IL-1 $\alpha$
	5.4e-08	TSP-1
	1.3e-08	TNF

GROUP	p-Value	Title
IPR003502 - IL-1 propeptide	7.1e-04	IL-1 $\beta$
	2.1e-07	IL-1 $\alpha$
IPR000975 - IL-1	3.2e-03	IL-1 $\beta$
	7.5e-06	IL-1 $\alpha$
5125 - cytokine activity	1.4e-02	IL-6
	4.5e-04	IL-1 $\beta$
	1.0e-05	IL-1 $\alpha$
	7.0e-05	IL-12
	3.8e-05	G-CSF3
	9.1e-06	IL-1R antagonist
TGF_Beta_Signaling_Pathway - GenMAPP	2.0e-02	Inhibin beta-A
	9.6e-04	TSP-1
	1.1e-04	TNF
	1.2e-05	Follistatin
IPR003573 - Interleukin-6/G-CSF/MGF	2.8e-04	IL-6
	1.5e-05	G-CSF3
6955 - immune response	1.2e-01	guanylate nucleotide binding protein 2
	7.4e-03	interferon-induced protein with tetratricopeptide repeats 1
	7.7e-04	CD14 antigen
	1.2e-04	guanylate nucleotide binding protein 1
1.6e-05	colony stimulating factor 3 (granulocyte)	

GROUP	p-Value	Title
50875 - cellular physiological process	1.4e-02	CXCL1 (GRO/MIP-2/KC)
	8.6e-03	E26 avian leukemia oncogene 2, 3' domain 3.
	3.4e-03	musculoaponeurotic fibrosarcoma oncogene family, protein F (avian.)
	6.9e-04	Jun-B oncogene
	5.2e-05	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100
7166 - cell surface receptor linked signal transduction	8.3e-02	IL-12
	5.4e-03	Plaur urokinase plasminogen activator receptor
	8.7e-04	IL-1 receptor antagonist
	5.4e-05	C-type lectin domain family 1, member b
8283 - cell proliferation	1.5e-02	IL-1 $\beta$
	1.8e-04	IL-1 $\alpha$
	7.6e-05	TNF
6953 - acute-phase response	2.7e-03	IL-6
	9.3e-05	serum amyloid A 3
8219 - cell death	2.2e-02	TGF- $\beta$ 1
	3.2e-04	TNFR-1 $\beta$
	1.1e-04	clusterin

**Table 7.5** Functional classes of genes significantly downregulated by DCs incubated with *P. chabaudi* AS-infected erythrocytes for 6h, as determined by the iGA approach (Breitling *et al.*, 2004a).

GROUP	p-Value	Title
IPR001208 - MCM	1.5e-02	minichromosome maintenance deficient 5, cell division cycle 46 ( <i>S. cerevisiae</i> )
	2.2e-04	minichromosome maintenance deficient 4 homolog ( <i>S. cerevisiae</i> )
	3.4e-06	minichromosome maintenance deficient 7 ( <i>S. cerevisiae</i> )
	2.5e-07	minichromosome maintenance deficient 6 (MMS5 homolog, <i>S. pombe</i> ) ( <i>S. cerevisiae</i> )
6260 - DNA replication	9.2e-02	minichromosome maintenance deficient 5, cell division cycle 46 ( <i>S. cerevisiae</i> )
	5.2e-03	ribonucleotide reductase M2 36
	4.0e-04	minichromosome maintenance deficient 4 homolog ( <i>S. cerevisiae</i> )
	4.7e-05	minichromosome maintenance deficient 7 ( <i>S. cerevisiae</i> )
	8.6e-06	retroviral integration site 2
	2.4e-06	minichromosome maintenance deficient 6 (MMS5 homolog, <i>S. pombe</i> ) ( <i>S. cerevisiae</i> )
GI_to_S_cell_cycle_Reactome - GenMAPP	1.1e-01	minichromosome maintenance deficient 5, cell division cycle 46 ( <i>S. cerevisiae</i> )
	1.3e-02	minichromosome maintenance deficient 4 homolog ( <i>S. cerevisiae</i> )
	1.8e-03	minichromosome maintenance deficient 7 ( <i>S. cerevisiae</i> )
	1.6e-04	transcription factor Dp 2
	2.43e-05	myelocytomatosis oncogene
	6.8e-06	minichromosome maintenance deficient 6 (MMS5 homolog, <i>S. pombe</i> ) ( <i>S. cerevisiae</i> )
DNA_replication_Reactome - GenMAPP	7.0e-02	minichromosome maintenance deficient 5, cell division cycle 46 ( <i>S. cerevisiae</i> ) 36
	5.3e-03	minichromosome maintenance deficient 4 homolog ( <i>S. cerevisiae</i> )
	4.6e-04	minichromosome maintenance deficient 7 ( <i>S. cerevisiae</i> )
	5.8e-05	retroviral integration site 2
	1.1e-05	minichromosome maintenance deficient 6 (MMS5 homolog, <i>S. pombe</i> ) ( <i>S. cerevisiae</i> )
IPR00183 - Orn/DAP/Arg decarboxylase 2	4.4e-03	ornithine decarboxylase, structural 1
	1.3e-05	ornithine decarboxylase antizyme inhibitor

GROUP	p-Value	Title
6596 - polyamine biosynthesis	4.4e-03 1.5e-05	ornithine decarboxylase, structural 1 ornithine decarboxylase antizyme inhibitor
IPR004254 - Ely-III related proteins	1.4e-02 1.0e-0	progesterin and adipoQ receptor family member IX adiponectin receptor 1
6164 - purine nucleotide biosynthesis	1.5e-02 2.6e-03 1.3e-04	5-aminimidazole-4-carboxamide ribonucleotide formyltransferase /IMP cyclohydrolase, adenylosuccinate synthetase, non muscle adenylosuccinate synthetase like 1
5488 - binding	1.5e-01 9.9e-03 5.9e-04 1.4e-04	DNA segment, Chr 11, ERA10 Doi 333, expressed RIKEN cDNA 6330416G13 gene mitochondrial solute carrier protein uncoupling protein 2 (mitochondrial, proton carrier)
IPR003395 - SMC protein, N-terminal	3.2e-02 6.2.7e-03 2.2e-04 1.8e-04	chondroitin sulfate proteoglycan SMC (structural maintenance of chromosomes 1)-like 1 ( <i>S. cerevisiae</i> ) SMC4 structural maintenance of chromosomes 4-like 1 (yeast) SMC2 structural maintenance of chromosomes 2-like 1 (yeast)
Circadian_Exercise - GenMAPP	6.7e-02 3.7e-03 1.9e-04	nuclear receptor coactivator ornithine decarboxylase antizyme inhibitor basic transcription element binding protein 1

## 7.3 Discussion

The use of a whole mouse genome expression array allowed the investigation of over 39,000 genes, 587 of which were found to be upregulated and 723 downregulated in bone marrow-derived DCs following treatment with pRBCs. Due to time constraints, a detailed analysis of this data was not possible. I therefore selected to discuss some genes, which encode molecules that may potentially affect DC function and are therefore worth further investigation.

### 7.3.1 Molecules involved in the modulation of DC functions

#### 7.3.1.1 Eicosanoids

The cyclooxygenases, COX-1 and COX-2, are enzymes involved in thromboxane and prostaglandin synthesis (Harizi and Gualde, 2004). Particularly, COX-2 is an inducible gene that generates high levels of prostaglandins during inflammation, whereas COX-1 is constitutively expressed in most tissues and generates prostaglandins for physiological homeostasis (Seibert and Masferrer, 1994). In the present study, COX-2 gene expression was found to be the 4<sup>th</sup> most upregulated gene in pRBC-treated DCs (Table 7.2), whereas COX-1 was downregulated (Table 7.3). This observation is particularly interesting in the current study, as PGE<sub>2</sub> is known to affect the maturation, cytokine production, Th cell polarising ability and migration of DCs (Harizi *et al.*, 2003; Harizi *et al.*, 2001; Harizi *et al.*, 2002). Although PGE<sub>2</sub> has been shown to exert its action by binding to four different types of G-protein-coupled receptors, EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub> and EP<sub>4</sub> (Narumiya *et al.*, 1999), EP<sub>2</sub> and EP<sub>4</sub> have been reported to play a central role in the modulation of DC functions by PGE<sub>2</sub> (Harizi *et al.*, 2003; Meja *et al.*, 1997). The involvement of PGE<sub>2</sub> in the observed suppression of DC function has been previously investigated by carrying out a preliminary experiment using an EP<sub>4</sub>-selective antagonist (chapter 6). The results shown in chapter 6 suggested that the PGE<sub>2</sub>-EP<sub>4</sub> signalling pathway did not play a role in the modulation of DC function, as treatment of DCs with the EP<sub>4</sub> antagonist did not rescue the ability of DCs to mature in response to LPS stimulation *in vitro*. However, since

PGE<sub>2</sub> also exerts its effects through the EP<sub>2</sub> receptor, the involvement of this molecule, as well as other prostaglandins, in the observed suppression of pRBC-treated DCs cannot be excluded yet. Furthermore, prostaglandins might act directly on T cells to inhibit their proliferation. In fact it has been reported that PGE<sub>2</sub>-EP<sub>4</sub> signalling in T cells potently suppresses their activation and proliferation (Narumiya, 2003), providing a possible mechanism used by the parasite to induce inappropriate T cell responses, as previously described in chapter 4. Thus, the observation that COX-2 gene expression is upregulated in pRBC-treated DCs further reinforces the possibility that prostaglandins might play a significant role in the observed suppression of DC function.

Formation of prostaglandins is determined by the relative expression of pro-inflammatory cytokines, which increase COX-2 gene expression and anti-inflammatory cytokines, which instead decrease COX-2 expression (Harris *et al.*, 2002). There is also evidence that prostaglandins regulate chemokine receptor expression and migration of DCs. In the presence of IL-1 $\alpha$  and TNF- $\alpha$ , PGE<sub>2</sub> has been reported to upregulate the expression of CCR7 and enhance DC migration in response to CCL19 or CCL21 (Harizi *et al.*, 2002). Therefore, the observed upregulation of the genes encoding the pro-inflammatory cytokines IL-1 $\alpha/\beta$  and TNF- $\alpha$  (Table 7.4) is consistent with the upregulation of COX-2, which in turn concurs with the increased expression of many chemokine ligands found in pRBC-treated DCs (Table 7.4).

Gene expression of 12-lipoxygenase, an enzyme that catalyses the production of pro-inflammatory leukotrienes from arachidonic acid (Harizi and Gualde, 2004), was also found to be increased in pRBC-treated DCs (Table 7.2). The relevance of this enzyme in the progression of many human diseases such as cancer, atherosclerosis and rheumatoid arthritis, has been demonstrated in the past (Yoshimoto and Takahashi, 2002). In a murine model of helminth infection, 12/15-lipoxygenase was found to be responsible for the suppressive activity of myeloid cells by generating lipid mediators, which triggered PPAR- $\gamma$  activation (Brys *et al.*, 2005). Although the involvement of PPAR- $\gamma$  activation in the suppression of pRBC-treated DC function has been previously excluded in the current study (chapter 6), 12-lipoxygenase might exert its suppressive effects on DCs by using a

mechanism other than PPAR- $\gamma$  activation. In this regard, it has been shown that lipoxin production, which is catalysed by lipoxygenase, selectively affected chemokine receptor signalling in murine DCs during *T. gondii* infection, resulting in defective DC migration and decreased IL-12 responses (Aliberti *et al.*, 2002).

Therefore, the observed upregulation of COX-2 and lipoxygenase gene expression in pRBC-treated DCs suggest a possible role of these gene products in the modulation of DC function, providing the basis for further investigation.

### 7.3.1.2 Thrombospondin-1

Thrombospondin-1 (TSP-1) was found to be the 17<sup>th</sup> most upregulated gene in pRBC-treated DCs (Table 7.2). TSP-1 is an extracellular matrix glycoprotein, which was first discovered as a stored protein of  $\alpha$ -granules that was released upon platelet activation. The first studies of its function therefore focused on its roles in platelet aggregation and fibrin clot formation and also its effects on the endothelial and smooth muscle cells of blood vessel walls (Tucker, 2004). These studies identified a multiplicity of functions including effects on cell attachment, motility, proliferation, cell-cell contact and cell aggregation (Tucker, 2004).

DCs have been shown to be an abundant source of TSP-1, both in the steady state and during activation by danger signals (Doyen *et al.*, 2003). More importantly, endogenous TSP-1 has been reported to actively render DCs refractory to subsequent stimulation thus contributing to the arrest of the inflammatory response (Doyen *et al.*, 2003). This notion is particularly interesting in the present study, as it was previously shown that pRBCs inhibit the ability of DCs to mature in response to LPS treatment *in vitro* as observed by the reduced levels of MHC Class II expression on DCs as well as costimulatory molecules CD40 and CD86 (chapter 3).

In view of the results obtained following gene array analysis, it seems reasonable that pRBCs might stimulate DCs to release TSP-1, which will in turn act on the secreting DC

itself as well as bystander cells. Indeed further experiments will be required to confirm this hypothesis and understand the mechanism by which TSP-1 exerts its immunosuppressive effects on DCs. In this regard, it has been reported that ligation of TSP-1 with CD47 and CD36 inhibited cytokine production and maturation of DCs in response to bacterial stimulation (Armant *et al.*, 1999; Demeure *et al.*, 2000; Doyen *et al.*, 2003). TSP/CD47 and TSP/CD36 ligation was found to prevent the upregulation of costimulatory molecules, the loss of endocytic activity and the acquisition of an increased capacity to stimulate T cell proliferation and IFN- $\gamma$  production (Demeure *et al.*, 2000). These findings were also consistent with a report indicating that anti-CD36 monoclonal antibody delivered a negative signal to DCs, resulting in decrease of IL-12 production and phenotypic maturation (Urban *et al.*, 2001b). Moreover, TSP-1 has been shown to inhibit the expression of the early T cell activation marker CD69 through ligation of CD47, which is also a costimulatory receptor on T cells (Li *et al.*, 2001).

The ability of pRBC-treated DCs to prime naïve T cells *in vitro* has been analysed in chapter 4 where it was found that the expression of CD69 on T cells was not significantly affected by pRBCs. This finding therefore suggests that TSP-1 might not be involved in the modulation of T cell responses in the context of blood stage malaria infections. However, despite the observed upregulation of CD69, pRBCs were found to significantly affect the ability of DCs to induce efficient T cell responses, leading to the development of an anergic rather than an effector T cell phenotype. Interestingly, in a different report TSP-1 was shown to promote the induction of anergy in naïve T cell (Avice *et al.*, 2001), leaving open the possibility that this molecule might play an important role in the suppression of DC function and subsequent induction of inappropriate T cell responses during blood stage malaria infection.

### **7.3.1.3 Haeme Oxygenase-1**

Haeme oxygenase-1 (HO-1) is an intracellular enzyme that degrades haeme and inhibits immune responses and inflammation *in vivo* (Kikuchi *et al.*, 2005). In the present study, HO-1 was found to be upregulated in DCs treated with pRBCs (Table 7.2). The

upregulation of HO-1 gene expression seemed particularly interesting, as this molecule has recently been reported to block DC maturation and to inhibit pro-inflammatory immune responses (Chauveau *et al.*, 2005). Chauveau *et al.* presented evidence that induction of HO-1 expression in DCs interfered with the LPS-induced maturation process *in vitro*, resulting in the downregulation of the phenotypic maturation markers, as well as modulation of cytokine production critical for DC maturation and for efficient effector T cell responses (Chauveau *et al.*, 2005). In the present study these changes were consistently found in DCs following interaction with pRBCs, suggesting a possible role of HO-1 in the modulation of DC function during blood stage malaria. Furthermore, since HO-1 has been shown to degrade haeme to free divalent iron and carbon monoxide (CO) (Otterbein and Choi, 2000), it is possible that the inhibition of T cell proliferation and cytokine production previously observed in chapter 4 might be due to CO formation, as CO has recently been reported to inhibit lymphocyte proliferation (Song *et al.*, 2004). Therefore HO-1 is not only a marker for immature DCs; instead it appears to have the potential to affect DC functions by inhibiting T cell responses and inducing T cell anergy.

The induction of HO-1 in DCs following pRBC interaction may result as a mechanism to degrade the haeme present in native haemozoin (HZ), which was shown to consist of a core of polymerised haeme derived from host haemoglobin (Arese and Schwarzer, 1997). Several studies, conducted both *in vivo* and *in vitro* have shown that phagocytes are unable to digest HZ and alteration of certain important functions of the phagocytes may be related to persistence of undigested HZ in these cells (Schwarzer *et al.*, 1998; Schwarzer and Arese, 1996; Schwarzer *et al.*, 1993; Schwarzer *et al.*, 1992). Consistent with the above reports, this study also showed that HZ suppressed the LPS-induced maturation of DCs *in vitro* (chapter 5). Therefore, it is reasonable to assume that HZ might activate HO-1 due to the presence of haeme in its structure, leading to DC suppression. However, Schwarzer and colleagues demonstrated in an early study that HZ blocked the induction of HO-1 in human monocytes. However HZ did not result in a generalised impairment of the mechanism of HO-1 activity as, HZ-laden cells were still able to induce HO-1 activity when challenged with free haeme (Schwarzer *et al.*, 1999). Furthermore, alkali-solubilised HZ was shown to be an efficient inducer of HO-1 when

added to monocytes or monocyte-fed HZ, strongly suggesting that the haeme component of HZ is potentially capable of acting as an inducer of HO-1, but needs to be converted to soluble free haeme in order to show these properties (Schwarzer *et al.*, 1999). Therefore, the lack of HO-1 induction seems to be mainly due to the structural characteristic of HZ.

The above observations appear to contrast with the observed upregulation of HO-1 gene expression in the present study. However, besides being induced by haeme itself, in most cell types, HO-1 is inducible by inflammatory stimuli and oxidative stress (Kikuchi *et al.*, 2005). In fact IL-1, IL-6 and TNF- $\alpha$  were all shown to cause a marked induction of HO-1 mRNA in mouse liver (Rizzardini *et al.*, 1993). In this regard genes associated with the inflammatory response pathway were found to be upregulated in the present study, a result consistent with the upregulation of HO-1. Therefore HO-1 may play an important role in the suppression of DC function in the context of blood-stage malaria infection. Indeed, induction of HO-1 in tissue macrophages and monocytes has been reported in fatal *P. falciparum* malaria (Clark *et al.*, 2003). Nevertheless, whether pRBCs actually modulate DC function by inducing HO-1 activity remains to be determined and should therefore be the focus of future studies.

### **7.3.2 Molecules involved in the control of DC apoptosis**

#### **7.3.2.1 CD47**

CD47 is a cell-surface molecule expressed on nearly all haematopoietic cells (Brown and Frazier, 2001) and has been shown to be involved in DC apoptosis (Johansson *et al.*, 2004) as well as inducing cell death in many other different cell types including B cells (Mateo *et al.*, 1999), activated T cells (Pettersen *et al.*, 1999) and endothelial cells (Freyberg *et al.*, 2000). In the present study, CD47 gene expression was found to be downregulated in pRBC-treated DCs (Table 7.3). This observation is consistent with previous finding shown in chapter 3 where pRBCs were found not to induce apoptosis DCs *in vitro*.

As mentioned earlier, it has been reported that CD47 also played a significant role in modulating DC function through interaction with TSP-1 (Armant *et al.*, 1999; Demeure *et al.*, 2000; Doyen *et al.*, 2003). The downregulation of CD47 gene expression would therefore suggest that, if TSP-1 truly mediated the observed suppression of DC function, it might use a mechanism other than CD47 ligation. Alternatively, it is possible that upregulation of CD47 gene expression might occur at an earlier/later time, as gene expression analysis was only carried out after a 6h incubation period of DCs with pRBCs. However, at the present time, the importance of TSP-1 and CD47 in the modulation of DC function in the context of blood-stage malaria infections can only be speculated upon and should be the focus for future studies.

### 7.3.2.2 Bcl-2 proteins

The Bcl-2 family of proteins includes the best-characterised regulators of apoptosis (Li *et al.*, 2004a). Proteins of this family directly regulate the release of mitochondrial apoptogenic factors (Li *et al.*, 2004a). Many of the pro-apoptotic family members, such as Bax, Bid, Bim and Bmf, are localised in the cytoplasm. Apoptotic stimulation results in their translocation to the mitochondria and induction of the release of apoptogenic factors, such as cytochrome c (Li *et al.*, 2004a). This will in turn activate the cysteine protease family (known as caspases), probably by inhibiting anti-apoptotic members of the family and activating multi-domain members like Bax (Antonsson *et al.*, 2000; Antonsson *et al.*, 2001; Li *et al.*, 2004a).

In the present study, the expression level of pro-apoptotic genes was downregulated, whereas anti-apoptotic genes were upregulated, further supporting the viability of DCs following interaction with pRBCs (Hou and Van Parijs, 2004). For instance, the pro-apoptotic genes, Bcl-2-like protein 11 and BNIP3 were downregulated (Table 7.3). The latter finding is particularly interesting not only because it supports the viability of DCs in the culture system used but also because BNIP3 has been reported to induce T cell apoptosis by binding to CD47 on T cells (Lamy *et al.*, 2003). The ability of pRBC-treated DCs to induce T cell responses *in vitro* has been previously investigated in chapter 4

where T cell viability was not found to be affected. Thus, the downregulation of BNIP3 gene expression in pRBC-treated DCs further reinforces the idea that pRBCs do not induce DCs to deliver apoptotic signals to T cells following their interaction.

Tyrosine-3 Mono-oxygenase (also known as 14-3-3 theta) is a cytoplasmic protein, which has been reported to bind directly to Bax and to inhibit its translocation and integration into the mitochondrial membrane, therefore blocking the release of apoptogenic factors (Nomura *et al.*, 2003). Thus, over-expression of Tyrosine-3 Mono-oxygenase could be expected to inhibit Bax-induced apoptosis. In the present study however Tyrosine-3 Mono-oxygenase gene expression was found to be downregulated (Table 7.3), possibly indicating induction of Bax-induced apoptosis in pRBC-treated DCs. Nevertheless, cytochrome c and caspase 3, which are expected to be activated following Bax translocation into the mitochondria (Antonsson *et al.*, 2000), were both found to be downregulated (Table 7.3). Nevertheless, DC viability was further substantiated by the downregulation of many other pro-apoptotic genes such as Tetratricopeptide repeat domain 11 (Fis 1) (Table 7.3) (Lee *et al.*, 2004), Homeodomain interacting protein kinase 2 (Hipk2) (Table 7.3) (D'Orazi *et al.*, 2002; Zhang *et al.*, 2003), and Tripartite motif protein 27 (Table 7.3) (Dho and Kwon, 2003). Furthermore Clusterin, which has been associated with cell survival (Pearse *et al.*, 1992) was found to be upregulated in pRBC-treated DCs (Table 7.2), consistent with the observed viability of DCs in the experimental culture tested. Indeed these ideas remain entirely speculative at the present time and they might be over simplified. In fact the involvement of so many different genes encoding the various apoptotic signals emphasises the complexity of the interactions that might take place during this phenomenon.

Collectively, DNA microarray analysis of pRBC-treated DCs emphasised the induction of a strong pro-inflammatory response occurring during the initial interaction of DCs with pRBCs, a finding typically reported during malaria infection (Stevenson and Riley, 2004). Nevertheless, as explained above, some mediators of inflammation might in turn inhibit DC function, perhaps at a later time point during infection, thus providing a

possible mechanism used by the parasites to modulate the immune response to their advantage.

The possible involvement of TSP-1, HO-1 and prostaglandins in the observed suppression of DC function represent only a few interesting molecules, which have been highlighted herein as a result of gene array technology. Undoubtedly, the microarray data might contain information on many other genes encoding molecules, which could potentially induce DC suppression following pRBC interaction. However due to the limited amount of time available, detailed analysis of these results could not be extensively carried out. Nevertheless, the data presented in this chapter shed light on potential molecules that might be involved in the modulation of DC function, providing the basis for future functional studies. Experiments using inhibitors of the molecules described above and analysis of protein production will be required to gain a better understanding of the involvement of these molecules during blood-stage malaria infection.

## 8. Conclusions

## 8.1 Conclusions

During malaria infection there is significant depression of the host's immune response to concurrent infections (Bomford and Wedderburn, 1973; Cook, 1985; Mabey *et al.*, 1987; Whittle *et al.*, 1984) as well as the response to the parasite itself (Brasseur *et al.*, 1983; Ho *et al.*, 1986; Troye-Blomberg *et al.*, 1984). Furthermore, infection with *Plasmodium* species impinges on the success of vaccination against unrelated organisms (Greenwood *et al.*, 1972; McGregor, 1962; Williamson and Greenwood, 1978), a significant obstacle to initiatives aimed at expanding childhood immunisation in malaria endemic regions. Using the murine *P. chabaudi* AS model, I examined whether pRBCs can suppress the function of DCs, which are central to the initiation and regulation of the adaptive immune response (Adams *et al.*, 2005). Impairment of DC function may therefore inhibit the induction of adaptive immunity to heterologous antigens, providing a potential explanation for epidemiological studies linking endemic malaria with secondary infections and reduced vaccine efficacy.

The present study revealed that pRBCs do not directly activate bone marrow-derived DCs *in vitro*; rather, they inhibit the LPS induced upregulation of Class II MHC and costimulatory molecules (CD40 and CD86) on DCs, as well as the production of IL-12 and IL-10 cytokines (chapter 3). pRBCs also inhibit DC activation in response to CpG and CD40 ligation indicating that multiple signalling pathways are affected, causing a generalised suppression of DC function. Furthermore, interaction with pRBCs significantly reduces the ability of DCs to induce OVA-specific T cell proliferation and cytokine production (chapter 4). However, although pRBC-treated DCs have a reduced ability to activate an OVA-specific T cell hybridoma, indicative of defective antigen presenting function, these cells are still capable of inducing the expression of the early activation marker CD69 on OVA-specific T cells (chapter 4). This finding suggests that the observed proliferation defect is not due to the failure of T cells to recognise antigen and that the amount of antigen presented, although significantly reduced, is sufficient to activate naïve T cells. Nevertheless, pRBC-treated DCs induce an anergic rather than an effector T cell phenotype (chapter 4), a result which is consistent with the suppression of

immunity observed in malaria-infected patients (Bomford and Wedderburn, 1973; Cook, 1985; Lam *et al.*, 1991; Mabey *et al.*, 1987; Moormann *et al.*, 2005; Thursz *et al.*, 1995; Whittle *et al.*, 1984).

Since pRBCs were present in DC cultures during the assessment of the OVA-specific T cell proliferation, I was aware of the possibility that the parasites might directly induce inhibitory effects on the T cells rather than inhibiting their proliferation by affecting DC function. I initially addressed this issue by lysing the pRBCs following a 24h incubation period with DCs. However, this procedure induced DC activation, thus affecting the outcome of the proliferation assay. I therefore carried out an APC-independent T cell proliferation assay by coating tissue culture plates with anti-CD3/anti-CD28 antibodies (which mimic the signals that T cells would receive from functional DCs) prior to addition of pRBCs and T cells (100:1, PRBC:T cell). Nevertheless, the results obtained by using this system varied significantly among experiments, with some assays showing control RBCs being as inhibitory as pRBCs when compared to cultures that did not receive any erythrocytes. These different outcomes might be due to the large number of RBCs non-specifically blocking the interaction of T cells with the antibodies. Although the issue of whether pRBCs induce suppression of T cell proliferation by affecting DC function or by having a direct effect on the T cells could not be resolved *in vitro*, ongoing studies in our laboratory provide strong evidence supporting the former scenario. In fact, results from *in vivo* experiments have shown that DCs purified from spleens of infected animals and pulsed with OVA, could not induce effector OVA-specific T cell responses when transferred into healthy recipients, which received CFSE-labelled OVA-specific DO11.10 T cells (in press).

It is known that presentation of antigen to T cells is an essential step in the induction of primary immune responses (Obst *et al.*, 2005). Low levels of antigen presentation may fail to induce a response, or stimulate only moderate levels of clonal expansion (Gett *et al.*, 2003). If the magnitude of the peptide:MHC signal received by T cells (signal 1) is sufficiently strong, it may overcome the requirement for costimulation (signal 2) in T cell priming (Bachmann *et al.*, 1998) and it can also influence the Th1/Th2 polarisation of the

resulting T cell response (Constant and Bottomly, 1997). Thus high doses of peptide:MHC can polarise towards Th1, with lower doses more likely to induce Th2 responses (Guery *et al.*, 1996; Obst *et al.*, 2005). Therefore, the dose and duration of antigen presentation, can significantly affect the type of adaptive immune response induced. In this regard, it is still not known how much antigen (heterologous and homologous) is taken up by APCs during malaria infection and, more importantly, how much is subsequently presented to T cells and for how long this presentation persists. Clearly, these are important questions, which should be the focus of future investigations in order to gain a deeper understanding of how the malaria parasite suppresses the development of protective immune responses.

A new method for studying antigen processing and presentation has been recently described (Itano *et al.*, 2003) in which a protein antigen and a peptide:MHC II complex derived from this antigen could be monitored simultaneously *in vivo*. This system uses the YAe antibody, which specifically recognises the peptide E $\alpha$ <sub>52-68</sub> (derived from the MHC I-E molecule) in the context of I-A<sup>b</sup> (Murphy *et al.*, 1992; Rudensky *et al.*, 1991). Linking the E $\alpha$  peptide with an appropriate fluorescent protein (e.g. Green Fluorescent Protein-GFP) will allow the visualisation of both uptake and presentation of antigen. Furthermore, TcR transgenic TE $\alpha$  mice, whose CD4<sup>+</sup> T cells recognise the E $\alpha$ /I-A<sup>b</sup> complex, are also available (Grubin *et al.*, 1997). Thus, by using fluorescently labelled antigen, the YAe antibody and these antigen-specific CD4<sup>+</sup> T cells, it is possible to monitor the uptake and presentation of antigen as well as visualising the interaction between APCs and T cells both *in vitro* and *in vivo*.

The above system has been recently established in our laboratory and has been used to examine how pRBCs affect the ability of DCs to process and present heterologous antigen *in vitro*. When the proportion of DCs expressing E $\alpha$  peptide was measured, no significant difference between RBC and pRBC-treated DCs could be observed (O. Millington, personal communication). This does not necessarily contradict my findings in which pRBC-treated DCs were less able to activate hybridoma T cells since the two systems use different readouts of presentation; one is phenotypic, the other functional. As

yet, no functional analysis on T cell activation has been carried out using the E $\alpha$  system and this would clearly be the focus of future studies. However, it is intriguing to think that two experimental systems may respond differently to parasite inhibition since human studies have revealed that certain vaccines appear to induce protective responses irrespective of malaria status and hence the immunosuppressive effect of malaria infection may not extend to all antigens (Greenwood *et al.*, 1972). In fact, while diminished antibody response to tetanus toxoid and to the O antigen of *S. typhimurium* were reported in children with acute malaria, their antibody response to the H antigen of *S. typhimurium* and their cellular immune responses were normal (Greenwood *et al.*, 1972). The E $\alpha$  system could therefore help us to gain a better insight in the uptake and presentation of heterologous antigens during malaria infection *in vivo* and depending on the outcome of the E $\alpha$ -specific T cell response induced by pRBC-treated DCs, it could perhaps aid in understanding why T cell responses to certain antigens are not affected during malaria. Furthermore, besides examining presentation of heterologous antigens, this system can be used to investigate presentation of parasite antigens *in vivo*. This can be achieved by developing transgenic parasites expressing E $\alpha$ -GFP (currently underway in our laboratory). The fluorescent moiety will allow tracking of parasites and antigen *in vivo*, while the expression of E $\alpha$  will help to investigate how this antigen is presented to the immune system and how T and B cells respond. Moreover, the anatomical location of uptake of E $\alpha$ -GFP-expressing parasites and presentation of E $\alpha$  by APCs can be identified by using laser-scanning cytometry (LSC) (Grierson *et al.*, 2005; Smith *et al.*, 2004) and, by using APC-specific markers, it is possible to characterise the phenotype of the APC engaged in antigen presentation as well as determining the magnitude and duration of antigen presentation during infection *in vivo*.

As mentioned earlier, the initial interactions between APCs and T cells determines the nature of the developing immune response and studies in our laboratory have shown that differences in the duration and/or frequency of these interactions underlie the outcome of priming versus tolerance (Zinselmeyer *et al.*, 2005). The movement of CD4<sup>+</sup> T cells in lymph nodes was imaged and compared during the induction of oral priming and tolerance. Primed T cells were shown to form larger and longer-lived clusters compared

to tolerised cells (Zinselmeyer *et al.*, 2005). This study was possible due to the use of multi-photon microscopy, which allows to image fluorescently labelled cells in intact tissue in real-time. The application of this technique during malaria infection is a logical extension of the present study and will enable us to investigate whether pRBC-treated DCs are capable of inducing the activation and clustering of T cells around the DCs and examine the subtle differences underlying the development of immune responses in malaria infected animals compared to controls. Gaining further knowledge of the mechanisms and requirements for the induction of immunological priming is critical to improve therapeutic strategies for the prevention and treatment of malaria and for the development of an effective malaria vaccine.

My initial experiments, which established that pRBCs do indeed inhibit DC function, were followed by studies aimed at investigating the mechanisms of suppression. Fixation of pRBCs immediately after harvesting enabled me to examine whether parasite viability and development was a necessary requirement for the observed inhibition of DC function. Isolation of pRBC-ghosts enabled me to analyse whether parasite-derived antigens expressed in the erythrocyte membrane played an important role in modulating DC function. Finally purification of haemozoin, a byproduct of haemoglobin digestion and the use of a transwell system enabled me to analyse the involvement of haemozoin and soluble factors potentially secreted by pRBCs in the suppression of the LPS-induced activation of DCs *in vitro*. While neither parasite growth or parasite-derived antigens expressed in the erythrocyte membrane are responsible for the observed modulation of DC maturation, haemozoin, and soluble factors secreted by pRBCs, contribute significantly to the immunosuppressive effects induced in DCs *in vitro*. A deeper understanding of the mechanisms employed by haemozoin to induce its effects is a necessary step for finding ways to induce haemozoin degradation, in an attempt to rectify the functional impairment of DCs and should therefore be the focus of future studies. Similarly, identification of parasite-derived soluble factors and investigation of their mechanism of action would be worthwhile. In this regard, treatment of pRBC-derived supernatants with proteases and lipases would provide a first step in their identification. This could be followed by purification and identification of potential proteins and/or

lipids. For instance, proteins may be purified by 2-D gel electrophoresis and either enzymatically or chemically cleaved (Gevaert and Vandekerckhove, 2000). The obtained peptide mixtures can then be analysed by mass spectrometric techniques, which will retrieve possible candidate proteins (Gevaert and Vandekerckhove, 2000). Similarly, analytical technologies have been developed for the detection and quantification of lipid species. Lipid extracts can be separated on a chromatographic column and eluted species identified and quantified by on-line mass spectroscopy (Hermansson *et al.*, 2005).

Although haemozoin and parasite-secreted factors were found to suppress DC function, I analysed potential mechanisms used by the parasite to exert these effects on DCs. I therefore investigated whether interaction with pRBCs would induce DCs to secrete immunosuppressive factors, which could potentially act in an autocrine manner and affect the function of the secreting cell itself or in a paracrine fashion and suppress the function of bystander cells (chapter 6). Since none of the most common immunoregulatory molecules selected for this study (NO, TGF- $\beta$ , PGE<sub>2</sub> acting through the EP4 receptor, PPAR- $\gamma$  and IDO) seemed to be involved in the suppression of DC activation, gene expression analysis seemed the most appropriate approach to gain an overall view of the molecular events involved during DC-pRBC interaction. In this regard, microarray analysis of pRBC-treated DCs shed light on some possible molecules that might play a role in the observed suppression of DC function, such as TSP-1 and HO-1 (chapter 7). Future work using enzyme inhibitors/blocking antibodies/receptor blockers should be carried out to validate this information. Furthermore, although genomics studies may provide important information on the expression of genes encoding proteins that might be involved in the modulation of DC function, protein production cannot be fully characterised by gene expression analysis alone. Alternative splicing and post-translational modification of proteins give rise to a large increase in protein diversity resulting in fewer protein-coding genes than there are proteins. For these reasons, proteomics (i.e. the large scale study of proteins) is a useful tool and would therefore be the 'next step' following genomic analysis. The 2-D Fluorescence Difference Gel Electrophoresis (DIGE) system allows the separation of up to three protein samples on a single 2-D gel. Protein samples are labeled with different fluorescent dyes, mixed and run

on the same gel, which is then viewed by fluorescence at different wavelengths, much like a microarray (Westermeier and Marouga, 2005). This method leads to highly accurate qualitative and quantitative results as gel-to-gel variations are eliminated (Westermeier and Marouga, 2005).

The identification of suppressive agents secreted either by the parasites or the DCs themselves and understanding their mechanism of action could have important implications for vaccine design and immunotherapy and might aid in the development of effective DC-based vaccines against malaria. Furthermore, these suppressive agents may have broader applications as potential therapeutic molecules in settings where immunosuppression would be clinically beneficial, such as in the case of autoimmune diseases. For instance, it would be interesting to analyse the effects of haemozoin administration on diseases like rheumatoid arthritis. In this regard, experiments similar to those carried out using ES-62 on the collagen induced arthritis model (McInnes *et al.*, 2003) would be ideal for examining its therapeutic potential.

Similar to haemozoin, ES-62, a glycoprotein secreted by the rodent filarial nematode *Acanthocheilonema viteae*, was shown to exhibit broad immunomodulatory activities *in vitro* that were in general anti-inflammatory (Harnett *et al.*, 2003). In fact, ES-62 reduced antigen-driven B and T lymphocyte proliferation, inhibited the ability of macrophages to produce Th1/proinflammatory cytokines such as IL-12, TNF- $\alpha$ , and IL-6, modulated DC maturation to preferentially elicit Th2-polarised responses, and induced spleen cells to produce IL-10 (Harnett and Harnett, 2001). Filarial nematode infection was also shown in some cases to modulate the human immune response to heterologous antigens, including vaccines (Stewart *et al.*, 1999). *In vitro* and *in vivo* studies using the collagen-induced arthritis model clearly demonstrated that ES-62 could potently suppress inflammation (McInnes *et al.*, 2003), providing powerful proof of the concept that molecular characterization of the host-parasite relationship offers therapeutic potential in the context of autoimmune diseases.

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Research article

## Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function

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### Abstract

**Background:** Dendritic cells (DCs) are central to the initiation and regulation of the adaptive immune response during infection. Modulation of DC function may therefore allow evasion of the immune system by pathogens. Significant depression of the host's systemic immune response to both concurrent infections and heterologous vaccines has been observed during malaria infection, but the mechanisms underlying this immune hyporesponsiveness are controversial.

**Results:** Here, we demonstrate that the blood stages of malaria infection induce a failure of DC function *in vitro* and *in vivo*, causing suboptimal activation of T cells involved in heterologous immune responses. This effect on T-cell activation can be transferred to uninfected recipients by DCs isolated from infected mice. Significantly, T cells activated by these DCs subsequently lack effector function, as demonstrated by a failure to migrate to lymphoid-organ follicles, resulting in an absence of B-cell responses to heterologous antigens. Fractionation studies show that hemozoin, rather than infected erythrocyte (red blood cell) membranes, reproduces the effect of intact infected red blood cells on DCs. Furthermore, hemozoin-containing DCs could be identified in T-cell areas of the spleen *in vivo*.

**Conclusions:** *Plasmodium* infection inhibits the induction of adaptive immunity to heterologous antigens by modulating DC function, providing a potential explanation for epidemiological studies linking endemic malaria with secondary infections and reduced vaccine efficacy.

## Background

Malaria is the major parasitic disease of humans throughout the tropics and subtropics, mainly affecting children under 5 years of age and causing 500 million clinical cases and up to 2.7 million deaths each year [1]. In addition to infection-induced mortality, malaria is also associated with public-health problems resulting from impairment of immune responses. Although this immunosuppression may have evolved as a mechanism by which the parasite can prevent immune-mediated clearance [2-8], it leaves malaria-infected individuals or experimental animals more susceptible to secondary infections, such as non-typhoidal *Salmonella* [9], herpes zoster virus [10], hepatitis B virus [11], Moloney leukemia virus [12] and nematode infection [13], as well as Epstein-Barr virus reactivation [14-17]. Because the efficacy of heterologous vaccines can also be suppressed in malaria-infected patients [18-21], children showing clinical signs of malaria are rarely immunized until after anti-malarial chemoprophylaxis, which can improve the response to vaccination [22]. In a recent study of a new conjugate vaccine against pneumococci, efficacy was reduced during the malaria transmission season [23], demonstrating the possible impact of malaria infection on large-scale vaccine regimes. Certain vaccines, however, seem to induce protective responses irrespective of malaria status and the immunosuppressive effect of malaria infection might thus not extend to all antigens [20]; studies *in vivo* are required to investigate this controversy further. Several animal studies have described suppression of immune function by *Plasmodium* parasites *in vitro* and *in vivo* [24-34], but the mechanisms involved remain unclear.

Dendritic cells (DCs) have a crucial role in the activation of T cells and consequently in the induction of adaptive immune responses and immunity [35,36]. There is evidence that many pathogens have evolved mechanisms that subvert DC function, thereby modulating the host's immune response to their advantage [37,38]. Recent studies have revealed that DCs are important in malaria infection, particularly during the early events of induction of the protective immune response to infection [39,40]. It has been reported that red blood cells (RBCs) infected with schizont-stage *Plasmodium falciparum* activate plasmacytoid DCs as

detected by increased expression of the antigen CD86 and the cytokine interferon- $\alpha$  (IFN- $\alpha$ ) *in vitro* [41]. In contrast, the asexual erythrocytic stages of *P. falciparum* were shown to impair the ability of human DCs to undergo maturation *in vitro* [42]. Indeed, peripheral blood DCs of *P. falciparum*-infected children showed reduced levels of the major histocompatibility complex (MHC) molecule HLA-DR compared with uninfected controls [43], suggesting a reduced activation state. Thus, the ability of malaria parasites to inhibit maturation of DCs could be involved not only in parasite-specific immunosuppression but also in the suppression of responses to heterologous antigens such as vaccines and unrelated pathogens [2,19,20]. As human malaria parasites are host-specific, however, observations on the effect of human malaria on DCs are largely limited to studies *in vitro*.

Here, we describe the mechanism underlying this suppression of immunity *in vitro* and *in vivo*. DC activation is dynamically altered by parasitized erythrocytes (pRBCs), partly because of deposition of the malarial pigment hemozoin (HZ) within these cells. Following presentation of heterologous antigen by pRBC-exposed DCs, there is less expansion of CD4<sup>+</sup> 'helper' T cells that are essential for the induction of adaptive immunity. Subsequently, migration of T cells to lymphoid follicles is abrogated, leading to defective B-cell expansion and differentiation and a failure of the antibody response. These studies explain why immunity to malaria is slow to develop and why protection against secondary infections is reduced in *Plasmodium*-infected individuals.

## Results

### Suppression of heterologous immune responses during malaria infection

We first examined the response to a heterologous antigen during *Plasmodium chabaudi* (AS strain) infection (Figure 1a) to determine whether this murine model reflected the clinical immunosuppression observed with *P. falciparum* infection [18-21]. Mice were immunized with the model antigen ovalbumin (OVA) and lipopolysaccharide (LPS) to act as adjuvant at various times after infection, and OVA-specific serum immunoglobulin G (IgG) was measured 21 days later.

#### Figure 1 (see figure on the following page)

Suppression of immunity by *P. chabaudi* infection. (a) BALB/c mice were infected with  $10^6$  *P. chabaudi* (AS strain) parasites by intra-peritoneal injection and the proportion of peripheral blood cells parasitized (parasitemia) was monitored by Giemsa's stain of peripheral blood smears. (b) Uninfected (squares) or *P. chabaudi*-infected (circles) BALB/c mice were immunized with OVA/LPS at the indicated times after infection. Three weeks after immunization, sera were analyzed for OVA-specific IgG. Data represent the mean of three mice per group  $\pm$  1 standard deviation (s.d.) and are representative of two similar experiments (\* $p \leq 0.05$ , \*\* $p \leq 0.005$  uninfected versus *P. chabaudi*-infected). (c) Spleen cells from uninfected (open bars) or *P. chabaudi*-infected (filled bars) BALB/c mice immunized with OVA/LPS 10 days post-infection were re-stimulated *in vitro* as indicated and supernatants assayed for levels of IFN- $\gamma$  (left) and IL-5 (right) after 48 h.

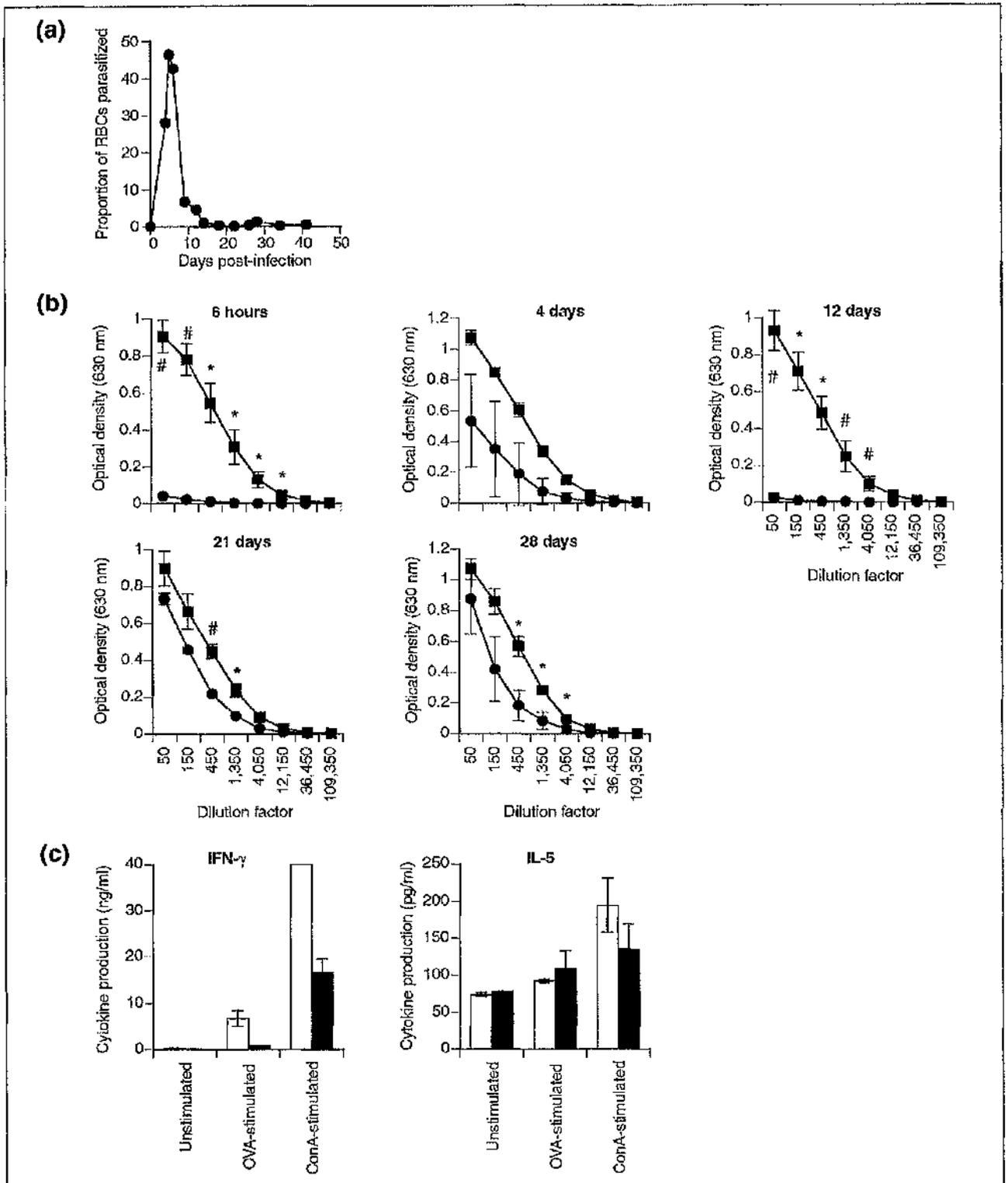


Figure 1 (see legend on the previous page)

Uninfected, immunized animals generated OVA-specific IgG responses, but OVA and LPS administered 6 hours and 12 days after infection with *P. chabaudi* produced significantly reduced levels of IgG (Figure 1b). Interestingly, suppression was lower in mice immunized during increasing levels of parasite infection (parasitemia; Figure 1b, 4 days). By 21 days post-infection, infected animals had regained immune responsiveness and mounted antibody responses of a similar magnitude to those seen in uninfected controls (Figure 1b, 21 and 28 days). Production of OVA-stimulated and concanavalin A (ConA) mitogen-stimulated T-cell cytokines was reduced in cultures of splenocytes taken from mice infected with *P. chabaudi* 10 days before immunization (Figure 1c). Thus, as described for *P. falciparum* in humans, *P. chabaudi* infection in mice induces suppression of immune responses, although these studies reveal that, in the animal model at least, this is a highly dynamic phenomenon.

#### Modulation of DCs *in vitro* by infected erythrocytes

DC activation is central to induction of adaptive immunity [35], and previous studies have suggested that several protozoan pathogens have evolved mechanisms to suppress this response and consequently to reduce immune-mediated protection [44]. Human DCs cultured with *P. falciparum*-infected erythrocytes are hyporesponsive to stimulation with LPS and less capable of stimulating CD4<sup>+</sup> T-cell responses [42]. This observation remains controversial, however, as studies using murine models have suggested that DCs may be activated during increasing parasitemia *in vivo* [45] and following culture *in vitro* with parasite schizont-infected erythrocytes [40].

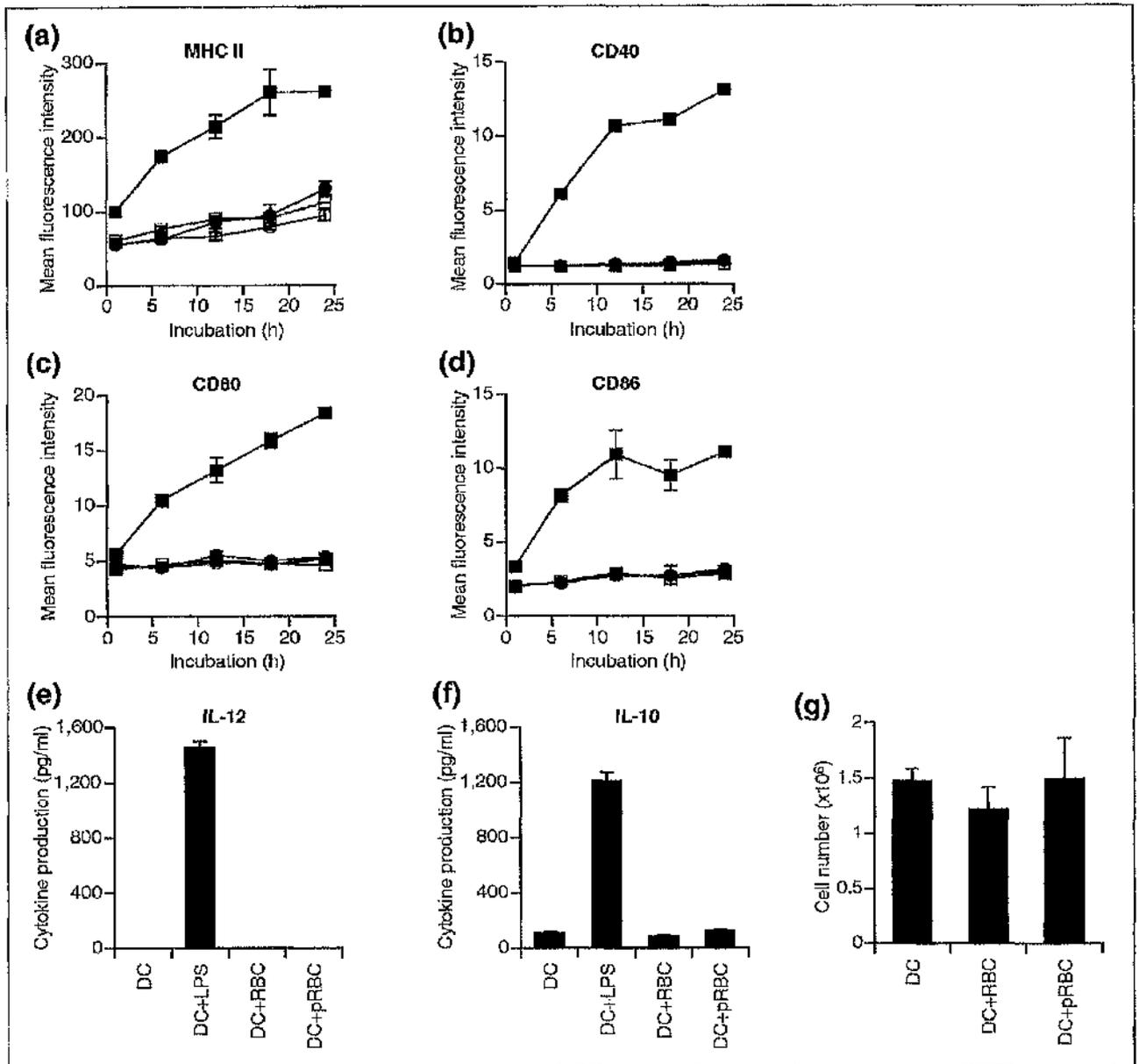
As the results above indicated that immune responsiveness *in vivo* is dynamically regulated during infection, we studied the ability of *P. chabaudi*-infected erythrocytes to modulate DCs directly, by examining the expression of MHC class II and co-stimulatory molecules on DCs. Bone-marrow-derived DCs were incubated with infected erythrocytes and the expression of surface markers examined at various times over the following 24 hours. Our results show that DCs expressed very low levels of surface MHC class II and the co-stimulatory molecules CD40, CD80 and CD86 when cultured in growth medium alone, thus confirming the immature state of these DCs in culture (Figure 2a-d). Stimulation with LPS promoted a significant increase in the expression level of all co-stimulatory molecules within 6 hours. DCs incubated with RBCs or pRBCs did not, however, increase expression of MHC class II, CD40, CD80 and CD86, indicating that malaria parasites do not induce DC activation directly. Analysis of cytokine production showed that DCs exposed to RBC or pRBCs produce small, though detectable, amounts of both interleukins IL-12 and IL-10 that are a thousand-fold lower than those observed

after LPS stimulation (Figure 2e,f), suggesting that the presence of parasites does not result in DC activation. The viability of treated DCs and control cells was quantified after 24 hours of culture by trypan blue exclusion (Figure 2g) and propidium iodide (PI) and annexin V staining (data not shown) and was not significantly affected by pRBCs.

Having established that pRBCs at a ratio of 100:1 do not directly induce DC maturation, we examined whether pRBC-treated DCs retained their ability to mature in response to LPS treatment *in vitro*. DCs were exposed to RBCs or pRBCs for 24 hours and subsequently challenged with LPS. After 18 hours of LPS stimulation, the expression levels of MHC class II, CD40, CD80 and CD86 increased significantly (Figure 3a-d). DCs pre-incubated with pRBCs and subsequently challenged with LPS, however, showed significantly lower levels of expression of MHC class II, CD40, and CD86 compared with those observed when cells were not treated with pRBCs or were pre-incubated with RBCs before the LPS challenge (Figure 3a-d). Kinetic studies demonstrated that the ability of pRBCs to induce this hyporesponsive state in DCs required at least 6 hours pre-incubation before the addition of LPS (data not shown). Cytokine production following treatment with LPS showed that, although DCs treated with pRBCs could still produce appreciable levels of IL-12 and IL-10 in response to LPS, the amount produced was significantly lower than that produced by DCs pre-incubated with RBCs (Figure 3e,f). As the interaction between CD40 on DCs and its ligand CD40L on T cells *in vivo* is known to be crucial in the production of bioactive IL-12 and upregulation of adhesion and co-stimulatory molecules [46,47], we stimulated bone-marrow-derived DCs with CD40L-transfected fibroblasts (Figure 3g,h). DCs treated with RBCs significantly upregulated CD40 expression in response to CD40L and produced high levels of the inducible IL-12p40 subunit. CD40 ligation, however, did not rescue the reduced maturation of DCs treated with pRBCs, although, as previously observed with the LPS treatment, these cells still produced IL-12 p40 but to a lesser extent than the control groups.

#### Modulation of DCs *in vivo* during malaria infection

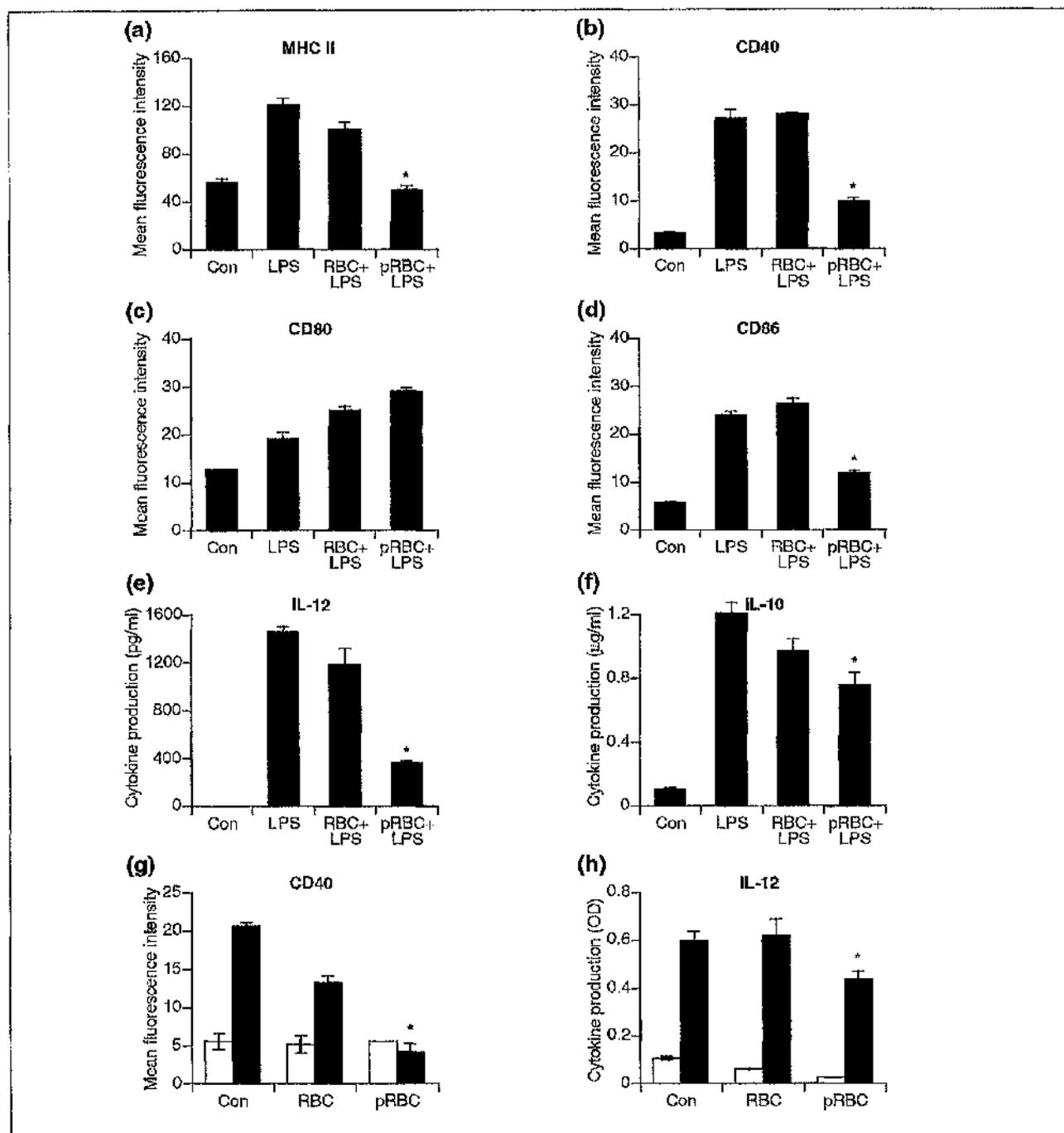
As our results suggested that malaria-infected erythrocytes might modulate the responsiveness of DCs *in vitro*, we next investigated the activation status of splenic DCs *in vivo* during a time-course of infection with *P. chabaudi*. DCs isolated from spleens of mice 4 days after infection showed a moderately activated phenotype, as demonstrated by increased expression of CD40 and CD80 (Figure 4a), confirming previous reports [45]. DCs isolated from infected animals 12 and 20 days after infection, however, showed a reduced level of activation, with lower levels of CD40, CD80, CD86 and MHC class II molecules on their surface



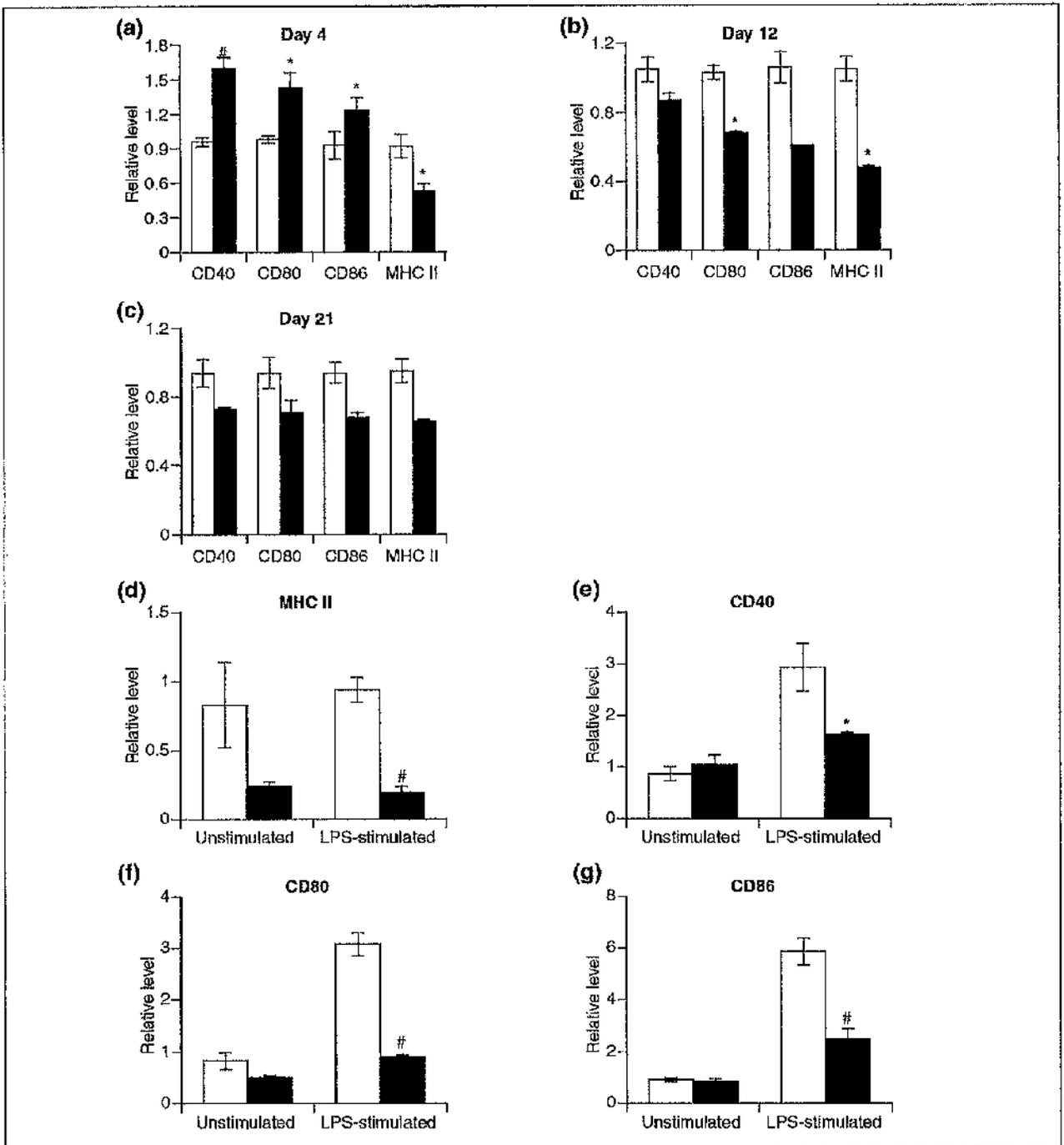
**Figure 2**  
*P. chabaudi*-infected erythrocytes do not activate DCs directly *in vitro*. DCs ( $2 \times 10^6$ ) were cultured with  $2 \times 10^8$  infected erythrocytes (DC+pRBC; filled circles) or with an equal number of uninfected erythrocytes (DC+RBC; empty circles). Control DCs remained unstimulated (DC; empty squares) or were stimulated with  $1 \mu\text{g/ml}$  of LPS (DC+LPS; filled squares). Results show the mean fluorescence intensity of (a) MHC class II, (b) CD40, (c) CD80, and (d) CD86, as determined by FACS analysis of gated CD11c<sup>+</sup> cells at various times of co-culture. Supernatants were analyzed for concentrations of (e) IL-12 (p40 subunit) and (f) IL-10 secreted by DCs after 24 h of incubation. (g) The number of viable DCs was determined by trypan blue exclusion. Results show the mean value  $\pm$  standard error (s.e.) of triplicate samples per group.

compared with DCs from uninfected animals (Figure 4b,c). Whereas DCs from uninfected mice upregulated CD40, CD80 and CD86 following LPS stimulation (Figure 4d-g), DCs isolated from the spleens of *P. chabaudi*-infected mice

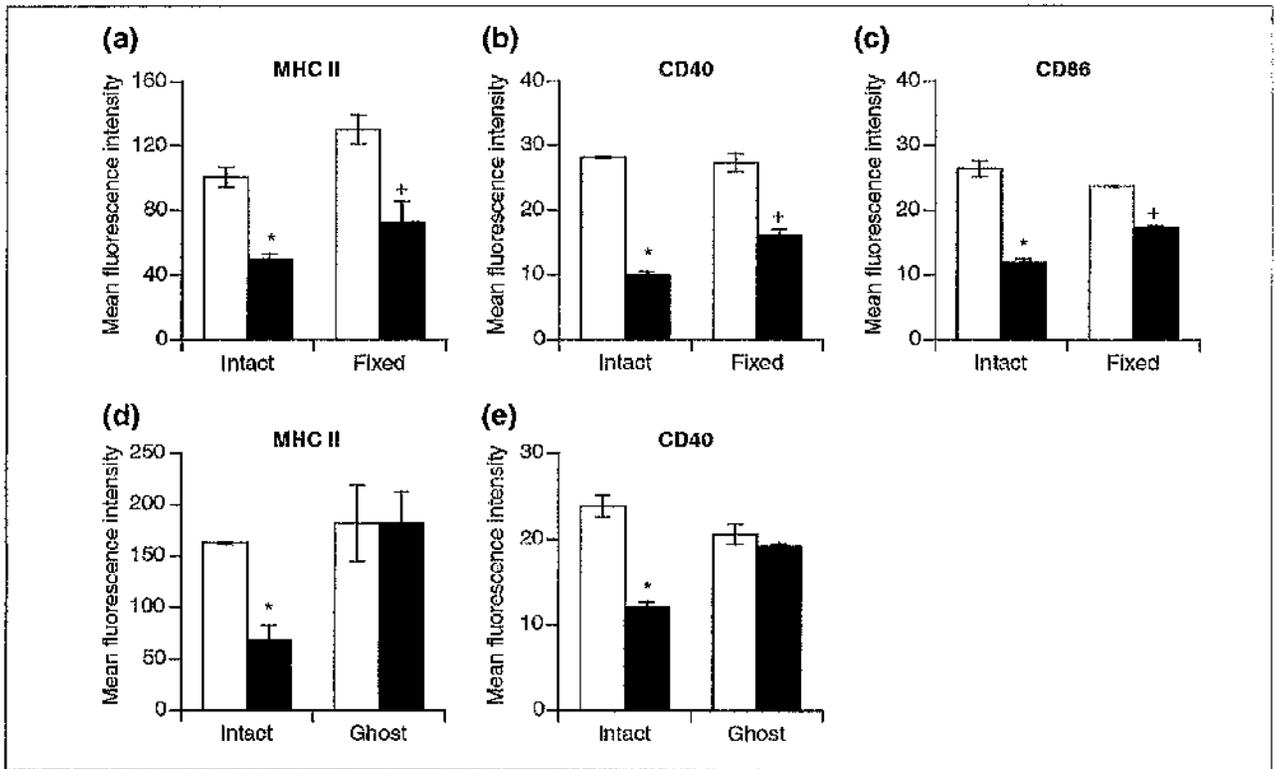
remained refractory to *in vitro* LPS-induced maturation, with reduced levels of these molecules following stimulation. Thus it seems that, *in vivo*, DCs are activated soon after infection, and the level of activation on DCs is reduced

**Figure 3**

*P. chabaudi*-infected erythrocytes inhibit the LPS and CD40L-induced maturation of DCs. DCs were cultured with infected (pRBC) or uninfected (RBC) erythrocytes for 24 h, as described in Figure 2, then treated with 1 µg/ml of LPS for a further 18 h. (a-f) Results were analyzed as described in Figure 2 (\* $p \leq 0.05$  pRBC versus RBC). (g,h) DCs ( $1 \times 10^6$ ) were cultured with  $1 \times 10^8$  infected erythrocytes (pRBC) before stimulation with CD40L-expressing fibroblasts (filled bars) or control fibroblasts (open bars) at a 1:1 ratio of fibroblasts:DCs. Untreated DCs (Con) and uninfected erythrocyte-treated DCs (RBC) were used as controls. DCs were incubated with fibroblasts for a further 18 h before analyzing CD40 expression and cytokine production. CD40 expression on DCs is shown as the mean fluorescent intensity on CD11c<sup>+</sup> cells. IL-12 production is shown as the mean optical density (OD) read at 450 nm. Results show the mean value  $\pm$  s.e. of triplicate samples per group (\* $p \leq 0.05$  pRBC versus RBC).



**Figure 4** Modulation of DC activation *in vivo* during *P. chabaudi* infection. (a-c) Splenic CD11c<sup>+</sup> DCs were isolated from *P. chabaudi* infected mice at various times (filled bars) or from uninfected controls (open bars) and analyzed by flow cytometry for the indicated markers. Data are expressed as mean fluorescence intensity relative to uninfected samples  $\pm$  1 s.d. (\* $p \leq 0.05$  uninfected versus *P. chabaudi*-infected). (d-g) Splenic DCs from uninfected (open bars) or *P. chabaudi*-infected (filled bars; 12 days post-infection) BALB/c mice were restimulated *in vitro* with LPS (1  $\mu$ g/ml) for 18 h before analysis by flow cytometry for (d) MHC class II, (e) CD40, (f) CD80 and (g) CD86. Data are expressed as mean fluorescence intensity relative to uninfected, unstimulated samples  $\pm$  1 s.d. (\* $p \leq 0.05$ , # $p \leq 0.005$  uninfected versus *P. chabaudi*-infected for the same treatment).

**Figure 5**

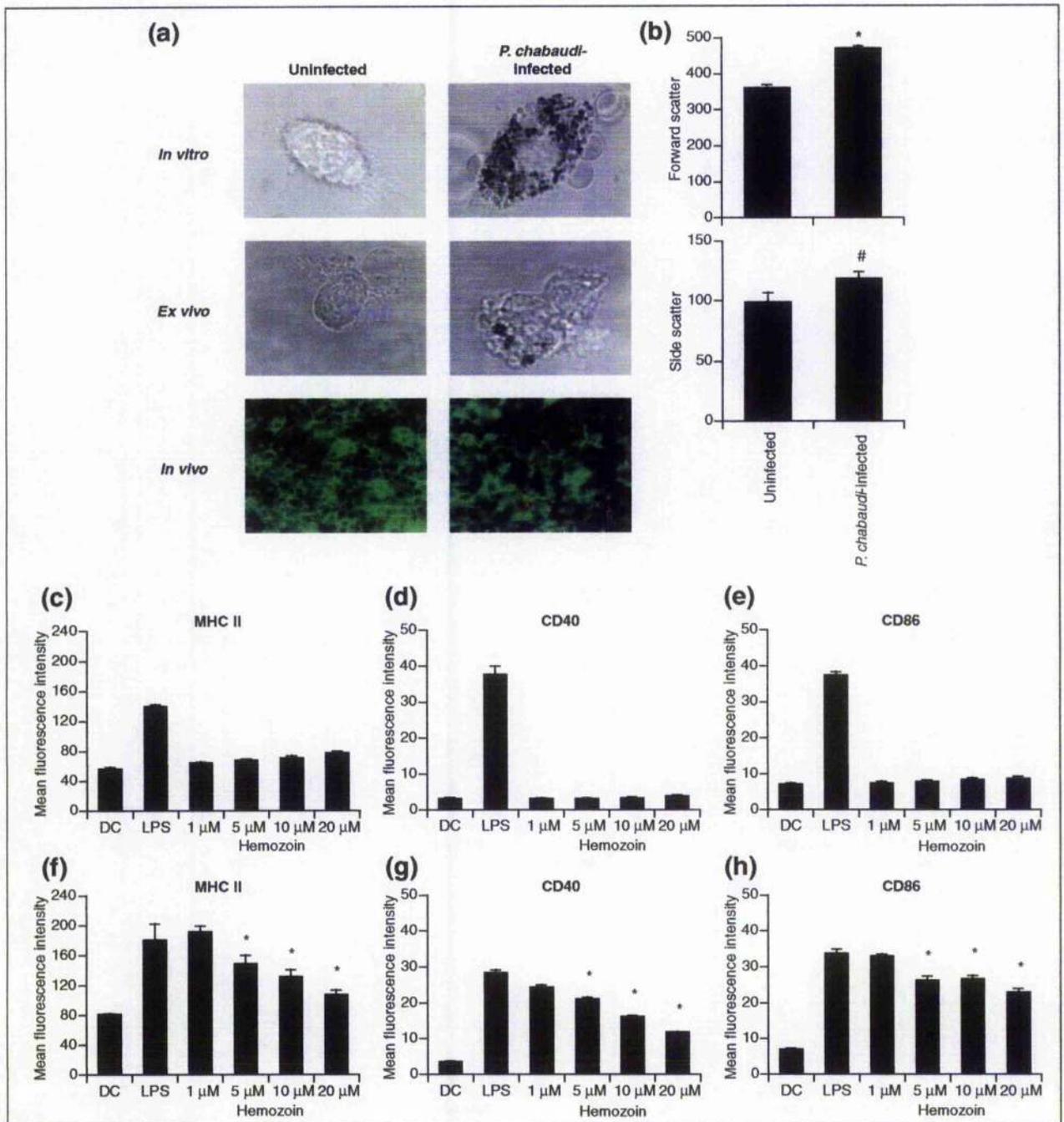
Identification of *P. chabaudi* components that inhibit LPS-induced DC maturation. (a-c) DCs ( $2 \times 10^6$ ) were treated for 24 h with uninfected RBC (open bars) or infected RBC (pRBC; filled bars) that were untreated or fixed before stimulation with LPS (1  $\mu\text{g}/\text{ml}$ ) for 18 h. DC activation was characterized by analysis of (a) MHC class II, (b) CD40, and (c) CD86 expression on CD11c<sup>+</sup> DC surfaces by FACS (\* $p \leq 0.05$  pRBC versus RBC, + $p \leq 0.05$  fixed pRBC versus fixed RBC). (d,e) DCs ( $5 \times 10^5$ ) were incubated with Intact erythrocytes or RBC ghosts from uninfected (open bars) or infected erythrocytes (filled bars), for 24 h before stimulation with LPS for 18 h. DC activation was characterized by analysis of (d) MHC class II and (e) CD40 expression on the DC surface by FACS (\* $p \leq 0.05$  pRBC versus RBC).

following the peak of infection (days 12-20), and this cannot be abrogated by microbial stimulation *ex vivo*.

#### Identification of *P. chabaudi* components that induce DC hyporesponsiveness

As we had demonstrated that malaria infection modulates key aspects of DC function, we wanted to examine the possible mechanisms involved. Initially, to determine whether maturation of the parasite from the trophozoite stage to the schizont stage *in vitro* or some metabolic process within the pRBCs was required for induction of DC hyporesponsiveness, pRBCs were fixed with paraformaldehyde and incubated with DCs for 24 hours before the addition of LPS (Figure 5a-c). The expression levels of MHC class II, CD40 and CD86 were significantly reduced when DCs were co-cultured with fixed pRBCs before LPS challenge, confirming that trophozoite-infected erythrocytes downregulate DC activation in response to LPS treatment and suggesting that

growth and maturation of the parasite into schizonts is not required for suppression. In an attempt to understand the mechanisms involved in the parasite-mediated modulation of DC function, we investigated the role of selected parasite components on the LPS-induced maturation of DCs. We addressed this question initially by analyzing the effect that parasite proteins expressed on the erythrocyte surface membrane have on DC function. DCs were exposed to RBC membranes (ghosts) isolated from infected or uninfected erythrocytes. After 24 hours of culture, DCs were challenged with LPS and the expression of co-stimulatory molecules analyzed 18 hours later (Figure 5d,e). Our results clearly show that ghosts isolated from pRBCs did not alter the ability of DCs to respond to LPS treatment *in vitro*, as observed by the levels of MHC class II and CD40, demonstrating that proteins expressed on the surface membranes of pRBCs are apparently not essential for the modulation of DC function.



**Figure 6**  
 Deposition of HZ in DCs suppresses maturation. **(a)** HZ content in bone-marrow-derived DCs (*In vitro*), purified DCs (*Ex vivo*) and spleen sections (*In vivo*) was visualized by light microscopy (top images) or by false-coloring malaria pigment viewed in bright-field image (red) and superimposing over the fluorescent CD11c image (green). **(b)** CD11c<sup>+</sup> DCs were analyzed for size and granularity by flow cytometry 12 days post-infection with *P. chabaudi* or in uninfected controls. Data are expressed as the mean forward scatter or side scatter of triplicate samples ± s.d. (\**p* ≤ 0.05, #*p* ≤ 0.005 uninfected versus *P. chabaudi*-infected). **(c-e)** 2 × 10<sup>6</sup> DCs were cultured with 1 μM, 5 μM, 10 μM and 20 μM of HZ. After 24 h, the level of expression of **(c)** MHC class II, **(d)** CD40 and **(e)** CD86 on CD11c<sup>+</sup> cells was determined by FACS analysis. **(f-h)** After 24 h culture with HZ, 1 μg/ml LPS was added to DCs and the levels of **(f)** MHC class II, **(g)** CD40, and **(h)** CD86 were analyzed 18 h later by FACS. All results are shown as the mean fluorescence intensity on CD11c<sup>+</sup> DCs in triplicate samples ± s.e. (\**p* ≤ 0.05 HZ versus LPS).

Having established that parasite proteins expressed on the erythrocyte cell membrane are not responsible for the modulation of LPS-induced maturation of DCs *in vitro*, we focused our attention on HZ, a by-product of hemoglobin digestion. We observed that bone-marrow-derived DCs cultured *in vitro* with infected erythrocytes accumulated intracellular malarial pigment (Figure 6a). Flow cytometric analysis of splenic DCs, identified by CD11c expression, also demonstrated an increase in the size and granularity of DCs during infection (Figure 6b), and DCs isolated *ex vivo* as well as DCs in spleen sections showed conspicuous HZ deposition (Figure 6a).

In order to assess the role of HZ in the parasite-induced modulation of DC function, we initially analyzed its ability to activate DCs directly *in vitro* (Figure 6c-e). Even at the highest dose (20  $\mu$ M), HZ did not induce DC maturation, as the levels of MHC class II, CD40 and CD86 were the same as the levels expressed by untreated controls. We then examined whether HZ-treated DCs still responded to LPS treatment *in vitro* (Figure 6f-h). Our data clearly demonstrate a dose-dependent inhibition of the LPS-induced maturation of DCs by HZ, as seen by the reduced levels of MHC class II, CD40 and CD86. Taken together, these results indicate that HZ, rather than pRBC membranes, is a key factor involved in the suppression of murine DC function *in vitro* and *in vivo*.

#### Failure of pRBC-treated DCs to induce T-cell effector function

The above experiments clearly implicated HZ-mediated suppression of DC function in the failure of antibody production and in the delayed acquisition of protective immunity seen during malaria infection *in vivo*. To examine the functional consequences of malaria on DC function directly, we examined the ability of affected DCs to activate naive, OVA-specific T-cell receptor-transgenic T cells. These cells allow monitoring of the antigen-specific CD4<sup>+</sup> T-cell response as their antigen specificity is known and they can be tracked using a clonotypic antibody directed against their T-cell receptor. One of the earliest cell-surface antigens expressed by T cells following activation is CD69, which is detectable within an hour of ligation of the T-cell receptor complex [48]. Interestingly, there was no significant difference in the percentage of OVA-specific T cells expressing CD69 between RBC-treated and pRBC-treated groups (Figure 7a), showing that T cells interacting with modulated DCs are equally activated.

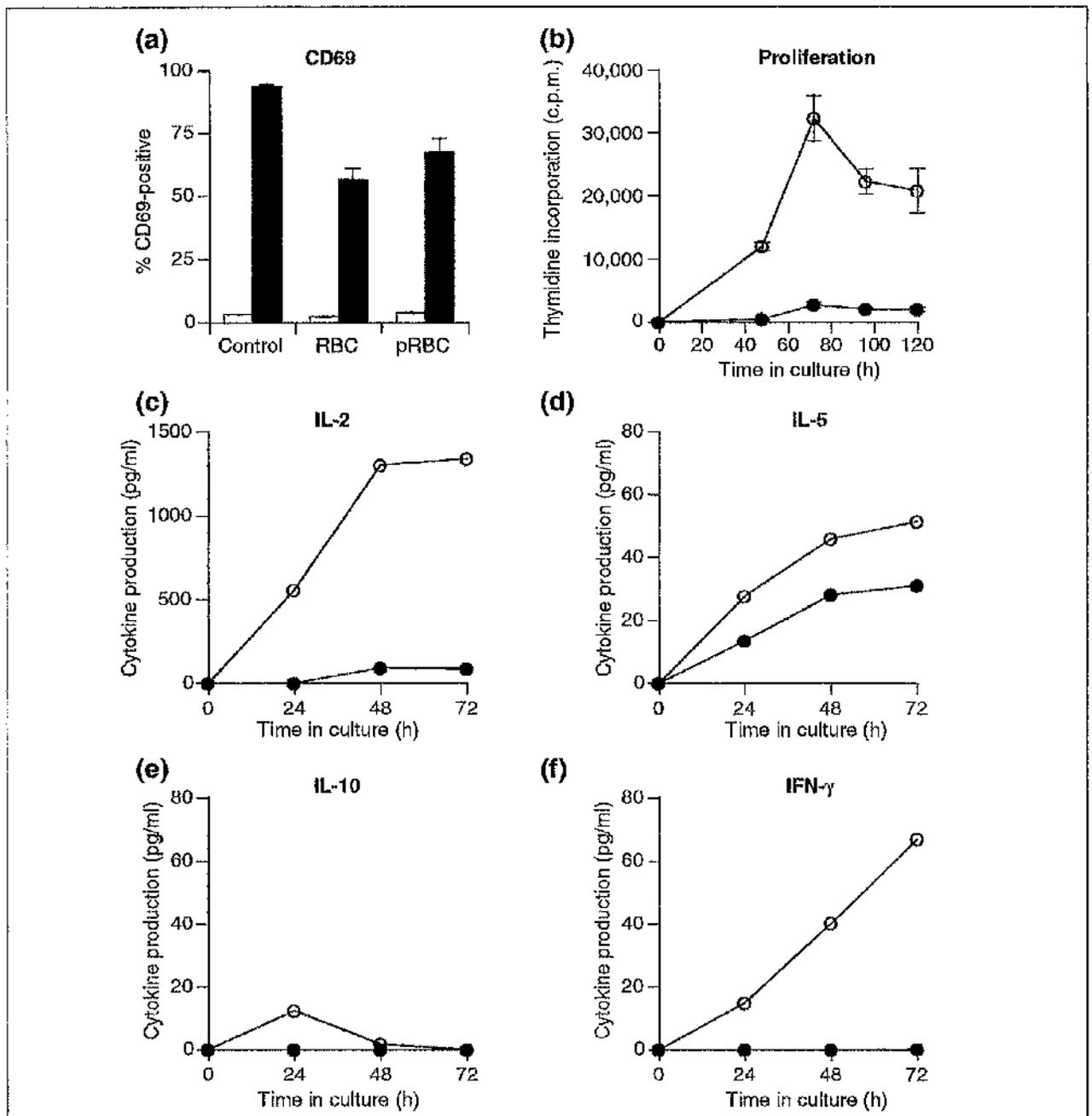
To investigate whether treatment of DCs with pRBCs could alter the dynamics of the T-cell proliferative response, we harvested the T cells at 48, 72, 96 and 120 hours of culture (Figure 7b). The ability of pRBC-treated DCs to induce T-cell proliferation was dramatically reduced compared to

the control group throughout the observation period (see Figure 7b). Analysis of T-cell production of IL-2, IL-5, IL-10 and IFN- $\gamma$  (Figure 7c-f) revealed that each cytokine was downregulated in the pRBC-treated groups compared with RBC-treated controls. The observed reduction in T-cell proliferation and cytokine production could not be explained by T-cell death, as similar levels of necrotic and apoptotic T cells were detected in both conditions by FACS analysis of propidium iodide and annexin staining (data not shown). Thus, although DCs pre-treated with pRBCs *in vitro* can induce initial activation of naive CD4<sup>+</sup> T cells, causing upregulation of CD69, these T cells fail to proliferate effectively and have a reduced ability to secrete effector cytokines.

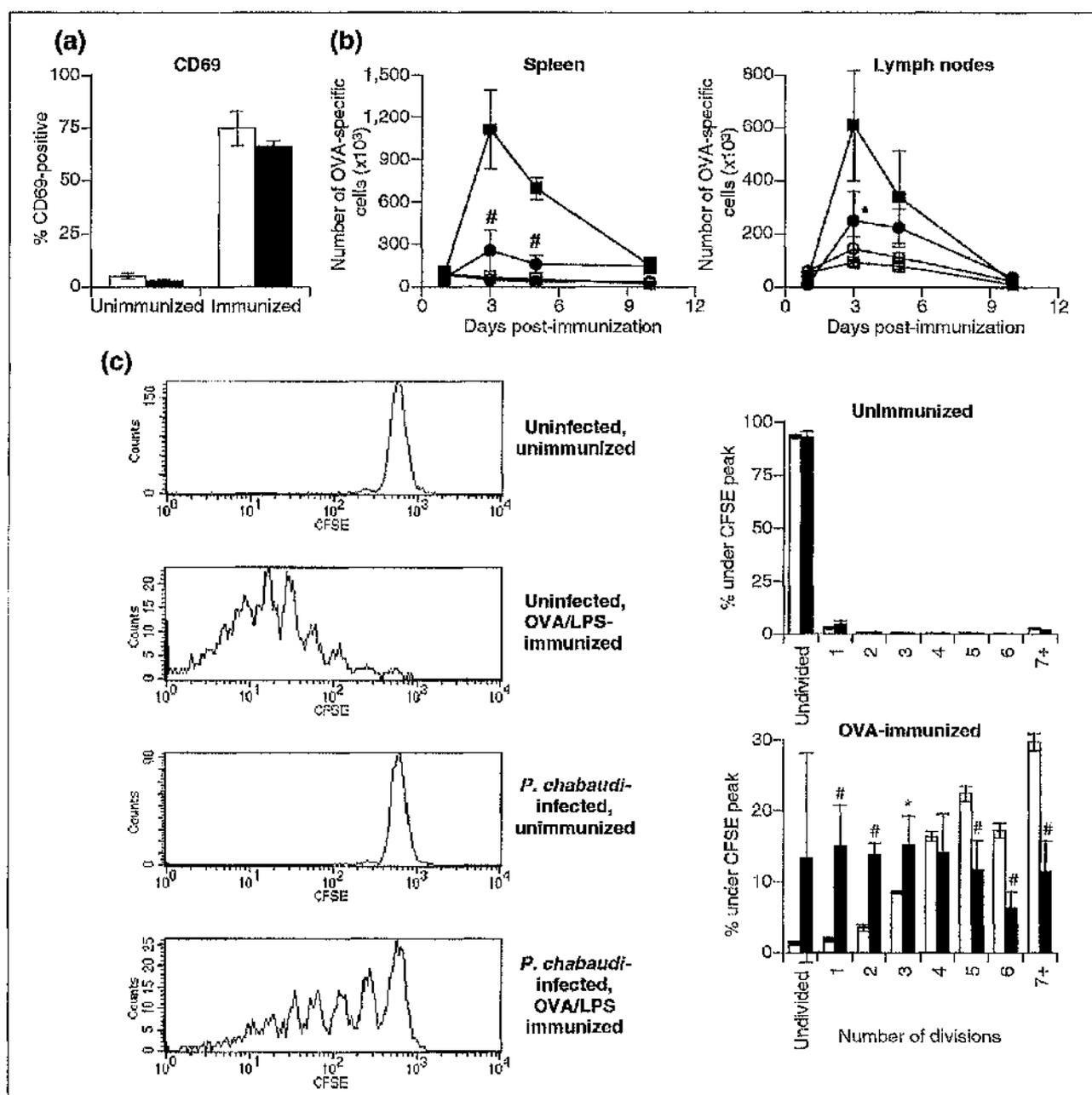
#### Suppression of T- and B-cell proliferation during malaria infection

To characterize fully the downstream effects of HZ-induced DC hyporesponsiveness on heterologous immune responsiveness, we directly investigated antigen-specific T-cell responses *in vivo* by transferring traceable OVA-specific CD4<sup>+</sup> T cells into recipient mice [49]. This allows us to follow the response of a small, but detectable, number of antigen-specific CD4<sup>+</sup> T cells during the induction of an adaptive immune response. Cells were transferred into recipients following the initial peak of parasitemia (day 12 of infection) and immunized 1 day later. In *P. chabaudi*-infected immunized mice, OVA-specific CD4<sup>+</sup> T cells underwent a similar initial activation to that of antigen-specific T cells in uninfected mice, as estimated by upregulation of CD69 (Figure 8a) and increased size/blastogenesis (also an indicator of activation; data not shown), confirming the *in vitro* observation (see Figure 7a). Antigen-specific CD4<sup>+</sup> T cells in lymph nodes and in spleen failed to expand to the same extent in infected mice as in uninfected controls (Figure 8b), however, partly because the antigen-specific CD4<sup>+</sup> T cells made a reduced number of divisions (Figure 8c). Interestingly, we did not detect increased apoptosis (determined by annexin staining) of OVA-specific T cells transferred into malaria-infected individuals (data not shown).

One of the most significant components of CD4<sup>+</sup> T-cell effector function is migration into primary lymphoid follicles to interact with, and provide help for, antigen-specific B cells [50]. To track the effect of malaria infection on these populations, we transferred B-cell-receptor transgenic B cells specific for hen egg-white lysozyme (HEL) taken from the MD4 transgenic mouse, together with the OVA-specific DO11.10 T cells and immunized with OVA coupled to HEL [51]. B-cell expansion in uninfected animals peaked 5 days after immunization (Figure 9a). Expansion of HEL-specific B cells was almost completely ablated in *P. chabaudi* infected animals immunized with OVA-HEL/LPS (Figure 9a), however, suggesting a defect in B-cell activation and/or T-cell help in infected mice.



**Figure 7**  
*P. chabaudi*-infected erythrocytes inhibit the ability of DCs to efficiently activate naive T cells *in vitro*. DCs ( $2 \times 10^5$ ) were treated with Infected (pRBC) or uninfected (RBC) erythrocytes for 24 h. DCs were then loaded with 5 mg/ml of OVA for 6 h.  $5 \times 10^5$  untreated (Control), RBC or pRBC-treated DCs and  $5 \times 10^5$  DO11.10 lymph node cells were then co-cultured. **(a)** CD69 expression assessed on DO11.10 T cells 24 h later by FACS analysis. Results are expressed as the percentage of antigen-specific cells expressing CD69 in cultures stimulated by OVA-pulsed DCs (filled bars) or by DCs only (open bars). Results show the mean of triplicate samples  $\pm$  s.e. **(b)** [ $^3$ H]thymidine was added for the last 18 h of culture; c.p.m., counts per min. Results show mean proliferation of OVA-specific T cells after incubation with DCs treated with uninfected RBCs (open circles) or pRBCs (filled circles) in triplicate samples  $\pm$  s.e. The concentrations of **(c)** IL-2, **(d)** IL-5 **(e)** IL-10, and **(f)** IFN- $\gamma$  secreted by OVA-specific T cells after incubation with DCs treated with uninfected RBCs (open circles) or pRBCs (filled circles) were measured in supernatants harvested after 24, 48, and 72 h of culture.

**Figure 8**

Suppression of CD4<sup>+</sup> T-cell expansion by *P. chabaudi* infection in vivo. (a) Uninfected (open bars) or *P. chabaudi*-infected (filled bars) BALB/c mice received DO11.10 T cells and remained unimmunized or were immunized with OVA/LPS 12 days post-infection. Expression of CD69 on splenic OVA-specific T cells was assessed 18 h post-immunization. Results shown are the proportion of OVA-specific CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells expressing CD69 and represent the mean of three mice per group  $\pm$  1 s.d. (b) Uninfected (squares) or *P. chabaudi*-infected (circles) BALB/c mice were transferred and immunized (filled symbols) or remained unimmunized (open symbols) and the absolute number of OVA-specific T cells in spleen (left) and lymph nodes (right) calculated at various times. Results show the mean number of CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells and represent the mean of three mice per group  $\pm$  1 s.d. (\* $p \leq 0.05$ , # $p \leq 0.005$  uninfected and immunized versus *P. chabaudi*-infected and immunized). (c) Representative CFSE profiles of OVA-specific T cells are shown (left) and data shown as the mean proportion of CD4<sup>+</sup>KJ1.26<sup>+</sup> cells under each CFSE peak in uninfected (open bars) and *P. chabaudi*-infected (filled bars) following immunization (bottom histogram) or in unimmunized controls (upper histogram). Data represent the mean of 3 mice per group  $\pm$  1 s.d. (\* $p \leq 0.05$ , # $p \leq 0.005$  uninfected and immunized versus *P. chabaudi*-infected and immunized).

### Failure of heterologous antigen-specific T-cell migration during malaria infection

Optimal expansion of antigen-specific B cells requires their cognate interaction with CD4<sup>+</sup> T cells [52,53]; we therefore examined the localization of OVA-specific CD4<sup>+</sup> T cells following immunization. Five days after immunization of uninfected mice, clonal expansion of antigen-specific T cells was evident by immunohistochemistry, and these cells had begun to migrate into B-cell follicles (Figure 9b). In *P. chabaudi*-infected mice, however, not only were there reduced numbers of OVA-specific T cells, but these cells were almost completely excluded from B-cell follicles (see Figure 9b). Migration was quantified using laser-scanning cytometry [54,55]. As shown in Figure 9c, the average proportion of OVA-specific CD4<sup>+</sup> T cells in B-cell follicles was significantly reduced in *P. chabaudi*-infected mice following immunization compared with uninfected, immunized animals. This demonstrates that in infected animals, CD4<sup>+</sup> T cells fail to migrate into follicles and therefore fail to interact with antigen-specific B cells, both essential steps in the induction of protective, adaptive immunity. Importantly, these failures in T- and B-cell function are similar to situations in which immune tolerance is induced [56-58], although few studies have directly examined the interaction of T and B cells *in vivo* during the course of infections. It should be noted that, in contrast to the early inflammatory stage of the infection, normal lymphoid architecture was apparent when these defects in migration were observed, with clearly distinguishable, intact B- and T-cell areas (see Figure 9b).

To investigate whether the failure of T-cell migration into follicles was simply due to a potential alteration in lymphoid architecture or chemokine gradients caused by malaria infection, OVA-specific CD4<sup>+</sup> T cells were initially activated *in vitro* with bone-marrow-derived DCs and pRBCs and then transferred into uninfected recipient mice. Expansion of T cells stimulated *in vitro* with DCs cultured with infected RBCs was reduced following transfer compared with expansion of OVA-specific T cells activated in the presence of uninfected erythrocytes (Figure 9d), and these cells also failed to migrate into B-cell follicles (data not shown).

To address whether the defect in T-cell migration was due to their activation in the context of parasite infection or to modulation of DC function, highly purified DCs from the spleens of malaria-infected animals or uninfected controls were pulsed with OVA and transferred into naive BALB/c recipient mice along with DO11.10 T cells labeled with the fluorescent dye 5,6-carboxy-succinimidyl-fluorescein ester (CFSE). Following transfer of OVA-pulsed DCs from uninfected mice, T cells divided efficiently, as seen by CFSE dilution (Figure 9e). In mice transferred with OVA-pulsed DCs purified from the spleens of malaria-infected mice,

however, OVA-specific CD4<sup>+</sup> T cells failed to divide to the same extent (see Figure 9e), suggesting that DCs exposed to malaria parasites are less capable of inducing effective T-cell responses. Furthermore, DCs purified from *in vitro* culture with pRBCs and pulsed with antigen were less able to induce an optimal T-cell response upon transfer to uninfected recipients (data not shown). Thus the defect in T-cell function and migration is primarily due to the modulation of DC function by the malaria parasite, and not simply the result of activation of T cells in the context of infection.

### Discussion

Several previous reports have suggested a generalized suppression of immune responses during infection with *Plasmodium* [2-10,12-21,24-34,59]. One possible mechanism could be impairment of the function of DCs, a cell type that is essential in the generation of the primary immune responses [42]. But the significance of this observation for *in vivo* studies, the implications for downstream immunological function, and what (if any) parasite component mediated this effect remained unclear. Here, we have shown that DCs are modulated by the malaria parasite and are suppressed by infection with *P. chabaudi* through the malarial pigment HZ. Importantly, *Plasmodium* infection also causes a significant defect in the induction of immune responses *in vivo*: expansion and migration of CD4<sup>+</sup> T cells is greatly reduced, resulting in a consequent reduction in the interaction between these T cells and B cells and in the help they can provide to the B cells. Despite severely impaired T-cell migration and effector function, early stimulation of antigen-specific CD4<sup>+</sup> T cells is not affected by malaria infection, as T cells stimulated *in vitro* and *in vivo* upregulate CD69, suggesting that, despite suppression of DC function, there is sufficient antigen presentation to induce initial T-cell activation.

Although the observed defect in CD4<sup>+</sup> T-cell function seems to be directly related to inhibition of DCs, it has also been suggested that optimal T-cell expansion and differentiation requires the interaction of T and B cells [60]. Thus it may be that the failure of DCs to activate properly and subsequently induce T-cell migration into B-cell follicles also results in the reduced expansion of CD4<sup>+</sup> T cells observed here. Nevertheless, the failure of optimal CD4<sup>+</sup> T-cell expansion and migration during infection clearly results in the abrogation of B-cell expansion and a subsequent absence of antibody production at specific time-points during infection. Thus, protective, systemic immunity against non-parasite antigens, and presumably against parasite antigens, fails to develop effectively.

Although alterations in the architecture of the spleen during infection with *P. chabaudi* have been described [61], these

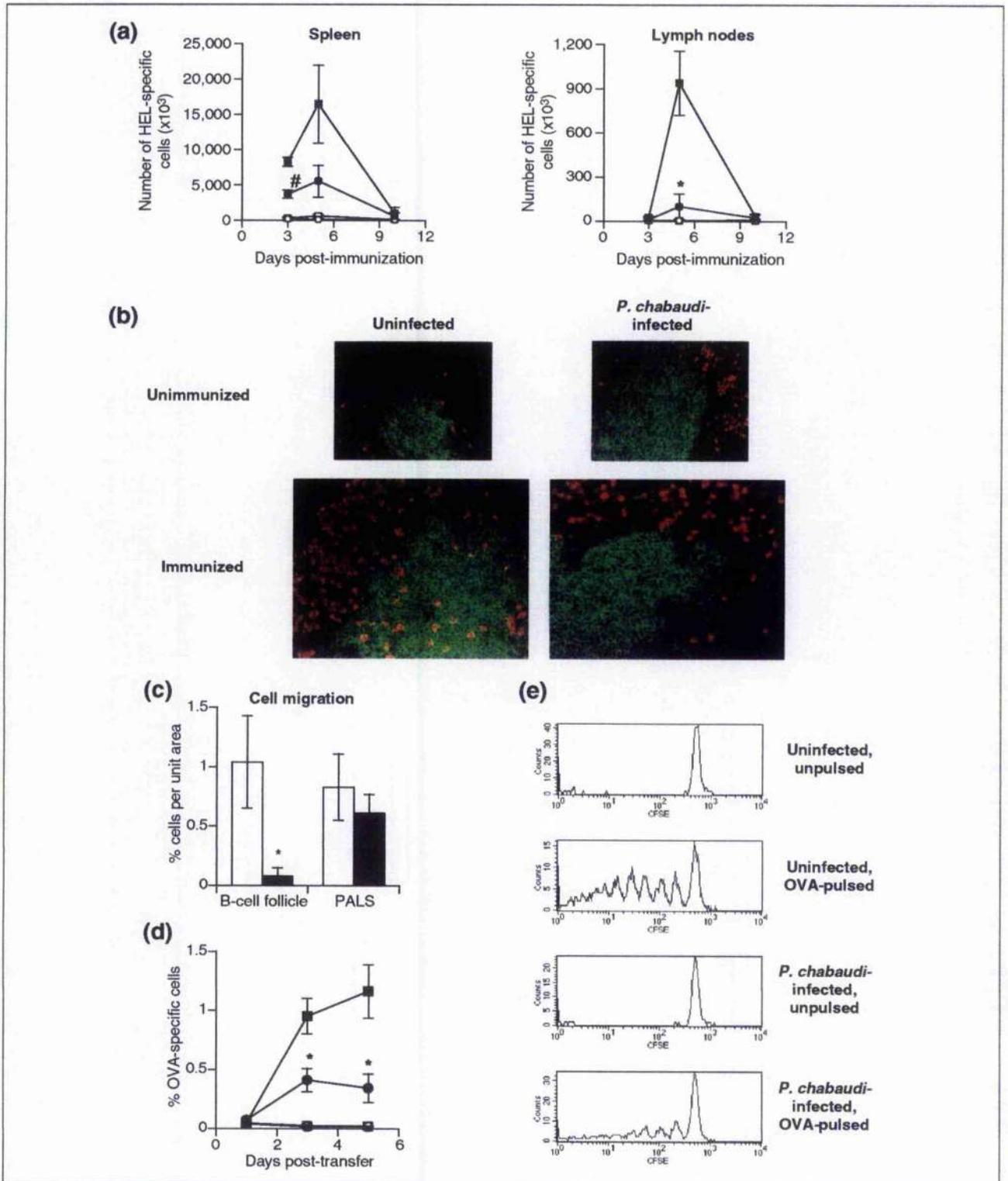


Figure 9 (see legend on the following page)

changes were less evident in our model, with intact B220<sup>+</sup> B-cell follicles still clearly discernible by day 17 of infection (see Figure 9b). Interestingly, infected mice immunized on day 4 of infection (when lymphoid structure is already disrupted [61]) were able to generate antibody responses, suggesting that despite the breakdown in architecture, immune priming is still normal in these mice.

Our important finding that activated T cells fail to migrate into follicles may in part be due to splenic alterations not visualized in our immunohistochemical staining for B220, or to alteration of chemokine gradients important for T-cell migration in infected mice. To address these issues, we transferred T cells that had been primed *in vitro* in the presence of malaria parasites into uninfected recipient mice with normal lymphoid architecture. In addition, we immunized naive, uninfected mice with OVA-loaded DCs which had been cultured *in vitro* with infected erythrocytes. In all of these situations we could separate the effect of parasites upon DCs or T cells from the described disruption of lymphoid architecture (see Figure 9). In each case, T cells primed in the presence of malaria parasites or T cells primed by DCs exposed to infection failed to differentiate fully following transfer into uninfected recipients. Thus, alteration of splenic architecture alone cannot account for our observations, suggesting that the failure in T-cell differentiation is due to DC modulation.

Another possible explanation for the observed defect in the induction of immunity could be through competition for access to antigen between adoptively transferred OVA-specific T cells and endogenous malarial antigen-specific T cells. Thus, a large dose of blood-borne parasite antigens

could impede the induction of other ('bystander') immune responses. But studies examining bystander responses in other parasitic diseases (for example, [62-65]), as well as the potent stimulatory capacity of mycobacteria-containing complete Freund's adjuvant [66], suggest that such inhibition of immunity does not occur, despite potentially large amounts of competing antigens. In our experiments, naive OVA-specific CD4<sup>+</sup> T cells activated *in vitro* and *in vivo* in the presence of infected erythrocytes upregulated CD69 to the same extent as T cells stimulated in uninfected controls, suggesting that sufficient access to antigen was available to initiate T-cell signaling cascades (see Figures 7 and 8). Furthermore, transfer of T cells activated in the presence of infected erythrocytes, or transfer of purified DCs from infected mice into uninfected recipients, transferred the immunosuppressive phenotype, suggesting that the effect cannot be ascribed to out-competition of the OVA-specific T cells by malaria-specific cells (see Figures 8 and 9).

In search of a mechanistic explanation for these observations, we initially focused on analyzing the effect that parasite proteins expressed on the erythrocyte membrane might have on DC function. It is known that the development of the parasites within erythrocytes is coupled with changes in the host cells, including the host-cell plasma membranes [67], and it is well established that parasites express 'neo-proteins' on the host-cell surface, some of which are reported to induce protective immunity [68-70]. Erythrocytes infected with the rodent-specific strain *P. chabaudi* adhere to specific cell types by interacting with molecules such as CD36 [71]; this is known as cytoadherence. Importantly, the interaction of *P. falciparum*-infected RBCs with CD36 has been shown to mediate suppression of DC

#### Figure 9

*P. chabaudi* infection causes reduced expansion of B cells and failure of CD4<sup>+</sup> T-cell migration through modulation of DCs. (a) Uninfected (squares) or *P. chabaudi*-infected (circles) BALB/c mice received OVA-specific CD4<sup>+</sup> T cells and HEL-specific B cells 12 days after infection and were immunized with OVA-HEL/LPS (filled symbols) 24 h later. Controls remained unimmunized (open symbols). Results shown are the absolute number of HEL-specific B cells in the spleen (left) and lymph nodes (LN, right) and represent the mean of 3 mice per group  $\pm$  1 s.d. and are representative of 3 similar experiments (\* $p \leq 0.05$ , \*\* $p \leq 0.005$  uninfected and immunized versus *P. chabaudi*-infected immunized). (b) Five days after immunization, spleens from the mice described in (a) were snap frozen and prepared for immunohistochemistry. Sections were stained using biotinylated-KJ1.26 followed by Streptavidin-AlexaFluor 647 to detect OVA-specific T cells (red) and B220-FITC to identify B-cell areas (green). Images shown are representative of 3 mice per group from 2 similar experiments. (c) Sections stained as in (b) were analyzed by laser-scanning cytometry. Results are expressed as mean proportion of OVA-specific T cells per unit area in the regions indicated. The number of OVA-specific T cells contained in identically sized regions of follicle and periarteriolar lymphoid sheath (PALS) was calculated and expressed as a proportion of total KJ1.26<sup>+</sup> cells in the section to avoid bias due to the difference in expansion between uninfected and infected spleens. Results represent triplicate readings of 3 mice per group  $\pm$  1 s.d. (\* $p \leq 0.05$  uninfected and immunized versus *P. chabaudi*-infected and immunized). (d) Bone-marrow-derived DCs were cultured with *P. chabaudi* pRBCs (circles) or RBCs (squares) for 18 h before pulsing with 5 mg/ml OVA (filled symbols). Controls remained unpulsed (open symbols). OVA-specific CD4<sup>+</sup> T cells were then added at a ratio of 1:1 and cultured for 72 h *in vitro*. T cells were isolated, washed and transferred into uninfected recipients immunized 48 h before transfer to synchronize the immune response. Clonal expansion was then assessed as described above. Results show the mean proportion of CD4<sup>+</sup>KJ1.26<sup>+</sup> T cells and represent the mean of 3 mice per group  $\pm$  1 s.d. (\* $p \leq 0.05$ , RBC-cultured, OVA-pulsed DCs versus pRBC-cultured, OVA-pulsed DC). (e) DCs were purified from spleens of uninfected or *P. chabaudi*-infected mice and pulsed with 5 mg/ml OVA for 2 h. Cells were then harvested, washed and  $5 \times 10^5$  DCs transferred into uninfected BALB/c mice along with CFSE-labeled OVA-specific T cells. The level of CFSE in OVA-specific CD4<sup>+</sup> cells was analyzed 5 days later, as described in Figure 8.

function [72]. Examination of the *P. chabaudi* genome has not, however, revealed any homologs of the *P. falciparum* protein PfEMP1 [73], which is important in sequestration [72]. Rather, a separate multigene family expressing surface antigens was identified in *P. chabaudi* [73], which may have a role in cytoadherence [74].

Another important difference between *P. chabaudi* (the rodent-specific strain used here) and the human-specific strain *P. falciparum* is the presence of surface complexes, known as knobs, in *P. falciparum*-infected erythrocytes, which strengthen interactions between pRBCs and receptors expressed on other cells [75]. *P. chabaudi*-infected pRBCs have no evident knobs [76]. Thus, although *P. falciparum* and *P. chabaudi* show specific differences in their mechanisms of cytoadherence, pRBC mediated DC suppression might occur through interactions with membrane molecules, such as CD36. We therefore exposed DCs to erythrocyte ghosts from infected or uninfected erythrocytes, before the LPS challenge. Ghosts isolated from pRBCs did not alter the ability of DCs to respond to LPS treatment *in vitro*, suggesting that parasite antigens expressed on the erythrocyte plasma membranes do not induce the suppression previously described following contact with DCs *in vitro*. In support of this finding is the observation that immunization of mice with pRBC ghosts can induce protection from parasite challenge [77], suggesting that protein structure is maintained on erythrocyte ghosts and that ghosts, unlike intact parasites, are not inherently immunosuppressive.

As fixed pRBCs suppressed the LPS-induced maturation of DCs whereas pRBC membranes did not, HZ seemed to be a good candidate to investigate when looking at the mechanism of parasite-induced modulation of DC function. Previous reports have suggested that HZ impairs the differentiation and functional capacity of human monocytes and murine macrophages through the production of IL-10 and/or the induction of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) [78-82]. The extrapolation of these results to DCs remains somewhat controversial, however, with other workers suggesting a proinflammatory role for HZ, possibly via the Toll-like receptor-9 [83-86]. Our results suggest that intracellular parasite components, including HZ, do indeed suppress DC maturation and function *in vitro*. The exact mechanism involved in this suppression remains undefined, although it is interesting that phagocytosis of HZ has been found to increase degradation of protein kinase C [87]. Thus, degradation of key intracellular signaling molecules may be one mechanism by which *Plasmodium* parasites suppress DC function. Together, these results suggest that in contrast to *P. falciparum*, intracellular HZ rather than *P. chabaudi*-derived membrane-expressed proteins is responsible for the suppression of APC function.

The results presented here clearly demonstrate that DC function is dynamically modulated *in vitro* and *in vivo* by asexual blood-stage malaria parasites. These findings support previous studies with *P. falciparum*-infected erythrocytes and human monocyte-derived DCs [42] as well as studies *in vitro* and *in vivo* of *Plasmodium yoelii* with murine DCs [88]. Other studies suggest, however, that *P. chabaudi* schizonts activate DCs *in vitro* [40]. Similarly, in previous studies [45,89], DCs isolated from *P. yoelii*-infected mice during peak parasitemia were found to be activated and to efficiently process and present antigen to naive T cells. In the present study, DCs exposed to trophozoite-infected erythrocytes show impaired maturation in response to stimulation, indicating that it is not only different parasites (*P. yoelii* versus *P. chabaudi*) but also different stages (trophozoites versus schizonts) that have different effects on DC function. Interestingly, the observed differences in the ability of pRBCs to stimulate DC maturation may arise through contamination of parasite material with mycoplasmas, which are known to contain potent Toll-like receptor (TLR) ligands that efficiently activate DCs [90-92].

It has recently been suggested that, during malaria infection *in vivo*, DCs are activated during early infection and then show TLR tolerance later in infection, becoming unresponsive to LPS stimulation [93]. We believe this not to be the case with *P. chabaudi*, however, because we see no evidence of direct maturation of the DCs by the parasite (see Figure 2). In addition, activated DCs show an increased ability to stimulate T-cell proliferation and cytokine production [36], neither of which were observed in the T-cell assays in the current study. Rather, we suggest that the transient increased expression of activation markers on DCs *ex vivo* reflects the high concentrations of pro-inflammatory cytokines caused by the early stage of infection [94]. In support of this, a recent report described that DCs activated through inflammatory cytokines without pathogenic stimulation upregulated markers of activation but were unable to drive CD4<sup>+</sup> T-cell differentiation [95].

The ability of DCs to interact with CD40L on T cells *in vivo* has also been used to explain the differences between *in vivo* and *in vitro* studies [45]. Interestingly, in our study we could not rescue DC maturation when pRBC-treated DCs were stimulated with CD40L-transfected fibroblasts, also suggesting that TLR tolerance (the refractory state of DCs to a second stimulation with a TLR ligand) is not involved in the failure of DCs to respond to stimulation. This suggests that the effects that *P. chabaudi*-infected erythrocytes exert on DC function *in vitro* might be more profound than those induced by *P. yoelii* infection. Whether these changes to the CD11c<sup>+</sup> population as a whole reflect changes in individual subsets of DCs awaits further investigation. Importantly, we

have shown that the immunosuppression seen is due to this inhibition of DC function rather than to suppression of T cells or breakdown of splenic architecture, as transfer of T cells activated in the context of parasites *in vitro* or of DCs from infected mice was sufficient to prevent subsequent T-cell differentiation in uninfected recipients (see Figure 9).

The results presented here demonstrate, for the first time, that suppression of immunity associated with *P. chabaudi* affects multiple populations of cells essential for development of immunity. DC function is impaired during parasite infection, as a result of ingestion of HZ, and although CD4<sup>+</sup> T cells specific for a non-parasite antigen become activated following immunization, they fail to expand clonally as efficiently as in uninfected controls. Crucially, these T cells subsequently show a defect in their ability to migrate into B-cell areas and, consequently, fail to provide effective help for B-cell expansion and antibody production. These results demonstrate an overall defect in priming of heterologous immune responses during *Plasmodium* infection and provide an explanation for increased secondary infections and the reduced efficacy of vaccines in areas where malaria is endemic.

## Materials and methods

### Animals and challenge infections

Female BALB/c mice were purchased from Harlan Olac (Bicester, UK). DO11.10 mice, with CD4<sup>+</sup> T cells specific for the OVA<sub>323-539</sub> peptide in the context of the MHC class II molecule I-A<sup>d</sup> recognized by the KJ1.26 clonotypic antibody [96] were obtained originally from N. Lycke, University of Göteborg, Sweden. MD4 mice containing HEL-specific B cells [51] were backcrossed onto the BALB/c background. All mice were maintained at Biological Services, University of Glasgow, under specific pathogen-free conditions and first used between 6 and 8 weeks of age in accordance with local and UK Home Office regulations.

To initiate a malaria infection, mice were inoculated with  $1 \times 10^6$  *P. chabaudi* AS-infected erythrocytes intra-peritoneally. Parasitemia was monitored by thin blood smears stained with Giemsa's stain. Peak parasitemia occurred at 5-6 days post-infection, after which time parasite levels declined and remained at low but usually detectable levels for the remainder of the experiments (see Figure 1a), as previously described [97]. Infected mice were held in a reverse light/dark cycle so that parasites harvested at 08:00 h were at the late trophozoite stage. For studies *in vitro*, blood was collected when parasitemia was 30-40%. Infected blood was recovered into heparin (10 IU/ml) by cardiac puncture and diluted in phosphate-buffered saline (PBS; Invitrogen, Paisley, UK) to the required concentration of pRBCs.

At various times following malaria infection, mice were immunized intravenously with 500 µg OVA (Sigma-Aldrich, Poole, UK), or a conjugate of OVA and HEL (Biozyme, Gwent, UK) [50], along with 50 ng LPS (from *Salmonella equi abortus*; Sigma-Aldrich).

### Preparation of bone-marrow DCs

DCs were prepared from bone marrow as previously described [98]. Cell suspensions were obtained from femurs and tibias of female BALB/c mice. The bone-marrow cell concentration was adjusted to  $5 \times 10^5$  cells/ml and cultured in six-well plates (Corning Costar, New York, USA) in complete RPMI (cRPMI: RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) (all from Invitrogen) and 10% fetal calf serum (FCS; Labtech International, Ringmer, UK) containing 10% of culture supernatant from X63 myeloma cells transfected with mouse granulocyte-macrophage colony stimulating factor (GM-CSF) cDNA. Fresh medium was added to the cell cultures every 3 days. On day 6, DCs were harvested and cultured at the required concentration for each individual experimental procedure, as described below. This technique generated a large number of CD11c<sup>+</sup> DCs largely free from granulocyte and monocyte contamination, as previously described [98].

### *In vitro* culture of DCs with fixed infected or uninfected erythrocytes

Blood from *P. chabaudi* AS-infected mice was washed twice in PBS before being resuspended in cRPMI for addition to DCs. For fixation, infected blood was washed three times in PBS and resuspended in 0.5% paraformaldehyde for 30 min at 4°C. Fixed erythrocytes were then washed in PBS, resuspended in 0.06% Gly-Gly (Sigma-Aldrich) for 5 min at 4°C and washed twice more in PBS before being resuspended in cRPMI for addition to DC. After 24 h culture, DCs were stimulated with 1 µg/ml LPS and the expression of cell-surface molecules was analyzed 18 h later by flow cytometry. To confirm complete fixation, we showed that  $2 \times 10^7$  fixed, infected erythrocytes could not establish infection when injected intra-peritoneally into a female BALB/c mouse.

### *In vitro* culture of CD40L-transfected fibroblasts with DCs

The cell lines 3T3-CD40L and 3T3-SAMEN [46] were kind gifts from P. Hwu (NCI, Bethesda, USA). Cells were grown in cRPMI in T75 tissue culture flasks (Helena Biosciences, Gateshead, UK) and, when confluent, harvested and distributed in six-well plates at  $2.5 \times 10^5$  cells/ml of cRPMI. Bone marrow-derived DCs were cultured with infected or uninfected erythrocytes at a ratio of 1:100. After 24 h, DCs were harvested, resuspended at  $1 \times 10^6$  cells/ml and cultured in a 1:1 ratio with either 3T3-CD40L or 3T3-SAMEN cells for a

further 24 h. The level of CD40 expression on DCs was analyzed by flow cytometry and culture supernatants collected for IL-12 cytokine analysis.

#### T-cell stimulation *in vitro*

Bone-marrow DCs were centrifuged at 450 x g, resuspended at  $1 \times 10^6$  cells/ml and 500  $\mu$ l aliquots were distributed into 24-well tissue culture plates (Corning Costar) with pRBCs or RBCs. After 24 h incubation at 37°C in 5% CO<sub>2</sub>, DCs were antigen-loaded for 6 h with 5 mg/ml OVA (Worthington Biochemical, Freehold, USA). OVA-specific T cells were isolated from the mesenteric and peripheral lymph nodes of DO11.10 transgenic mice [96] on the SCID background and cultured at a 1:1 ratio with DCs. T-cell proliferation was assessed after 48, 72, 96 and 120 h of culture and assessed by incorporation of [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci/well) for the last 24 h of culture. Cells were harvested using a Betaplate 96-well harvester (Wallac Oy, Turku, Finland) and [<sup>3</sup>H]thymidine incorporation measured on a Betaplate liquid scintillation counter (Wallac).

#### Cytokine assay

For the detection of IL-12 (p40 and p70) and IL-10, OptELIA™ enzyme-linked immunosorbent assay (ELISA) kits (Becton Dickinson, Oxford, UK) were used according to the manufacturer's instructions. For T-cell cytokines, Mouse Th1/Th2 6-Plex kit (Biosource, Nivelles, Belgium) was used according to the manufacturer's instructions. For analysis of cytokine production *ex vivo*, single-cell suspensions of spleen cells were prepared by rubbing through Nitex mesh (Cadisch & Sons, London, UK) in RPMI 1640 medium. After washing, cells were resuspended at  $4 \times 10^6$  cells/ml in cRPMI, either alone or with 1 mg/ml OVA or 5  $\mu$ g/ml concanavalin A (ConA; Sigma-Aldrich) and supernatants sampled after 48 h. These were stored at -20°C until analysis by standard sandwich ELISA protocol (antibodies used: for IFN- $\gamma$  capture, R4-6A2; for IFN- $\gamma$  detection, XMG1.2; for IL-5 capture, TRKF5; for IL-5 detection, TRKF4; Pharmingen, Oxford, UK) and the levels of cytokine in supernatants calculated by comparison with recombinant cytokine standards (R & D Systems, Abingdon, UK).

#### Flow cytometry

Aliquots of  $1 \times 10^6$  cells in 12 x 75 mm polystyrene tubes (Falcon BD, Oxford, UK) were resuspended in 100  $\mu$ l FACS buffer (PBS, 2% FCS and 0.05% NaN<sub>3</sub>) containing Fc Block (2.4G2 hybridoma supernatant) as well as the appropriate combinations of the following antibodies: anti-CD4-PerCP (clone RM4-5), anti-CD11c-PE (clone HL3), anti-CD40-FITC (clone 3/23), anti-CD69-PE (clone H1.2F3), anti-CD80-FITC (clone 16-10A1), anti-CD86-FITC (clone GL1), anti-MHC-II (clone 2G9), anti-B220-PE (clone RA3-6B2), PE-hamster IgG isotype control, FITC-rat IgG2a,  $\kappa$  isotype

control and FITC-hamster IgG1,  $\lambda$  isotype control (anti-TNP) (all Pharmingen), biotinylated KJ1.26 antibody or biotinylated HEL. Biotinylated antibodies were detected by incubation with fluorochrome-conjugated streptavidin (Pharmingen). After washing, samples were analyzed using a FACSCalibur flow cytometer equipped with a 488 nm argon laser and a 635 nm red diode laser and analyzed using CellQuest software (both BD BioSciences, Oxford, UK).

#### Preparation of erythrocyte ghosts from infected and uninfected mouse blood

Ghosts from infected and uninfected erythrocytes were generated as previously described [67]. Briefly, blood was collected into heparin by cardiac puncture and washed three times in PBS. Infected and uninfected erythrocytes were concentrated in PBS supplemented with 113 mM glucose (Sigma-Aldrich) and 3% FCS. Infected erythrocytes were incubated in an equal volume of glycerol buffer (10% glycerol (Sigma-Aldrich) supplemented with 5% FCS in PBS) for 1 h at 4°C. Parasites and ghosts were separated in a continuous Percoll (Amersham Biosciences, Little Chalfont, UK) gradient ( $\rho$ : 1.02-1.10 g/cm<sup>3</sup>) in intracellular medium buffer (IM: 20 mM NaCl, 120 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM glucose, 5 mM Hepes pH 6.7) by centrifugation at 5,000 x g for 30 min. Ghosts were then washed in IM buffer and layered on a two-step Percoll gradient ( $\rho$ : 1.01+1.02 g/cm<sup>3</sup>) to separate them from ghosts that might still contain parasites. Ghosts from uninfected erythrocytes were obtained by adding a 40-fold volume of phosphate buffer (5 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 1 mM PMSE, 0.01% azide, pH 8.5). The suspension was centrifuged at 32,000 x g for 30 min. Ghosts from infected and uninfected erythrocytes were then washed three times in PBS before being resuspended in cRPMI for addition to DCs at a ratio of 100:1.

#### Hemozoin preparation

HZ was isolated from supernatants obtained from cultures of *P. falciparum* gametocytes, kindly provided by Lisa Ranford-Cartwright, (Division of Infection and Immunity, University of Glasgow, UK). Endotoxin-free buffers and solutions were used throughout. Supernatants were centrifuged for 20 min at 450 x g. The pellet was washed three times in 2% SLS and resuspended in 6 M guanidine HCl. Following 5-7 washes in PBS, the pellet was resuspended in PBS and sonicated for 90 min using Soniprep 150 (Sanyo Scientific, Bensenville, USA) at an amplitude of 5-8  $\mu$ m to minimize aggregation and maintain the HZ in suspension. Total heme content was determined as previously described [99] by depolymerizing heme polymer in 1 ml of 20 mM NaOH and 2% SDS, incubating the suspension at room temperature for 2 h and then reading the optical density at 400 nm using a UV-visible Helios spectrophotometer (Thermo Spectronic, Cambridge, UK). DCs were pulsed

with 1-20  $\mu\text{M}$  HZ - a concentration range similar to that seen when DCs were cultured at a 1:100 ratio with pRBCs.

#### Assessment of antigen-specific antibody responses

Peripheral blood was collected and the plasma was separated by centrifugation at 450  $\times$  g for 10 min and stored at  $-20^\circ\text{C}$  until analysis. OVA-specific IgG was measured by standard sandwich ELISA using a peroxidase-conjugated anti-mouse total IgG (Sigma-Aldrich).

#### Adoptive transfer of antigen-specific lymphocytes

Lymph nodes and spleens were homogenized and the resulting cell suspensions washed twice by centrifugation at 400  $\times$  g for 5 min and resuspended in RPMI. The proportions of antigen-specific T cells were evaluated by flow cytometry, and syngeneic recipients received  $3 \times 10^6$  antigen-specific cells. In some experiments, cells were labeled with the fluorescent dye CFSE (Molecular Probes, Oregon, USA) immediately before use [100]. The level of CFSE in cells was analyzed by flow cytometry and expressed as the mean proportion of antigen-specific T cells under each CFSE peak.

#### Immunohistochemistry

Spleens were frozen in liquid nitrogen in OCT embedding medium (Miles, Elkart, USA) in cryomoulds (Miles) and stored at  $-70^\circ\text{C}$ . Tissue sections (8  $\mu\text{m}$ ) were cut on a cryostat (ThermoShandon, Cheshire, UK) and stored at  $-20^\circ\text{C}$ . Sections were blocked and stained as previously described [55], using B220-FITC to stain B-cell areas and biotinylated-KJ1.26 to detect OVA-specific DO11.10 cells, and visualized using Streptavidin-Alexa Fluor 647 (Molecular Probes). All photographs were taken at 20 $\times$  magnification.

To visualize HZ deposition in DCs, cells were photographed using an Axiovert S-100 Zeiss microscope using a 63 $\times$  oil-immersion lens by normal bright-field imaging. To image HZ in splenic DC, 8  $\mu\text{m}$  sections were cut as described above and stained with biotinylated-CD11c followed by Streptavidin-FITC and finally tyramide-488 (PerkinElmer, Boston, USA). Images were then taken of bright-field and green fluorescence and the images merged by inverting and then false coloring the bright-field image such that deposited HZ appeared red and CD11c appeared green.

#### Laser-scanning cytometry

Sections were stained as described above. Sections were then scanned on a laser-scanning cytometer equipped with argon, helium, neon, and ultraviolet lasers (Compucyte, Cambridge, USA) and visualized with the Openlab imaging system (Improvision, Coventry, UK). The localization of transgenic T cells and B-cell follicles were plotted. Using these tissue maps the number of transgenic T cells in defined gates was calculated for three gates in periarteriolar

lymphoid sheath (PALS) and three B-cell follicle gates per section. Data are plotted as the mean proportion of transgenic T cells in each gate relative to the number of transgenic T cells in the entire section and are the mean of triplicate readings from three mice per group.

#### Isolation of DCs from spleen

Spleens were excised and single-cell suspensions obtained as described above. In some experiments, cells were stimulated with 1  $\mu\text{g}/\text{ml}$  LPS for 18 h before analysis by flow cytometry. To obtain purified DCs from spleens of mice, single-cell suspensions were labeled using a CD11c isolation kit (Miltenyi Biotec, Biscy, UK) according to the manufacturer's instructions. DCs were then purified using two MS magnetic columns (Miltenyi Biotec) and found to be 85-95% pure by flow cytometric analysis.

#### Statistical analysis

Results are expressed as mean  $\pm$  standard error or standard deviation as indicated. Significance was determined by one-way ANOVA in conjunction with the Tukey test using Minitab. A  $p$ -value of less than 0.05 was considered significant.

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