

ABSTRACT

The immuno-pathology of malaria is associated with the parasite ability to adhere to various endothelial cells in body tissues and organs. Dendritic cells (DCs) play an important role in innate immunity and have the capacity to link innate and adaptive immunity. We analysed the proportions of CD36 positive cells on monocytes, peripheral blood plasmacytoid dendritic cells (pDCs) and on monocyte-derived dendritic cells (MoDCs) and whether different CIDR molecules bound to CD36 expressed on dendritic cells; MC CIDR and CSA CIDR. All cells showed high proportion of being CD36 positive cells with over 90% of monocytes and pDCs population being positive and 60% on MoDCs. Binding of MC CIDR to dendritic cells corresponded with the proportion of CD36 positive cells. There was no or minimal binding by CSA CIDR to monocytes and dendritic cells with <4% positive CSA CIDR cells in both groups. Binding of biotinylated MC CIDR to monocytes and dendritic cells was blocked by pre-incubation of cells with MC CIDR. Pre-incubation of dendritic cells with MC CIDR resulted in modulation of dendritic cell maturation process even after stimulation with lipopolysaccharide (LPS) because dendritic cells did not up-regulate expression of CD40, CD54, CD83, CD86 and HLA DR. These data suggest that cytoadhesion of malaria parasite *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) to DCs via CD36 may modulate DC maturation process which could have the effect of dampening down host immune response to malaria infection.

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DECLARATION

I declare that no portion of the work referred to in the dissertation has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Signature:

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LIST OF ABBREVIATIONS

CSA.....	chondroitin sulfate A
DCs.....	dendritic cells
MoDCs.....	Monocyte-derived dendritic cells
pDCs.....	Plasmacytoid dendritic cells
LPS.....	Lipopolysaccharide
PfEMP-1.....	<i>Plasmodium falciparum</i> erythrocyte membrane protein-1
CIDR-1.....	Cysteine-rich interdomain region 1
MC.....	Malayan Camp
NK.....	Natural killer
IRBCs.....	infected red blood cells
PBMCs.....	Peripheral blood mononuclear cells.
FITC.....	Flourescent iso-thiocyete
TCR.....	T cell receptor
Ig.....	Immunoglobulin
IFN.....	Interferon
IL-	Interleukin
MHC.....	Major histocompatibility complex
HLA.....	Human leukocyte antigen
ICAM-1.....	intercellular adhesion molecule-1
DBL.....	duffy-like binding ligand
TNF.....	tumour necrosis factor
TLRs.....	Toll-like receptors
GM-CSF.....	Granulocyte-macrophage colony stimulating factor

THE AUTHOR

I completed my BSc in 1999 in Immunology and biochemistry from Kenyatta University in Kenya with a score of 2.1. The same year I was employed as a research assistant with KEMRI/Wellcome Trust research Programme in Kilifi, Kenya to work in the malaria immunology Lab. For the last 7 years I have been honoured to work with leading malaria research groups in the world. I was attached to Dr Michael Griffiths on a project that looked at the genetic susceptibility of severe malaria in Children. I was trained in DNA and RNA isolation and had an in-depth genotyping techniques experience at the Wellcome Trust centre for Human Genetic, University of Oxford. I have also worked with Dr Alex Rowe from Edinburgh University on the role of rosetting in malaria, Dr Fredrik Pattersson from Karolinska institute in Stockholm, Sweden on the adhesive features of malaria. From 2004 I have been attached to Dr Britta Urban from centre of vaccinology and tropical medicine, Churchill hospital, University of Oxford looking at the effect of malaria parasites on dendritic cells, focussing on the cellular interaction between the parasite and dendritic cells.

Some of the publications;

Pain A, Urban BC, Oscar K, Casals-Pascal C, **Shafi J**, Marsh K, Roberts DJ. A non-sense mutation in Cd 36 gene is associated with protection from severe malaria. *Lancet* 2001;357:1505-03

Heddini A , Petterson F, Kai O , **Shafi J**, Obiero J, Chen Q, Barragan A, Wahlgren M , Marsh K. Fresh isolates from children with severe plasmodium falciparum malaria bind to multiple isolates. *infect immun* .2001 sep;69(9) :5849-56

Rowe JA, **Shafi J**, Kai OK, Marsh K, Raza A. Non immune IgM but not IgG binds to the surface of Plasmodium falciparum-infected erythrocytes and correlates with resetting and severe malaria. *Am J Trop Med Hyg* 2002 Jun; 66(6): 692-9

Micheal J Griffiths, **Shafi MJ**, Popper SJ, Hemingway CA, Kortok MM, Wathen A, Rockett KA, Mott R, Levin M, Newton CR, Marsh K, Relman DA, Kwiatkowski DP. Genomewide Analysis of the Host Response to Malaria in Kenyan Children. *J Infect Dis*. 2005 May 15;191(10):1599-1611.

CHAPTER 1

INTRODUCTION

1.1 Malaria – overview

Malaria one of the worlds leading cause for morbidity and mortality is caused by the protozoan intracellular parasite of genus *Plasmodia*. The disease is distributed globally and is a leading public health concern in tropical and sub-tropical regions. Malaria accounts for over 2 million deaths annually with the heavy burden being felt in sub-Sahara Africa which contributes to over 90% of the global deaths associated with malaria (Snow *et al.*, 2005) The most susceptible groups are children under the age of five years, pregnant mothers during their first pregnancy and individuals not immune to malaria. In human malaria infections four *plasmodia* species are responsible- *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium falciparum*. Most mortality is attributed to *P. falciparum* the lethal of the four human infecting plasmodium species with children under the age of five years, non-immune adults and pregnant women at greatest risk. Malaria in Africa pose as a major drawback both socially and economically (Chima *et al.*, 2003). The impact of malaria in Africa in economical terms is estimated to cost a large amount of resources that could have been used to fund development issues. It is estimated that African countries spend over \$2 billion annually due to malaria infections directly or indirectly (Sachs *et al.*, 2003). Not only there is lost productivity as a consequence of deaths and illness due to malaria, education standards are also impaired leading to intellectual underdevelopment which directly impacts on Africa's human resources (Fernando *et al.*, 2003). In endemic areas, infection with *P. falciparum* results in a range of outcomes from asymptomatic infection, through mild disease, severe disease and death. Immunity is exposure-related and therefore age-related and occurs rapidly to severe non-cerebral disease, more slowly to mild disease and probably never to asymptomatic infection (Gupta *et al.*, 1999). The emergence

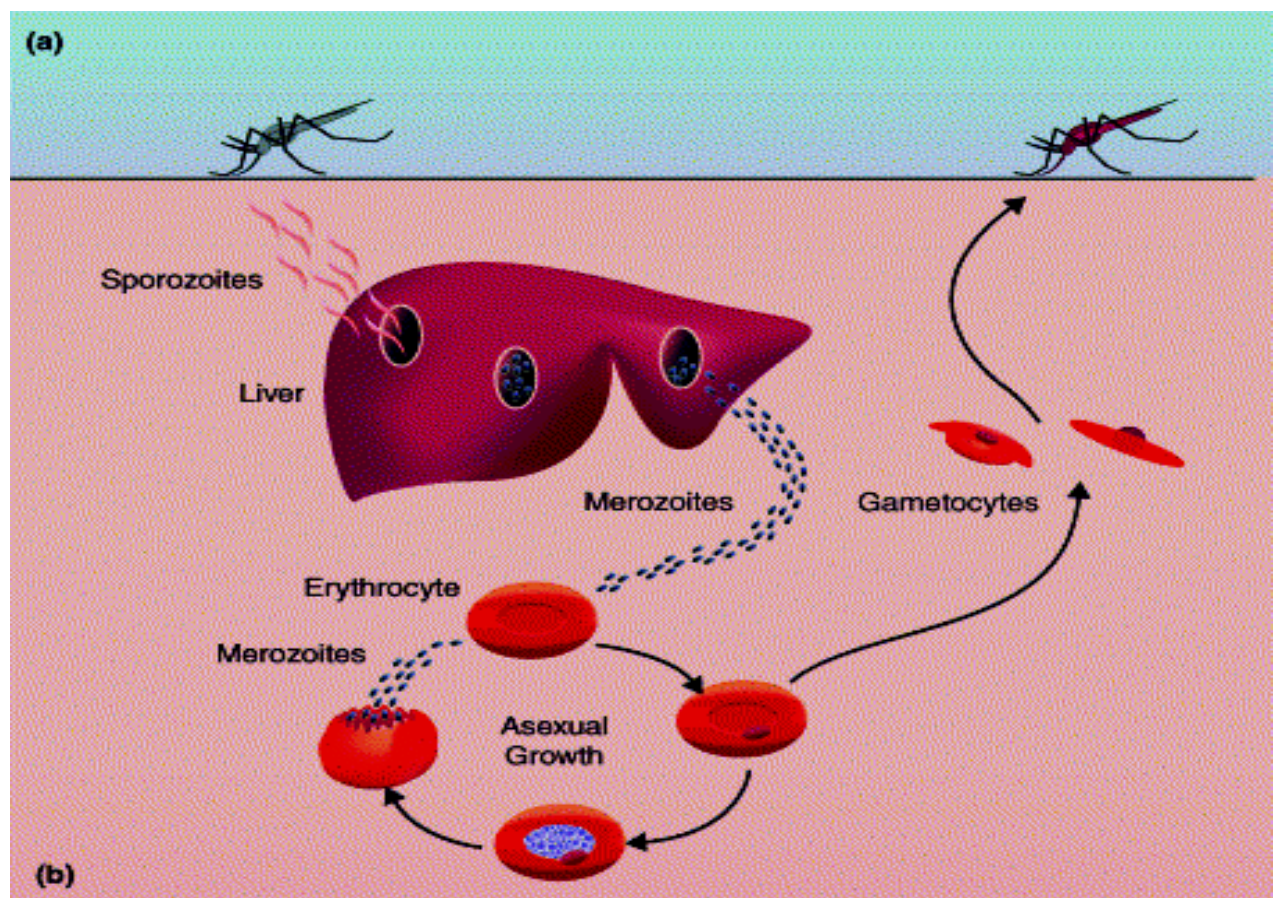
of the HIV epidemic in malaria endemic areas poses a particularly difficult challenge. Although the direct effects of HIV infection on malaria associated morbidity and mortality are not clearly established, by placing an unprecedented demand on the already little health resources, HIV is bound to have a major negative impact on malaria interventions strategies.

1.2 *Plasmodium falciparum* life cycle

The life cycle of malaria parasite involves both the vector stage in mosquitoes and the definite human host. During a blood meal, the female *anaopheles* mosquito having the malaria parasites in its salivary glands injects the parasites into the human host. In less than 60 minutes, the sporozoites migrate to the liver and infect the hepatocytes. This starts the phase of exo-erythrocytic schizogony in that once inside the hepatocytes, the malaria sporozoites undergo asexual form of multiplication and modification. Between 5 to 14 days in the hepatocytes, an infective sporozoite can yield as much as 30,000 merozoites. In this phase of exoerythrocytic schizogony, there is no clinical associated form of illness as a result of hepatocyte infection. Once they reach maturity, the mature form of the parasite, schizont rupture and empty its contents into the blood stream to begin the erythrocytic phase of development that occurs primarily in the red blood cells. This phase of development is responsible for clinical manifestation of malaria disease. Once inside the red blood cells, the malaria merozoites replicates the asexual amplification and this multiplication may yield as much as 36 merozoites per single merozoite infecting red blood cell. The merozoite will under various development forms reaching the mature schizont stage. Bursting of the schizont releases the merozoites that may infect new red blood cells and repeat the erythrocytic phase of the malaria life cycle. During the repeated erythrocytic cycle, some of the ring trophozoites fail to mature into schizonts, a proportion undergo morphological changes and sexual differentiation to become gametocytes. The gametocytes will be in form

of male and female forms. During a blood meal by the mosquito anopheline vector, the gametocytes are fed up and migrate to the midgut of the mosquito where fertilization occurs resulting in production of zygotes. The zygote transforms into ookinets and later form oocytes that matures, divide and burst to release sporozoites. The sporozoites migrates to the mosquito salivary glands where the next infection of humans start after another blood meal.

Figure 1.1: Life cycle of *Plasmodium species*



During blood meal, the female *Anopheles* mosquito vector harbouring the malaria parasites injects the sporozoites into the human host (a). Within hepatocytes, replication takes place within 10-14 days to generate approximately 10000 merozoites per hepatocyte. The pre-erythrocytic shizont ruptures, releasing merozoites into the blood circulation, and enter and multiply within erythrocytes. The parasites can multiply approximately eight-fold every 48 hours and in few days the number of malaria parasites may be increased exponentially. During the erythrocytic phase, a proportion of the parasites differentiate into male and female gametocytes. During the next blood meal by the mosquito, the male and female gametocytes are taken up to complete the parasite life cycle. (Modified from Urban and Roberts, Curr Opin Immunol 14(4): 458-65).

1.3 Malaria pathogenesis

Among the four species of human malarial parasites, *Plasmodium falciparum* stands out as the most pathogenic form that is responsible for the severe manifestation of malaria disease. It is associated with the blockage of the central nervous system leading to cerebral malaria, increased red blood cell destruction causing anemia and pulmonary acidosis due to kidney malfunctioning. Two features, important for disease severity and pathogenesis, separate *P. falciparum* from the other human malaria parasites: the unique property to increase the parasite's presence exponentially by infecting new red blood cells of all ages and the evasion of host immune responses by cytoadhering to body organs and endothelium through sequestration. Sequestration is an important process that the malaria parasite uses to avoid destruction by the host via the spleen. The infected erythrocyte (IRBC) binds to body organs and host endothelium using parasite-derived molecules that are inserted on the surface of IRBCs. Adhesion to endothelial cells (cytoadherence) (Newbold *et al.*, 1997) and to uninfected erythrocytes (rosetting), as well as the aggregation of infected cells (clumping), are adhesive traits of *P. falciparum* infected erythrocytes which are studied *in-vitro* and that are believed to reflect the events bringing about sequestration and accumulation of parasites in vascular beds.

Malaria impact is profound in children below the age of five years as they are yet to develop full immune status. In malaria endemic areas, immunity against malaria parasites is acquired after numerous challenges to the parasites living young children with few or no exposure at greater risk of malaria. Majority of malaria deaths in these areas are confined to children below five years of age. The next susceptible group is the immuno-compromised pregnant women especially during their first pregnancy. The ability of the malaria parasites to sequester in the intervillous space of the placenta have been attributed to a number of severe complications during and after pregnancy including; abortions, premature delivery, low birth

weight and in rare cases, death of the newborn (Beeson *et al.*, 2004). The generation of protective antibody responses against the placental malaria parasites in subsequent pregnancies enables the women to avoid the severe complications seen during first pregnancy (Beeson *et al.*, 2004). A lot of work still needs to be done to elucidate the actual mechanism of protection against placental malaria in subsequent pregnancies.

Another key pathogenic feature of *falciparum* malaria is the increased inflammatory responses seen in severe malaria cases which is thought to be mediated by the secretion of pro-inflammatory cytokines TNF- α , IFN- γ , IL-1 and IL-6. A reported increase in TNF- α secretion has been reported in malaria positive persons as compared to malaria free controls (Grau *et al.*, 1998, Molyneux *et al.*, 1990). The increased production of pro-inflammatory cytokine TNF- α has been linked to malaria parasite schizogony stage (Kwiatkowski *et al.*, 1989).

1.4 Malaria- the disease

Malaria as a disease exhibits a variety of symptoms and signs that overlaps with other tropical diseases. The features of malaria are complicated and varied depending on the form of the disease. General symptoms include raised body temperature in some cases above 39°C, general body weakness with muscle pains, joint pains, occasional vomiting and headache. The severe form of malaria may lead to malaria-associated anemia, cerebral malaria, respiratory complications and body bases imbalance leading to metabolic acidosis. Where cerebral malaria is seen, obstruction of the occlusates leading to the brain may precipitate seizures and fatal cases leading to malaria associated coma. Splenomegaly, the enlargement of the spleen is one of the common features of acute malaria infections in children (Mackintosh *et al.*, 2004). There is no single symptom of malaria and clinical picture is normally confirmed by having confirmatory laboratory results to detect the

presence of the malaria parasites or parasite products in patients. Most severe complications in malaria infections are attributed to *Plasmodium falciparum* species, the most lethal of the four human infecting malaria parasites.

1.5 Global distribution of malaria

The baseline factors that determine the probability of malaria transmission in an area are the presence of climatic and ecological conditions appropriate for the malaria vector *anopheline* mosquitoes and the development of malaria sporozoites in the vector. At least 80mm of rainfall annually and an average temperature of over 18°C for at least five continuous months are necessary for malaria transmission. However, limits set by climatic conditions may be modified by human activities such as agriculture, urbanization, mass population movement, and malaria control programmes. The burden of clinical malaria globally is estimated to affect more than 300 million people with reported estimate of over 2 million deaths annually (Snow *et al.*, 2005). Due to availability of favorable weather conditions for parasite growth in the mosquito vector, malaria is dominant in both tropics and sub-tropics areas.

By 2002 malaria was estimated to be endemic in more than 90 countries with about half of the world population being at risk of exposure to the disease (Hay *et al.*, 2004). Malaria distribution spreads from central to South American countries, South-east Asia and sub-Saharan Africa. Over 80% of malaria cases worldwide were reported in Africa in 2002 (WHO report, 2003). The heavy presence of malaria in Africa has been attributed to the poor vector control programmes associated with poverty levels and lack of public health policy to fight the transmission of the disease. The emergence of insecticide resistant mosquito vector is a major threat to the fight against malaria transmission and distribution in malaria endemic

countries.

1.6 Malaria Control

Malaria control in endemic countries should target the reduction of the malaria burden to the communities. Malaria control should not aim to eliminate malaria. Complete elimination of the malaria parasite and thus the disease would constitute eradication. While eradication is more desirable, it is not currently a realistic goal for most of the countries where malaria is endemic. The burden of malaria is heavily felt in countries in sub-Saharan Africa (Hay *et al.*, 2005). Malaria control is carried out through the following interventions, which are often combined; Case management involving diagnosis and administration of anti-malaria drugs for infected persons, prevention of malaria transmission through mosquito control and finally prevention of disease by administration of anti-malarial drugs to particularly vulnerable population groups such as pregnant women and children under the age of 5 years. Infection is prevented when malaria-carrying *Anopheles* mosquitoes are prevented from biting humans. Vector control targets reduction in contacts between mosquitoes and humans. These measures may involve destruction of larval breeding sites, insecticide spraying inside houses and these activities require organized teams and resources that are deficient in malaria endemic countries. An alternate approach to the use of insecticide treated bed-nets (ITN), combines mosquito control and individual control remains the effective approach in controlling the vector (Binka *et al.*, 2006). This intervention can often be conducted by the communities themselves and has become a major intervention in malaria control worldwide. However Malaria control is made difficult by several technical and administrative problems. The emergence of insecticide resistant mosquito vectors makes vector control a difficult tool to employ together with emergence of malaria parasites that are resistant to common anti-malarial drugs (Toure *et al.*, 2004). This calls for the use of substitute drugs that in most cases are very expensive, less safe and not easy to administer. On the other hand, insecticide

resistance observed in mosquitoes decreases the efficacy of interventions that rely on insecticides such as insecticide-treated bed nets and insecticide spraying. This coupled with inadequate health infrastructures in endemic poor countries yield poor interventions approaches against malaria.

1.7 Immunology of malaria

1.7.1 Introduction

Most episodes of infection with *P. falciparum* in malaria-endemic areas lead to mild clinical symptoms and after repeated exposure children eventually develop immune responses that protect against severe disease (Gupta et al., 1999). This immunity does not fully prevent infection, however, but decreases the rate and severity of the disease, with low-grade infections continuing to occur with few or no clinical symptoms. Many targets for the humoral-immune responses are polymorphic or clonally variant antigens exposed on the surface of merozoites or infected erythrocytes, (Dodoo *et al.*, 2001). During acute infection, individuals develop-specific antibody responses to antigens of the parasite variant they are exposed to. Maximum protection is generally achieved by late childhood to early adulthood. Over this time children in endemic areas develop an immunity that reduces the frequency and severity of disease, however immunity is not sterile. Immunity is a combination of variant-specific and non-variant responses (Bull *et al.*, 1999). In previous studies it has been shown that antibodies directed against the surface exposed protein PfEMP-1 agglutinate IRBCs in a variant-specific manner, associated with protection against disease (Newbold *et al.*, 1992). As explained below PfEMP1 are variant antigens, and cumulative exposure to a broad range of different *P. falciparum* serotypes leads to the development of a large number of anti-PfEMP-1 antibodies to cover most antigenic variants (Bull et al., 2002). Mechanisms of

cellular immunity thought to play a role in malaria includes direct activities of immune cells, such as phagocytosis and cytolysis, as well as soluble mediators, such as cytokines and nitric oxide, secreted or induced by these cells. Such immunity can be specific or non-specific, and may require or be enhanced by antibody. Important immunologic cells thought to play a role in immunity to malaria includes; T cells, macrophages, B cells, Natural killer cells (NK) and dendritic cells.

1.7.2 Antibody response important for malaria protection

Humoral responses are important in protection against malaria. Evidence for *in-vivo* protection against malaria by antibodies comes from passive transfer experiments both in animal models (Groux *et al.*, 1990) and humans. Passive transfer of immunity to malaria in human was first demonstrated in a series of experiments carried out in early 1960s (Cohen *et al.*, 1961). In these experiments, intra-muscular administration of sera from immune African adults into Gambia and East African children suffering from severe malaria caused a marked drop in parasitemia within five days. Sera from malaria non-immune Europeans did not show the parasitocidal effect, indicating that the antibodies in African sera were malaria specific (Cohen *et al.*, 1960). In addition it was shown that antibodies that protected against malaria could be obtained from cord blood thus demonstrating the maternal transfer of anti-malaria antibodies (Edozien *et al.*, 1962). Sabchareon and colleagues repeated these experiments. He treated Thai malaria patients with intravenous IgG from malaria immune African adults. A marked drop in parasitaemia within 24 hours of treatment was observed in the patients (Sabchareon *et al.*, 1991). The faster rate of response in the Thai experiments compared to the earlier ones is probably due to the different route of administration of the immune serum. *In-vitro*, antibodies from immune individuals have been shown to inhibit sporozoites invasion of hepatocytes (Pasquetto *et al.*, 1997), prevent merozoites invasion of

red blood cells (Vande Waa *et al.*, 1984), depress parasite growth (Brown *et al.*, 1980), and promote parasite phagocytosis by macrophages (Groux *et al.*, 1990). In addition, immune serum can disrupt rosetting (Wahgren *et al.*, 1990; Treutiger *et al.*, 1992) and the binding of infected erythrocytes to endothelial cell ligands (Iqbal *et al.*, 1993; Ricke *et al.*, 2000), two processes that are implicated in the pathogenesis of severe malaria. However it is not clear how the *in-vitro* activates correlates with *in-vivo* mechanisms.

Despite the evidence cited, there is lack of a correlation between total antibody titres and malaria protection (Marsh *et al.*, 1989). The majority of malaria antibodies are probably directed against cellular debris released when schizonts burst and are of little consequence. However, even antibodies against antigens that are deemed to be important for parasite survival often do not correlate with protection (Hoffman *et al.*, 1987). There are several reasons why this could happen. The immunodominant regions of many malaria antigens consist of tandem amino acid repeats, altering the number, which is an easy way to generate polymorphisms that may help the parasite escape immune recognition (Day *et al.*, 1991). At the same time, polymeric antigens can cross-link B cell antigen receptors and induce T cell independent antibody production that is characterised by IgM dominance and poor affinity maturation and memory cells induction. Besides being short-lived and ineffective, T cell independent responses can also thwart protective responses to adjacent critical epitopes through epitope inhibition (Schofield *et al.*, 1991).

Under a variety of *in-vitro* situations, malaria antibodies are often ineffective against parasites in absence of effector cells and may even promote parasite growth (Shi *et al.*, 1991). Despite exhibiting potent anti-parasitic activity *in-vivo*, the antibodies used in transfer experiments showed no activity *in vitro* except in presence of monocytes (Bouharoun-Tayoun *et al.*, 1990, Sabchareon *et al.*, 1991). Conversely antibodies that do not protect *in vivo* were unable to interact with monocytes *in vitro* (Groux *et al.*, 1990). Thus the

ability of antibodies to cooperate with effector cells may be more important than their quantity. It has been observed that humoral responses to malaria show pronounced skewing towards cytophilic antibodies IgG1 and IgG3, unlike responses to other antigens where IgG1 and IgG2 dominate (Ferrante *et al.*, 1997). This bias has been reported severally in responses against ring-infected erythrocyte surface antigen (RESA) (Dubois *et al.*, 1993), merozoites surface antigens (MSA 1/2) (Rzepczyk *et al.*, 1997) and schizont antigens (Piper *et al.*, 1999). *In-vitro* work has shown that while cytophilic antibodies cooperate with monocytes in inhibiting parasites, non-cytophilic subclasses antagonise this cooperation. Data from field studies indicate that young children and non-immune adults have a high proportion of non-cytophilic antibodies (Wahlgren *et al.*, 1983), while cytophilic antibodies are associated with protection against infection (Ferreira *et al.*, 1996) and better prognosis during acute malaria episodes (Sarhou *et al.*, 1997). Taken together, these data suggests that acquisition of immunity to malaria may involve a shift in responses from non-cytophilic to cytophilic antibodies (Bouharoun-Tayoun *et al.*, 1992).

1.7.3 Cellular immune responses to malaria

B cells

The involvement of B cells in malaria infections is of important interest as B cells have ability to present antigen as well as activate production of antibody secreting plasma cells. The CIDR-1 domain of PfEMP1 has been implicated in mediation of B cells activation *in vitro* and induction of hypergammaglobulinaemia two distinct features of *P. falciparum* infections. It has been shown that IRBC of the laboratory isolate FCR3S1 attach to B cells and induce their activation from malaria non-exposed controls (Donati *et al.*, 2004). This interaction is thought to be mediated by the *Plasmodium falciparum* erythrocyte membrane protein-1 and involve the extracellular portion of the cysteine-rich interdomain region-1

(CIDR-1). Stimulation with recombinant CIDR-1 derived from the var gene FCR3S1 induced multiplication and changes in B cell resulting in induction of activation molecules and production of cytokines accompanied by production of immunoglobulin M (Donati *et al.*, 2004). However the effect on B cells seems to be mediated by binding of this specific CIDR- 1 sequence to Immunoglobulin molecules rather than binding to CD36.

Macrophages

In addition to the modulation of DC function by IRBC, there is evidence suggesting that macrophage function is modulated in malaria infections. Macrophages are essential effector cells of the host innate defence against malaria (Serghides *et al.*, 2003). Macrophage can activate T cells by presenting antigen in the context of MHC class II, and have the ability to ingest IRBC using opsonic or non-opsonic phagocytosis pathways. There is experimental evidence that non-opsonic phagocytosis contributes significantly to the uptake of IRBC by both rodent and human macrophages (Ayi *et al.*, 2005). Wild-type macrophages displayed an enhanced phagocytic activity for non- opsonized IRBCs, compared with those for CD36 null mouse and rat macrophages (Patel *et al.*, 2004). In addition, the uptake of ring-infected IRBCs by humans could be inhibited with anti-CD36 antibodies (Ayi *et al.*, 2005).

Haemozoin loading of macrophages, resulting from either the uptake of free malaria haemozoin or the phagocytosis of mature IRBCs, can lead to macrophage dysfunction (Schwarzer *et al.*, 1996). Dysfunction is characterized by an inability to repeat phagocytosis, generate an oxidative burst upon stimulation and activate protein kinase C after exposure to haemozoin (Schwarzer *et al.*, 1996). Collectively, these studies suggest that by modulating DC, macrophage and B cell function via CD36 or other receptors, the malaria parasite may subvert the immune response of the host to enhance its survival.

T cells and malaria infections

Humans T cells can be divided into two main groups depending on their surface markers and the class of HLA that they interact with. CD4⁺ T cells, which are restricted by class II HLA, provide help to B cells and other effector cells, and as such are also referred as T-helper cells. On the other hand, CD8⁺ T cells, which interact with class I HLA, are also called cytotoxic T cells (CTL) because they kill infected cells by various mechanisms. There is also a minor subset of T cells that express $\gamma\delta$ receptors rather than $\alpha\beta$ receptors, and whose interaction with MHC is still uncertain. The roles for these T cell subsets in immunity to malaria have been described either by indirect observations in humans or in animal models. The caveat here is that many animals in which immunity experiments have been carried out are poor models of human malaria.

CD4⁺ T cells

Traditionally mature CD4⁺ T cells are placed in two groups that are associated with distinct cytokine profiles. Production of interferon alpha/gamma (IFN- α/γ), lymphotoxin- α (TNF- β), IL-8, IL-12 defines type 1 helper cells (TH1) and is associated with a strong cell-mediated immunity, while production of IL-4, IL-6, IL-9, IL-10 and IL-13 defines type 2 (TH2) helper T cells associated with antibody production. However, because some T cells and non-T cells can produce both TH1 and TH2 cytokines, it may be more appropriate to talk of a type 1 (TR1) or type 2 response (TR2) (Clerici *et al.*, 1994). In malaria, TR1/TR2 dichotomy is most clearly seen in the mouse *P. chabaudi* model. In this model, TR1 dominates the early response of mice to acute *P. chabaudi* infection and parasite killing is mediated by IFN- γ , tumour necrosis factor (TNF- α) and nitric oxide (NO) secreted by activated TH1 CD4⁺, macrophages and natural killer cells. TR1 cytokines- NO, INF- γ and TNF- α are also thought

to mediate disease symptoms. On the other hand a shift toward TR2 leads to a less symptomatic chronic infections. Along with inhibiting both INF- γ and TNF- α , type 2 cytokines also stimulate B cells to secrete antibodies (Taylor-Robinson *et al.*, 1995, Fell *et al.*, 1998). The dual anti-parasite/pathogenic nature of TR1 is also evident in *P. berghei* infections (Rudin *et al.*, 1997). Other malaria-mouse models display variable tendencies towards either type of response during acute and chronic infections (Taylor-Robinson *et al.*, 1999).

The distinction between type 1 and 2 responses is less clear in human malaria. Increased IFN- γ is associated with the resolution of parasitaemia in acute malaria episodes (Winkler *et al.*, 1998) and a delay in re-infection (Luty *et al.*, 1999), while reduced levels accompany hyper-parasitemia in children (Winkler *et al.*, 1999). IFN- γ levels were also found to be higher in pregnant women who did not have placental malaria than in those with malaria (Moore *et al.*, 1999). These observations argue for a possible anti-parasite role of TR1 in human. On the other hand, IL-10 and IL-4 both type 2 cytokines have been associated with protection against malarial anaemia (Biemba *et al.*, 2000). Although reduced secretion of IFN- γ by immune T cells in response to malaria led to the conclusion that, reduced pathology in immune individuals may be attributable to down-regulation of TR1 cytokines (Chizzolini *et al.*, 1990).

CD8+ T cells (CTL)

Because hepatocytes, unlike red blood cells, express class 1 HLA, the liver stage of malaria parasites is thought to be a target for induction of CTL responses. Classical adoptive transfer and depletion experiments have confirmed the protective role of CTL in animal models (Schofield *et al.*, 1987, Weiss *et al.*, 1988). The fact that adoptively transferred CTL pre-primed with *P. berghei* failed to protect from infection by *P. yoelii* indicates that this

protection is species-specific (Romero *et al.*, 1989). Indirect evidence for CTL involvement in malaria immunity in man is borne in the association of some class 1 HLA alleles with protection against malaria (Hill *et al.*, 1991). Over 40 epitopes on the sporozoites and liver stage antigens of malaria parasites have been identified as epitopes for human CTL (Aidoo *et al.*, 2000). Some of the epitopes exhibit extensive polymorphism generated by non-synonymous mutations, an indication that they are under some sort of selection probably from host immunity (Hughes *et al.*, 1995). CTL could kill parasites by perforin-mediated lysis, FAS-induced apoptosis of infected cells (Lowin *et al.*, 1994), or via a cytokine pathway in which IFN- γ stimulates the host cell to kill the parasites through nitric oxide production.

$\gamma\delta$ T cells

In healthy individuals, the majority of T cells receptors are made up of α and β chains, however a minority of T cells, whose restriction is uncertain, express receptors made of γ and δ chains. Infection with malaria causes a marked increase in the proportion of $\gamma\delta$ T cells (Ho *et al.*, 1990) but the significance of this phenomenon is yet to be established. Most of the work indicating a role for $\gamma\delta$ T cells in resistance to malaria is based on mice that have immunological disruptions (Langhorne *et al.*, 1996, Yanez *et al.*, 1999) and may not necessarily reflect the situation in immunologically intact mice. Nonetheless, there is evidence that $\gamma\delta$ T cells from both malaria immune and non-immune individuals can produce IFN- γ , TNF- α in response to malaria infections (Ho *et al.*, 1990, Pichyangkul *et al.*, 1997 and McKenna *et al.*, 2000).

1.7.4 Malaria protection by haemoglobinopathies

Haldane in 1949 was the first to hypothesize that the reason certain red blood cell defects have an unexpectedly high prevalence in malaria endemic areas is because protection against malaria gave heterozygotes selective advantage over normal people (Yuthavong *et al.*, 1993,

Weatherall *et al.*, 1997). Such protection is most evident in sickle cell trait heterozygotes who enjoy over 90% protection against severe malaria (Hill *et al.*, 1991) while homozygous sicklers often die young from a variety of infections and effects of the defect (Molineux *et al.*, 1979). Protection by α^+ thalassaemia, though less (40%-60%) than sickle cell trait is still significant (Allen *et al.*, 1997). A large combined case-control study in Kenya and the Gambia found between 40-50% protection against severe and mild malaria in both female heterozygotes and male homozygote G6PD deficient individuals (Ruwende *et al.*, 1995) contrary to earlier assertions that only heterozygote G6PD deficient females were protected (Martin *et al.*, 1994)

The mechanisms by which haemoglobinopathies protect against malaria are poorly understood. Decreased parasite invasion and growth, possibly due to altered membrane and physiology in abnormal cells has been reported (Senok *et al.*, 1997). Although sickling of infected cells could physically injure the parasite or alter haemoglobin so that it is unavailable for the parasites, susceptibility of homozygote sicklers to malaria argue against this being the basis of protection in sickle cell trait (Nagel *et al.*, 1990). G6PD- and thalassaemic cells susceptibility to oxidative stress by parasites could mediate cell damage and kill the parasite in the process (Golenser *et al.*, 1989). High potassium levels in culture media abrogates the effect of oxidants on thalassaemic and G6PD- malaria cultures (Friedman *et al.*, 1979), thus loss of this cation from infected thalassaemic, G6PD- cells and sickle cells could be important in mediating parasite damage. At the same time infected abnormal red blood cells exhibited reduced cytoadherence and rosetting, two phenomena that have been implicated in pathogenesis of cerebral malaria (Carlson *et al.*, 1994 and Udomsangpetch *et al.*, 1993).

1.7.5 Effect of MHC and other gene polymorphisms on malaria susceptibility

Unlike haemoglobinopathies which have easily distinguished phenotypes, other genotypes that influence responses to malaria are less discernable and only through recent advances in molecular techniques have they been detected. The role of MHC genes that code for Human Leukocytes Antigens (HLA) in immunity to malaria is well established. T cells, which are central to specific immunity, only recognise foreign antigens that are presented in cooperation with self-HLA. The T cells can then mediate cytotoxic and inflammatory functions or stimulate B cells to produce antibodies. It has been postulated that the widely observed variation in immune responses to and outcome of malaria infection in individual in endemic areas might be associated with MHC restriction (Riley *et al.*, 1996). The clearest evidence for HLA association with malaria came from a large case-control study in Gambian children where possession of the class I HLA-Bw53 allele provided about 40% protection against severe malaria anaemia and cerebral malaria while the class II alleles DRB1*1302 protected against severe anaemia but not cerebral malaria (Hill *et al.*, 1992). Correlation between the geographic distribution (25-40% in West African and absent in Caucasians) of these alleles and malaria endemicity lends support to the malaria selection hypothesis.

Besides MHC, several other genes have been studied to determine their association with immunity to malaria infection. Of particular interest are the tissue necrosis factor (TNF) promoter genes and intracellular adhesion molecule 1 (ICAM-1) polymorphism. Mutations on the TNF promoter gene that affect malaria outcome have been identified; a point mutation at position 308 that is associated with increased risk of sequelae and death in children with cerebral malaria (McGuire *et al.*, 1994), and another mutation at position 238 that increases the risk of severe malaria anaemia (McGuire *et al.*, 1999). The exact mechanism by which these mutations exert their effects is yet to be established, although the

up-regulation of TNF- α leading to increased pathology has been considered. The two mutations are not linked suggesting that severe malaria anaemia and cerebral malaria are influenced by separate genetic factors linked to the TNF- α gene.

Further evidence of genetic control of immunity to malaria comes from population, twins and family pedigree studies (Hill *et al.*, 1997, Taylor-Robinson *et al.*, 1993). In a study among sympatric ethnic groups in West Africa, the Fulani tribe had different parasite prevalence, malaria antibodies titres and allelic profiles of genes involved in malaria outcome compared with the Mossi and Ramaibe groups. Permethrin impregnated bed nets had a higher impact on parasite rates among the Fulani than in the other two groups (Modiano *et al.*, 1998). A segregation analysis of blood infection levels among 44 Camaroonian families was consistent with a complex genetic control of malaria immunity that is not inherited in a mendelian manner (Garcia *et al.*, 1998). Another family study in Burkina Faso identified a region of the short arm of chromosome 5 that is associated with the control of parasitaemia. This region has genes that are implicated in the regulation of immune responses including cytokines IL-3, IL-4, IL-2 and macrophage stimulating factors (Rihet *et al.*, 1999). It is clear from these studies that genetic associations in malaria are very complex and a lot remains to be elucidated. Hopefully, the recent completed sequencing of the human and malaria parasite genomes will provide an opportunity for the rapid identification of other genes involved in immunity to malaria.

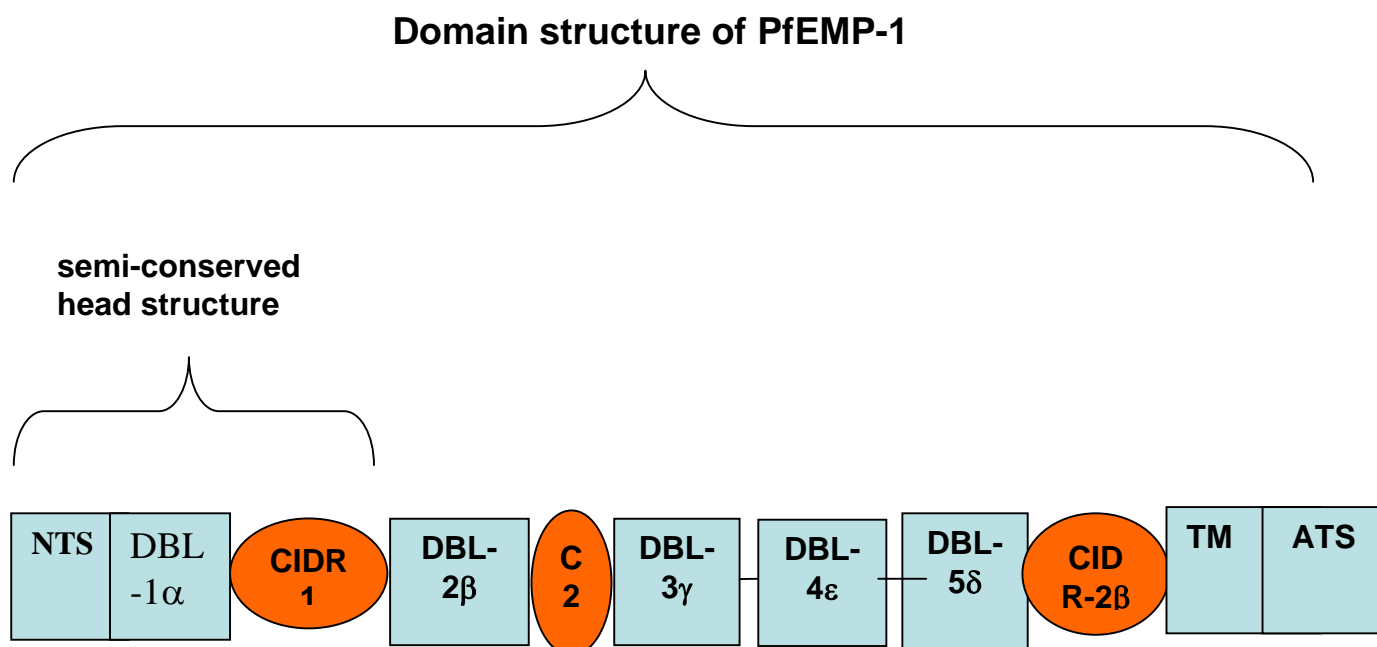
1.8 *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1)

More than 16 hours after they infect red blood cells, malaria parasites secrete and export proteins onto the surface of IRBC. One of these proteins PfEMP-1 is thought to play a significant role in malaria pathogenesis through attachment to host endothelium and other body organs and tissues in a process called cytoadhesion and also has been implicated in the

malaria parasite ability to evade host immune responses via antigenic variation. PfEMP-1 is a heterogenous protein ranging from 200 to 400 kDa. Apart from cyoadhesion and antigenic variation, PfEMP-1 has also been found to mediate the attachment of infected red blood cells to uninfected red blood cells (resetting) and also in the ability of IRBCs to attach to other IRBCs (agglutination).

PfEMP-1 is encoded by var genes, a highly polymorphic family of genes that undergo clonally antigenic variation (Kyles et al., 2001). Though very diverse, these genes have a similar basic structure consistent of two exons. The first exon codes for multiple extracellular domains that are homologous to the cysteine-rich domains of *P. falciparum* erythrocyte binding antigens (EBA 175) (Rodriguez et al., 2000) and the *P. knowlesi* duffy binding proteins (DABP) (Adams et al., 1992) and have therefore been termed Duffy binding-like domains (DBL). A short trans- membrane region precedes the second exon coding for a conserved sub- membrane acidic terminal segment (ATS) that probably anchors PfEMP1 at the knob (Voigt et al, 2000). The first extra cellular domain (-DBL1-) is relatively conserved and is adjacent to another semi-conserved region, the cysteine-rich inter domain region 1 (CIDR-1) (Smith et al., 2000). The relative conservation of these two regions is suggestive of functional constrain and in fact the two regions have been shown to be the bonding sites for CD36, PECAM/CD31, blood group antigens and glycosaminoglycans (Baruch et al., 1997;Smith, 1998 and Chen et al., 2000). PfEMP1 from different parasites isolates have different number of extracellular domains and this appears to influence the isolates binding phenotype.

Figure 1.2: *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) architecture and binding domains



The intracellular domain, acid terminal segment (ATS), is highly conserved and anchors PfEMP-1 to the red cell membrane. The extracellular part is highly variable but assembled from four domains: NTS, DBL, CIDR-1 and C2. CD36 is considered to be the major endothelial sequestration receptor, other receptors being used less frequently, but might be important in disease pathogenesis. Abbreviations: ATS, acidic terminal segment; CIDR-1, cysteine-rich Interdomain region-1; DBL, duffy binding-like domain; NTS, N-terminal segment; TM, transmembrane domain. (Figure modified from Bull et al, PLOS pathogen Vol 1 (3) 11 November 2005).

The malaria parasite protein PfEMP-1 is inserted on surface of infected cell during the second half of asexual blood-stage development. The mature parasites are absent in the peripheral circulation as they sequester in the capillaries of organs such as the heart (Luse et al., 1971), liver (Gilks et al., 1990) and brain (Aikawa et al., 1988). Sequestration is thought to contribute to malaria pathology by occluding blood flow in the affected organs while helping the parasite avoid splenic clearance. Attachment of IRBC to host endothelium receptors and other vital organs is mediated by PfEMP-1. Some of the receptors that bind to PfEMP-1 include; Intercellular adhesion molecule-1 (ICAM-1), heparin sulfate,

CD36, chondroitin sulfate A (CSA) and complement receptor 1 (CR1) (Chen *et al.*, 1998, Rowe, 1997, Baruch, 1997 Reeder, 1999 Smith *et al.*, 2000).

PfEMP-1 as the adhesive parasite ligand

Plasmodium falciparum infected red blood cells possess the unique ability to adhere to vital host organs and a range of host endothelium receptors during the second part of its life cycle. This ability is attributed to the parasite protein PfEMP-1. Advances in molecular biology techniques has enabled the mapping and elucidation of specific domains within PfEMP-1 protein that have a role in binding of IRBC to host receptors. Some of the receptors implicated in cytoadhesion include; heparin sulfate, CD36, chondroitin sulfate A, ICAM-1 and complement receptor-1 (Chen *et al.*, 1998, Rowe *et al.*, 1997, Baruch *et al.*, 1997, Reeder *et al.*, 1999 and Smith *et al.*, 2000). Most of the PfEMP-1 binding domains are located at the extracellular N-terminal point of PfEMP-1. Experimental findings have identified at least three domains to be involved in parasite binding ability to a number of host receptors namely; DBL1, DBL2 and CIDR-1 (Chen *et al.*, 2000). The CIDR-1 domain of PfEMP-1 has been implicated in mediating binding to CD36 receptor.

Binding of infected erythrocytes to CD36 is especially interesting as it is a feature of many parasite isolates (Newbold *et al.*, 1997 and Rogerson *et al.*, 1999). Antibodies raised against a recombinant protein corresponding to a portion of the CIDR-1 α domain of two var genes present within the Malayan Camp parasite isolate, blocked adherence of this strain to immobilised CD36 (Baruch *et al.*, 1995). Furthermore these antibodies immunoprecipitated the same fragment of CIDR-1 α as purified CD36 (Baruch *et al.*, 1996) and, then anti-CD36 monoclonal antibodies were shown to block adherence of parasitized erythrocytes to CD36 this occurred regardless of differences in the expressed PfEMP-1 protein (Barnwell *et al.*, 1989). This suggests that most variants of PfEMP-1 bind to the same region of CD36 and as such the CD36-binding domain of PfEMP-1 may have a conserved sequence and structure. Analysis of the sequences corresponding from diverse parasites to the CD36 binding region of PfEMP1, located within the CIDR-1 α domain, demonstrated that these sequences were not identical but did display some homology. This was most apparent by conservation of cysteine residues at either end of the fragment resulting in a highly conserved predicted three-dimensional structure (Baruch *et al.*, 1997). It is thought that this structure is maintained regardless of differences in amino acid sequence and that it is the conformation of the protein fragment rather than the sequence itself that is essential for function. Exceptions to CIDR-1 α mediating binding to CD36 are CSA-binding parasite isolates. PfEMP-1 was discovered to be a parasite ligand mediating binding to CSA in 1999 (Reeder *et al.*, 1999). Selection of infected erythrocytes on CSA *in vitro* leads to a loss of CD36 and ICAM-1 binding and the ability to form rosettes (Gamain *et al.*, 2001 Beeson *et al.*,

2004). As a consequence of binding to CSA via CIDR-1 α , CSA and CD36- binding are mutually exclusive phenotypes (Gamain *et al.*, 2002). Selection of a laboratory isolate for binding to CSA initiated a switch in the var gene expressed coinciding with a change in antigenic type. A specific var gene was transcribed in these CSA-selected parasites, different to the dominant var transcribed in the parental non-selected clone (Reeder *et al.*, 1999). This var gene was called CS2. The direct association of this PfEMP-1 variant and CSA binding was demonstrated by almost complete reduction in adhesion in the presence of antibodies raised against some of the domains of this protein, specifically anti- DBL3 γ and anti-CIDR-1 α antibodies. Interestingly the inhibitory effect of anti-DBL3 γ antibodies was specific to CSA binding but the anti-CIDR-1 antibodies also abrogated binding of other non-CSA selected parasite lines to CD36. In fact it has become clear that although the PfEMP1 variants expressed on the surface of CSA-binding parasites contain CIDR-1 domains they do not bind to CD36 (Reeder *et al.*, 1999). This suggests that the DBL γ -type and the CIDR-1 domains of parasites displaying binding to CSA may combine to form the CSA-binding region. Further work suggested that recombinant CIDR-1 domains on their own might be able to mediate binding to CSA in the absence of DBL γ -type domains. However, the expressed CIDR-1 protein in this particular case was isolated from a parasite line unable to bind CSA and so this adhesion characteristic may not underline in vivo mechanism. Although binding to CSA has been largely attributed to the DBL γ domains of PfEMP1, many expressed DBL γ domains do not bind CSA (Gamain *et al.*, 2004). There are varying amounts of homology and diversity in sequence amongst DBL domains without any clear conserved areas among those domains that do bind CSA (Gamain *et al.*, 2004). An analysis of 5 DBL sequences from placental isolates revealed a homology ranging from 39 to 55% (Khattab *et al.*, 2001). The suggestion from serological studies, (Fried *et al.*, 1998), that a conserved ligand may be responsible for placental binding and may thus be amenable to targeting in a pregnancy-associated-malaria specific vaccine was given support by the finding that one var gene was the dominant transcript in many placental isolates as well as many isolates selected for adhesion to CSA (Salanti *et al.*, 2003). This gene, called var2csa, has an atypical structure lacking both DBL and CIDR-1 domains instead possessing three DBLX-type and three DBL- γ -type domains (Salanti *et al.*, 2003). Until recently a CSA-binding domain had not been identified for this gene although substantial homology existed between the third DBL domain of var2csa and the minimal-binding region of the DBL domain of another commonly expressed CSA-binding var gene, FCR3varCSA (Gamain *et al.*, 2004). Recent work, however, has led to the identification of multiple CSA-binding domains within both 3D7 and A4 var2csa genes, including DBL2-X and DBL6. Of note though the third DBL domain, which as described showed homology to the CSA binding region of

FCR3varCSA, did not bind to CSA (Gamain *et al.*, 2005). Despite these encouraging results, the role of var2csa remains controversial. Analysis of expression of membrane proteins in placental and CSA-binding isolates showed that neither var2csa nor FCR3varCSA were preferentially displayed on IRBC (Fried *et al.*, 2004).

***P. falciparum* cysteine-rich Interdomain region-1 (CIDR-1) and CD36 binding**

CD36 is a 88-kDa glycoprotein expressed on different cells including dendrite cells and monocytes. It has been shown in-vitro that majority of field *P. falciparum* isolates bind to CD36 ((Newbold *et al.*, 1997, Roberts *et al.*, 1992). In malaria parasite cytoadherence to endothelium, CD36 has been shown to be the major receptor involved contributing to sequestration of IRBCs (Ho *et al.*, 1999). CD36 binding of IRBC in post-capillary venules is strong and long lasting (Cooke *et al.*, 1994). CD36 binding portion of PfEMP-1 has been shown to lie within the region, CIDR-1 α , between amino acids 139-184 of PfEMP-1 (Baruch *et al.*, 1999). The evidence for the CIDR-1 α of PfEMP-1 being the CD36 binding domain comes from the fact tryptic peptides containing the CIDR-1 α domain bound to CD36, and that anti-CIDR-1 α antibodies blocked the binding of IRBC to immobilised CD36 (Baruch *et al.*, 1999). Interaction between CIDR-1 α domain and CD36 receptor is important for parasite survival in the human host. Besides its implication in pathogenesis of malaria, CIDR-1 α -CD36 interaction has been implicated in inhibition of dendritic cell function (Urban *et al.*, 2001b; Urban and Roberts., 2002). DCs are important antigen presenting cells and many pathogens including malaria parasites have evolve ways of either inhibiting or modulating their functions. Although IRBC modulated DCs produce TNF-a and IL-10, they were unable to induce T cells activation and production of IL-12 (Urban *et al.*, 2001b).

1.9Dendritic cells

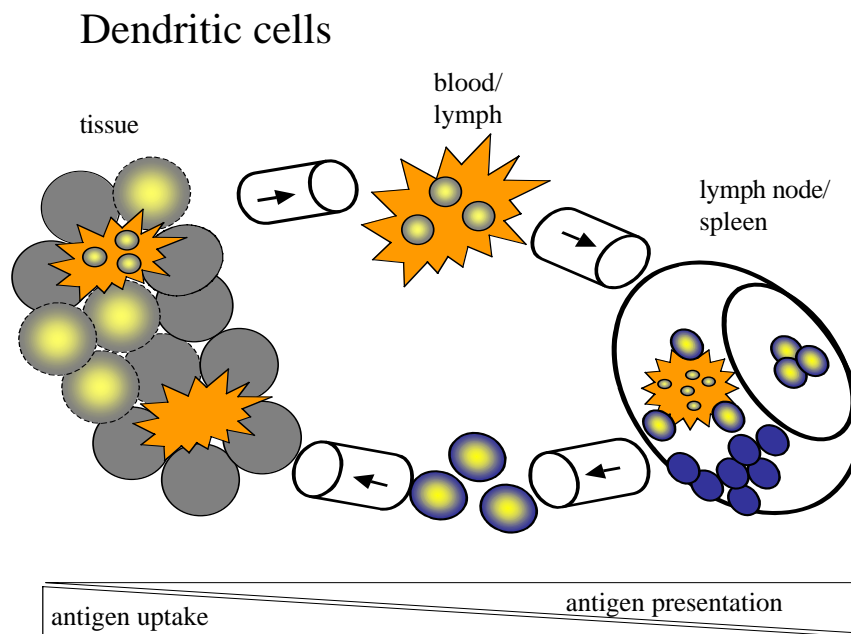
Dendritic cells (DCs) are a small population of white blood cells constituting less than 1% of the total white cell count in a normal person. First described by Ralph Steinman in the early 1970s

because their processes closely resembled the dendrites of nerve cells, dendritic cells have gained considerably attention in the last few years in the field of Immunology. DCs participate in initiation of immune responses, and maintenance of peripheral tolerance (Banchereau *et al.*, 2000). DCs are unique antigen presenting cells with the ability to activate naïve immune responses and hence capacity to prepare subsequent memory immune responses. DCs precursors originate in the bone marrow with the immature form actively circulating in peripheral blood circulation. The availability of dendrites gives the immature DCs ability to mount endocytic and phagocytic activities. At steady state, the immature form of DCs patrol their environment, take up protein and cellular debris, and a proportion migrate into secondary lymphoid tissues. Upon encounter with foreign antigen, DCs undergo a complex maturation process and become specialized in antigen presentation. This is achieved by up-regulation of cell surface MHC class I and II and surface molecules CD86, CD40, CD54, CD80 and CD83 (Banchereau *et al.*, 1998). Concomitantly, the DC down-regulates its antigen-capture mechanisms. DCs migrate to T cells rich areas in the lymphoid organs interact and present the antigen to T cells in the context of MHC molecules thus inducing immune responses (Sallusto *et al.*, 1998, Gerosa *et al.*, 2005).

In human peripheral blood, two major subpopulations of DCs can be identified by cell phenotype and morphology (Dzionek *et al.*, 2000). The DC subsets have distinct but overlapping functions, plasmacytoid DCs (pDCs) express HLA DR, CD123 and blood dendritic cell antigen 2 (BDCA2), and are the main producers of IFN- α , while myeloid DCs (mDCs) express HLA DR, CD11c and CD1c also known as blood dendritic cell antigen-1 (BDCA1) and are the main producers of IL-12 (Dzionek *et al.*, 2000, Patterson *et al.*, 1999, Heufler *et al.*, 1996). Studies on animal models of malaria suggest activation and migration of mDCs into the T-cell areas of the spleen upon malaria infection leading to induction of IFN γ -producing T cells (Stevenson and Riley., 2004). However in *in vitro* studies, adhesion of *P. falciparum*-infected human erythrocytes to mDCs via CD36 can modulate

mDC maturation and function whereby they fail to produce IL-12 and activate T cells but produce large amounts of IL-10 instead (Urban *et al.*, 2001). Plasmacytoid and myeloid DCs differ not only in their location and cytokine secretion but also in the expression of pattern recognition receptors (PRRs), such as TLRs and scavenger receptors, as well as Fc receptors, providing the ability for each DC subset to respond to a distinct but overlapping repertoire of pathogens (Kaisho and Akira., 2003).

Figure 1.3 Antigen uptake and presentation by dendritic cells



DC circulating precursors arise from their source in the bone marrow. During the immature state DCs have high phagocytic and endocytic abilities. At steady state, the immature form of DCs patrol their environment, take up protein and cellular debris, and a proportion migrate into secondary lymphoid tissues. Upon encounter with foreign antigen, DCs undergo a complex maturation process and become specialized in antigen presentation. This is achieved by up-regulation of cell surface molecules including MHC class I and II and CD40, CD80, CD54 and CD86. Concomitantly, DC down-regulates its antigen-capture mechanisms. DCs then move and home to secondary lymphoid organs and using the costimulatory and MHC molecules select for T cells that are specific for the antigens. The antigen presentation initiates immune responses. DCs have the ability to present antigens to specific CD8⁺ cytotoxic T cells via MHC class I or MHC class II select CD4⁺ T cells. DCs also possess unique ability to activate B cells, eosinophils, macrophages and natural killer cells via non-antigen specific ways. DCs secrete cytokines and chemokines that causes the effector cells to reside to areas of infections.

Dendritic cells and Malaria

Dendritic cells are the only antigen presenting cells that can act as a vital connection in immune responses with the ability to link the innate and adaptive responses and are responsible for presenting antigen, activating naive T-cells and enhancing antibody production (Liu *et al.*, 2001). Immature dendritic cells are highly phagocytic, internalising antigens by various pathways including those involving CD36 (Albert *et al.*, 1998). Antigen uptake then induces dendritic cell maturation process, characterized up-regulation of expression of DC surface molecules responsible for presentation of the microbe to antigen specific T cells. There is also production of cytokines by DCs that activate effector immune cells. During antigen uptake there is up-regulation of expression of CD80, CD40 and CD86. HLA DR is also upregulated to prepare DCs to present antigens to T cells. However in malaria infections, infected red blood cells (IRBCs) have been shown to modulate monocyte derived DCs maturation in-vitro by adhering to CD36 that is expressed on DCs (Urban *et al.*, 1999). DCs that were pre-incubated with IRBCs failed to mature even after activation stimuli tissue necrosis factor (TNF) or LPS were added (Urban *et al.*, 1999 and Urban *et al.*, 2001). The binding of IRBC through the *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) to CD36 expressed on DCs is thought to be the modulating agent that inhibits DC maturation. In children with malaria infection, the expression of HLA DR was dramatically reduced in either the mild or severe form of malaria as compared to non-malaria parasite children (Urban *et al.*, 2001).

Inhibition of DC maturation has two beneficial consequences for microbes: firstly, prevention of microbe-specific immunity and secondly--even more deviously--induction of microbe-specific tolerance, when immature DCs present microbial antigens in the absence of costimulatory signals. The first mechanism is thought to be dominant trend in *P. falciparum* infections.

Monocyte-derived Dendrite cells (MoDCs)

Early observations suggested that DCs could be generated *in vitro* by exploiting appropriate "progenitor" cells, such as monocytes (Peters *et al.*, 1991) and cultured in a conditioned medium with appropriate cytokine environment. A common approach in generating cells with DC properties has been culturing monocytes from peripheral blood mononuclear cells. Under the provision appropriate growth and differentiation cytokines, IL-4 and GM-CSF, monocytes give rise to a good proportion of cells with DC features. When stimulated with lipopolysaccharide or tissue necrosis factor, the monocyte-derived DCs are able to induce antigen-specific T cells responses *in-vitro*. GM-CSF is a central mediator for the generation of DCs *in vitro* (Inaba *et al.*, 1992) and was one of the first cytokines identified to have an effect on DCs. GM-CSF is a 20-30 kDa glycoprotein, which is synthesized by lymphocytes, monocytes, fibroblasts and endothelial cells. Its major function is to prolong cellular survival. It promotes the differentiation of monocytes to large macrophage like cells, increasing their metabolism and function as APC by enhancing MHC II expression (Fischer *et al.*, 1988) IL-4 is a 18-20 kDa glycoprotein that is produced primarily by activated T cells. It is felt to be the principle factor controlling the differentiation of monocytes into DCs, as monocytes cultured in the presence of IL-4 acquire a macrophage like DC morphology (Velde *et al.*, 1988). Upon exposure to IL-4, cells increase in size and develop extensive processes. Together both cytokines have the capacity of inducing a large pool of potent APC, from peripheral blood monocytes, or adherent apheresis cells. Both cytokines act to up-regulate DCs properties and function. Addition of both growth factors to blood monocytes cultures induces their differentiation into immature DC. These cells which efficiently capture and process antigen, express MHC I and II, CD1, B7 but not CD14.

OBJECTIVES

We set out to attain the following objectives;

1. To analyse the expression of CD36 on peripheral blood dendritic cells and monocyte-derived dendritic cells.
2. To analyse the binding of CIDR molecules to CD36 receptor expressed on monocytes, peripheral blood DCs and monocyte-derived dendritic cells (MoDCs)
3. To analyse whether CIDR binding is sufficient to mediate modulation of monocyte-derived dendritic cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Separation of peripheral blood mononuclear cells (PBMCs)

Buffy coats were obtained from the national blood services, Bristol. 20mls of the buffy coat was layered into equal volume of Lymphoprep (Nycomed, Oslo, Norway) reagent in a 50ml Falcon tube. Sample was then centrifuged at 2000rpm, 30minutes without brakes at room temperature. The peripheral blood mononuclear cells layer was then collected from the interphase by Pasteur pipette and transferred into a clean 50ml Falcon tube placed on ice. The PBMCs tube was filled with R2 medium and cells centrifuged at 1600rpm for 7minutes at 4C. The supernatant was discarded and the pellet resuspended in 5mls of R2 medium. The haemocytometer was then charged with 10ul of the PBMCs suspension and number of cells counted by microscopy.

2.2 Magnetic cell sorting (MACS)

In most experiments we used super magnetic beads coated with MAbs to either deplete or positively select for a particular cell phenotype. In all these cases, a powerful magnet fitted with necessary adapters (VarioMACS, Miltenyi Biotec, Germany) was used according to the manufacturers protocol.

2.3 BDCA-4 dendritic cell isolation

Positive selection of BDCA-4 positive plasmacytoid dendritic cells (pDCs) was done using a magnetic labelling system according to the manufacturers protocol (Miltenyi Biotec, Germany). The DCs isolation was done in two steps. Firstly B cells were magnetically labelled with CD19 microbeads and subsequently depleted by separation on a MACS

column (Miltenyi Biotec, Germany). In the second step, BDCA-4 blood DCs in the B cell depleted flow through fraction were indirectly labelled with biotin-conjugated BDCA-4 antibody and anti-biotin microbeads. Upon separation, the labelled BDCA-4 blood DCs are retained in the column and eluted after removing the column from the magnetic field. Briefly PBMC were resuspended in R2 medium at 10^8 cells per 200ul. To deplete B cells, 100ul of each of FcR blocking reagent, CD19 microbeads and BDCA-4 biotin antibody per 10^8 cells was added, mixed and incubated for 15 minutes at 4°C . The cells were then washed in 20 volumes of wash buffer and the pellet resuspended in 500ul of the wash buffer per 10^8 cells. The cell suspension was then loaded into a LS column (Miltenyi Biotec, Germany) equilibrated with wash buffer and placed in a strong magnetic field. To positively select BDCA-4 positive DCs, the flow through collected above was centrifuged and resuspended in 400ul per 10^8 cells and incubated at 4°C in the presence of anti-biotin microbeads. The cells were washed as above and then resuspended in a final volume of 500ul and then applied in to an LS column placed in a powerful magnetic field. BDCA-4 positive DCs were then flushed out of the column into a 15ml falcon tube using a plunger.

2.4 Purification of monocytes with CD14 microbeads

PBMC were separated and washed as described above. The PBMCs were resuspended in 2.5mls of wash buffer. CD14 microbeads (Miltenyl biotech) were then added at a concentration of 300ul per buffy coat, or 20ul of CD14 microbeads added per 1×10^7 PBMCs. The cells were incubated at 4°C in fridge for 30 minutes. After incubation cells were washed once in 20mls wash buffer by centrifuging at 1400rpm for 7 minutes at 4°C . The pellet was then resuspended in 3mls of wash buffer. The LS column was washed with 3mls of R2 medium before the loading of the cell suspension into the column. CD14 positive

monocytes were then flushed out of the column into a 15ml falcon tube using a plunger after removing the column from the magnetic field using 3mls of eluting buffer.

2.5 Binding assay with MC-CIDR-1, CSA-CIDR-1 and anti-CD36 antibodies

Monocyte-derived dendritic cells and peripheral DCs were centrifuged and the pellet resuspended in R2 medium to a concentration of 1×10^6 cells/ml. 100,000 cells were added to individual wells of 96-round bottom well plate (Nunclon) for the binding assay.

Cells were incubated with an increasing concentration of biotinylated MC or CSA-CIDR (0,0.1, 0.3, 1 and 3 μ g/ml). The cells were incubated on ice for 30 minutes and washed twice with 200 μ l of wash buffer. The cells were then stained with FITC-conjugated anti-biotin antibody and incubated for 30 minutes on ice. After the incubation, the wells were washed twice with wash buffer and finally resuspended in 200 μ l of fix buffer before run on flow cytometry.

2.6 Blocking of binding assay

Monocytes, monocyte-derived DCs and plasmacytoid DCs were centrifuged and the pellet resuspended in R2 medium to a concentration of 1×10^6 cells/ml. 100,000 cells were added to individual wells of 96-round bottom well plate (Nunclon) for the binding assay.

Cells were pre-incubated with medium alone, 1 μ g/ml of MC CIDR, CSA CIDR or anti-CD36 antibody. After 30 minutes incubation on ice, the cells were washed twice with 200 μ l of wash buffer. The cells were then incubated with 1 μ g/ml of biotinylated MC-CIDR before staining with FITC-conjugated anti-biotin antibody as described above. After the final incubation, the wells were washed twice with wash buffer and finally resuspended in 200 μ l of fix buffer ready for analysis with flow cytometry.

2.7 Monocyte-derived dendritic cells culture (MoDCs)

To induce differentiation to dendrite cells, monocytes were resuspended to 1×10^6 cells/ml in either R2 medium (serum) or X VIVO15 (for serum free medium). One million monocytes were transferred into individual wells of 12 well plates. The medium was supplemented with 50ng/ml each of IL-4 and GM-CSF. The plates were incubated at 37°C with 5% CO₂ concentration. The medium plus the cytokines were replenished every two days and monocyte-derived dendrite cells were harvested between day 5 and day 7.

2.8 Maturation assay

Between day 5 and day 7 of culture, monocyte-derived dendrite cells were harvested washed twice in cold R2 medium or X-VIVO 15 medium and resuspended at a final concentration of 1×10^6 cells/ml. One million cells were transferred into wells of a 12 well plate with or without LPS to induce maturation. The plates were incubated at 37°C with 5% CO₂ for between 36-48 hours. Alternatively MoDCs were pre-incubated in duplicate with different concentrations of MC-CIDR and CSA-CIDR. After two hours incubation, one well was treated with LPS to induce maturation whereas the other well left untreated.

2.9 Analysis of dendritic cells maturation by flow cytometry

36-48 hours after induction of maturation, dendritic cells were harvested from individual wells and washed twice in cold R2 medium. The pellet was resuspended in FACS buffer at a volume of 200ul of the staining buffer. 30ul of the cell suspension were transferred into individual wells of the 96-round bottom well plate (Nunclon) and mixed with 30ul of buffer (negative control) or 30ul of the primary antibodies against HLA DR, CD40, CD54, CD80, CD83 or CD86. After incubating for 30 minutes on ice the wells were washed twice in FACS buffer. FITC-conjugated anti-mouse Ig antibody (1:25 DAKO) was added to each

well. After incubation for 30 minutes on ice the cells were washed and suspended in 200ul of fix buffer ready for flow cytometry analysis.

2.10 Source of CIDR-1 molecules

Both MC CIDR and CSA CIDR were kind donations from Damien Cordery of Churchill, Oxford. The MC CIDR was isolated from the CD36 malaria parasite Malayan Camp MC*var*CIDR whereas the CSA CIDR was from a non-CD36 binding malaria parasite FCR3*var*CSACIDR as described (Gamain *et al.*, 2001).

2.11 Data analysis

After acquisition, flow cytometric data was further analysed by Flowjo software version 4.3 (Treestar, Inc., USA). Results from flow cytometry data were stored and formatted in Microsoft Excel (Microsoft Corporation, USA) and all graphs shown were plotted in PRISM software (PRISM company USA). Where appropriate, statistical analyses were done using Stata version 8.0 (Stat Corporation, USA). The relative fold increase in surface marker expression was calculated by dividing the mean fluorescence intensity (MFI) of LPS-exposed DCs by the MFI of immature DCs after subtracting values obtained with isotype-control antibody.

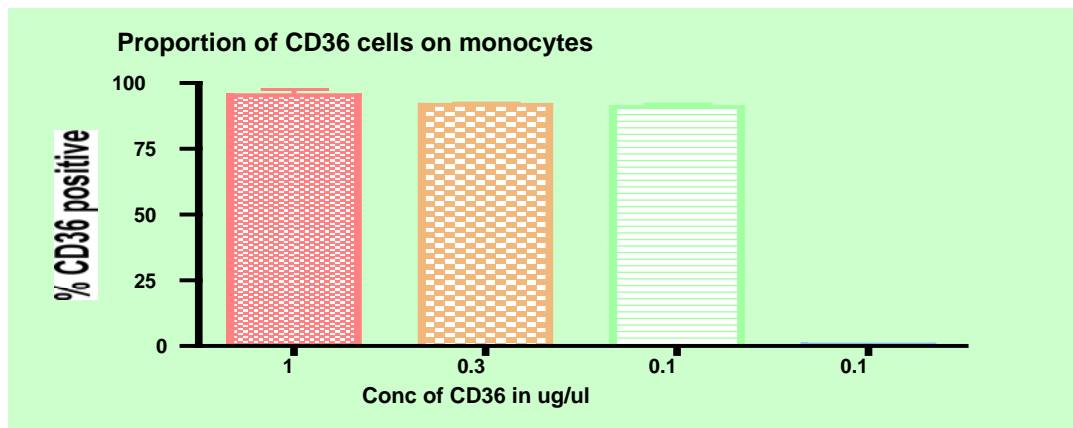
CHAPTER 3

RESULTS

3.1 Expression of CD36 on monocytes and dendritic cells

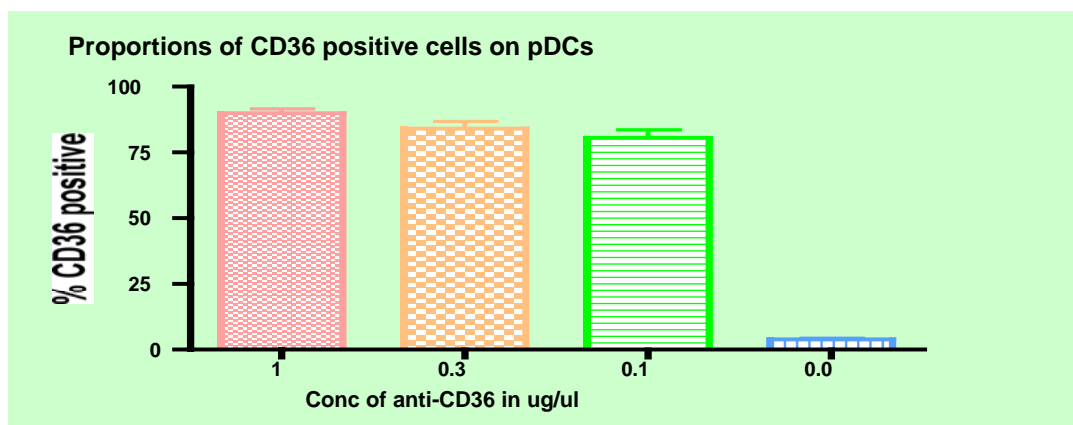
The CD36 expressed on endothelial cells have been thought to be an important receptor for malaria parasite cytoadherence. We were keen to analyse the proportion of CD36 positive cells on dendritic cells and monocytes and relate this to the ability of dendritic cells and monocytes to bind to different CIDR-1 molecules. Monocytes, plasmacytoid dendritic cells and monocyte-derived dendritic cells were incubated in different dilutions of anti-CD36 antibodies and the proportion of CD36 positive cells measured by flow cytometry. The proportion of CD36 positive cells was relative high in monocytes, monocyte-derived dendritic cells and peripheral blood plasmacytoid dendritic cells with a median of 92%, 60% and 85% of the CD36 positive cells respectively (Figures 3.1, 3.2, 3.3, Table 1). However, the proportion of CD36 positive cells was highest in monocytes followed by peripheral blood plasmacytoid dendritic cells. Monocyte-derived dendritic cells had the least proportion of CD36 positive cells at 60%. The expression of CD36 on dendritic cells is important as it enables the binding of dendritic cells and monocytes to the malaria parasite through the PfEMP-1 molecule.

Figure 3.1 Proportion of CD36 positive cells on monocytes



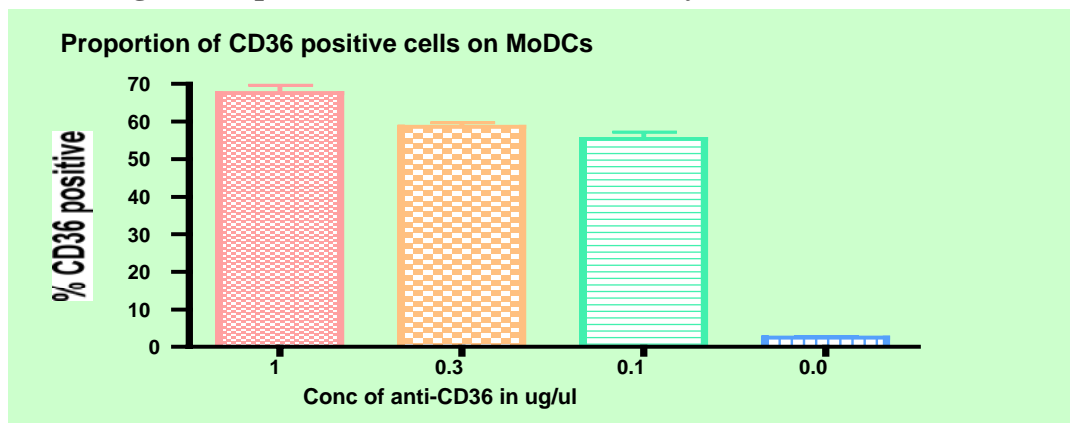
Monocyte-derived DCs were resuspended to a concentration of one million cells/ml. 100,000 cells were incubated with an increasing concentration of anti-CD36 antibody at (0, 0.1, 0.3, and 1ug/ml). The cells were stained with mouse anti-human FITC secondary antibody before analysis on flow cytometry. Shown are proportions of CD36 positive cells using different concentrations of the antibody. n=4. The bars indicate the standard error mean for 4 independent experiments.

Fig.3.2 Proportion of CD36 positive cells on plasmacytoid dendritic cells



Plasmacytoid dendritic cells were resuspended to a concentration of one million cells/ml. 100,000 cells were incubated with an increasing concentration of anti-CD36 antibody at (0, 0.1, 0.3, and 1ug/ml). The cells were then stained with mouse anti-human FITC secondary antibody before analysis on flow cytometry. Shown are proportions of plasmacytoid DCs CD36 positive cells at different concentrations of the antibody. n=4. The bars indicate the standard error mean for 4 independent experiments.

Fig.3.3 Proportion of CD36 cells on monocyte-derived dendritic cells.



Monocyte-derived dendritic cells were resuspended to a concentration of one million cells/ml. 100,000 cells were incubated with an increasing concentration of anti-CD36 antibody at (0, 0.1, 0.3, and 1ug/ml). The cells were incubated on ice for 30 then stained with mouse anti-human FITC secondary antibody fore analysis on flow cytometry. Shown are proportions of monocyte-derived DCs CD36 positive cells at different concentrations of the antibody. n=4. The bars indicate the standard error mean for 4 independent experiments.

Table 1: Proportions of CD36 positive cells

Conc of anti-CD36 ug/ml	monocytes	Plasmacytoid DCs	Monocyte-derived DCs
n	4	4	4
1.0	96 (94.4 – 97.60)	90 (87.5 – 92.40)	67 (64.6 – 70.60)
0.3	91 (90.4–95.70)	84 (80 – 88)	58 (57.25–60.10)
0.1	90 (84.50 – 88.30)	81 (76.20–84.80)	54 (52.45 – 58.20)
0.0	1.1 (1.30 – 1.78)	3.7(3.3 – 4.3)	2.6 (2.16 – 2.95)

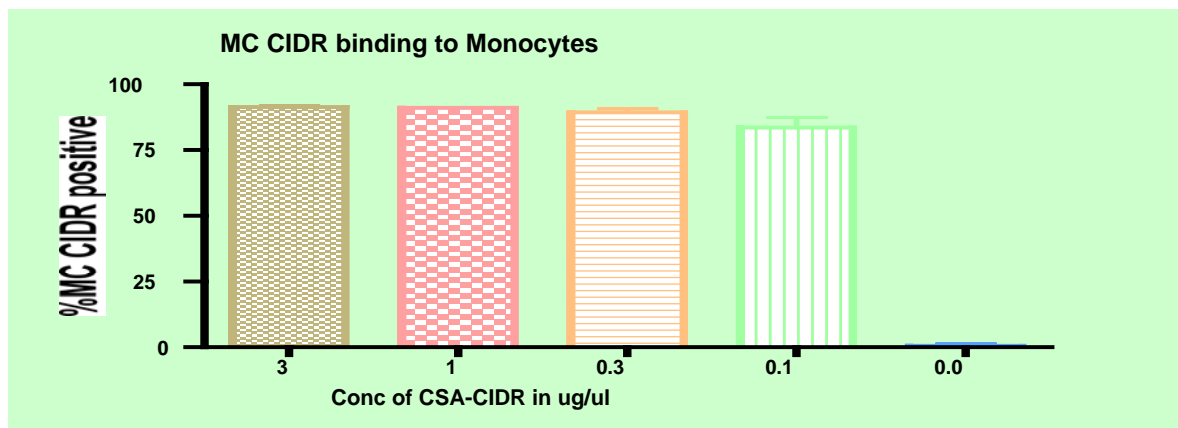
Shown are median and in parenthesis 25th and 75th percentile

3.2 Increased binding by MC CIDR-1 to monocytes, plasmacytoid dendritic cells and monocyte-derived dendritic cells

We wished to examine whether the expression of CD36 receptor on dendritic cells is associated with the binding of dendritic cells to MC CIDR-1 molecules. Monocytes, plasmacytoid dendritic cells and monocyte-derived dendritic cells were incubated in different concentrations of biotinylated MC CIDR-1 or CSA CIDR-1 and the proportion of MC CIDR-1 or CSA CIDR-1 positive cells measured by flow cytometry.

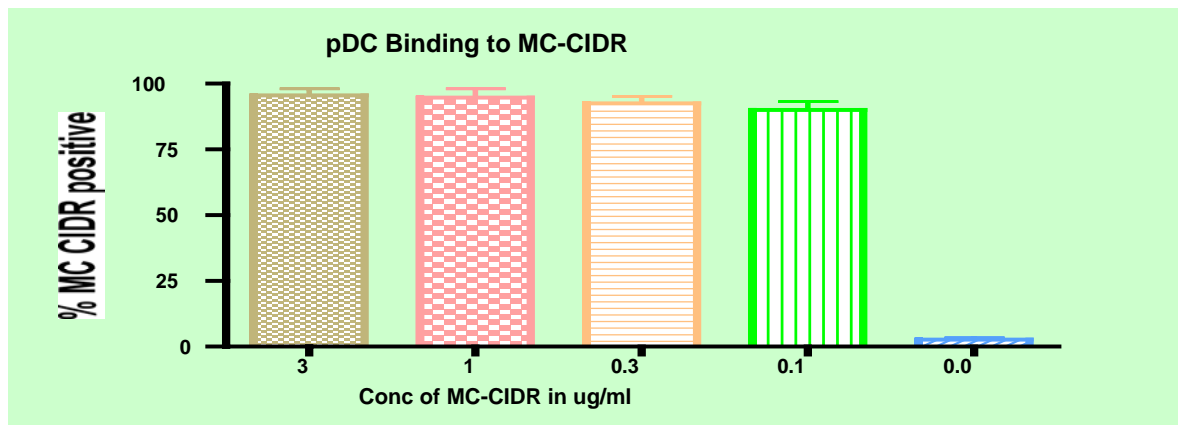
There was a higher proportion of MC CIDR-1 positive cells for monocytes and peripheral blood plasmacytoid dendritic cells at a median of >90.0% across all dilution used (figures 3.4, 3.5), while the monocyte-derived dendritic cells had a median of >65%. The binding of dendritic cells to MC CIDR-1 molecules gave comparable proportions to that of CD36 positive cells done earlier (table 1). These findings emphasise the importance of availability of CD36 as a malaria parasite CIDR-1 binding receptor on dendritic cells. Binding of CSA CIDR-1 molecules to monocytes, monocyte-derived dendritic cells and plasmacytoid dendritic cells was minimal and resembled that of negative control with the proportion of CSA CIDR-1 positive cells at below 4% in all cells across different dilutions (figures 3.7, 3.8, 3.9). These results show that CSA CIDR-1 molecules do not bind to CD36 expressed on these cells.

Figure 3.4: Binding of MC CIDR-1 to monocytes



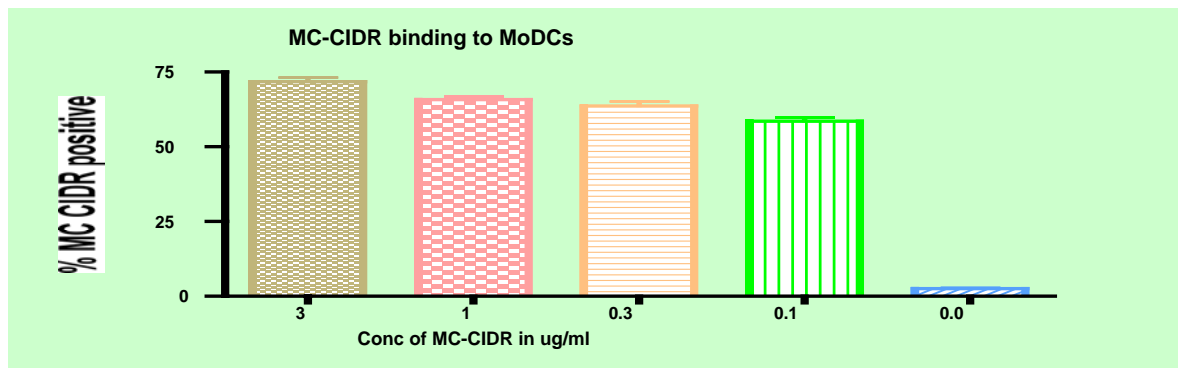
Monocytes were resuspended to a concentration of 1×10^6 cells/ml. 100,000 cells were incubated with an increasing concentration of biotinylated MC CIDR-1 (0,0.1, 0.3, 1 and 3ug/ml). The cells were stained with FITC-conjugated anti-biotin antibody before run on flow cytometry. Shown are proportions of monocytes CSA CIDR-1 positive cells at different concentrations of the protein. n=4. The bars indicate the standard error mean for 4 independent experiments.

Figure 3.5: Binding of MC-CIDR to plasmacytoid dendritic cells



Peripheral blood plasmacytoid dendritic cells were resuspended to a concentration of 1×10^6 cells/ml. 100,000 cells were incubated with an increasing concentration of biotinylated MC CIDR-1 (0,0.1, 0.3, 1 and 3ug/ml). The cells were then stained with FITC-conjugated anti-biotin antibody before analyzed on flow cytometry. Shown are proportions of plasmacytoid DCs MC CIDR-1 positive cells at different concentrations of the protein. n=4. The bars indicate the standard error mean for 4 independent experiments.

Figure 3.6: Binding of MC-CIDR to monocyte-derived dendritic cells



Monocyte-derived DCs and were resuspended to a concentration of 1×10^6 cells/ml. 100,000 cells were incubated with an increasing concentration of biotinylated MC CIDR-1 (0,0.1, 0.3, 1 and 3ug/ml). The cells were then stained with FITC-conjugated anti-biotin antibody before analyzed on flow cytometry. Shown are proportions of monocyte-derived DCs MC CIDR-1 positive cells at different concentrations of the protein. n=4. The bars indicate the standard error mean for 4 independent experiments.

Figure 3.7: Binding of CSA CIDR-1 to monocytes

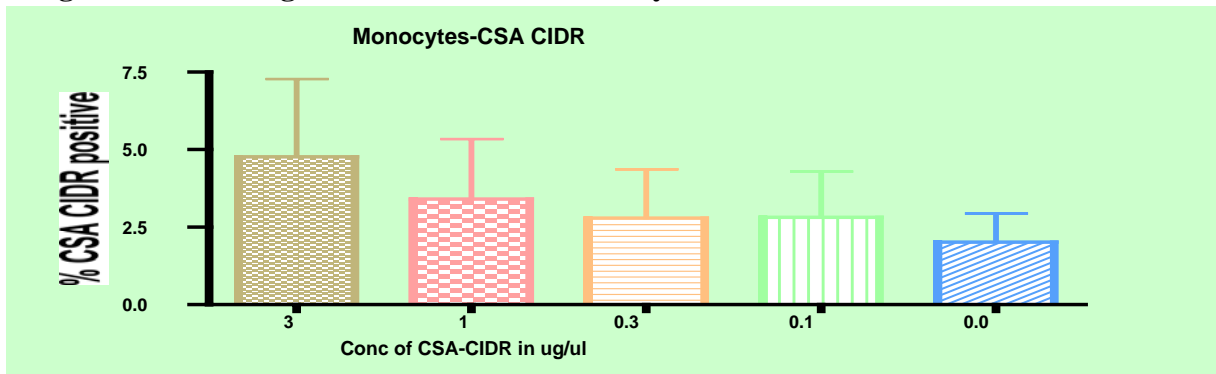
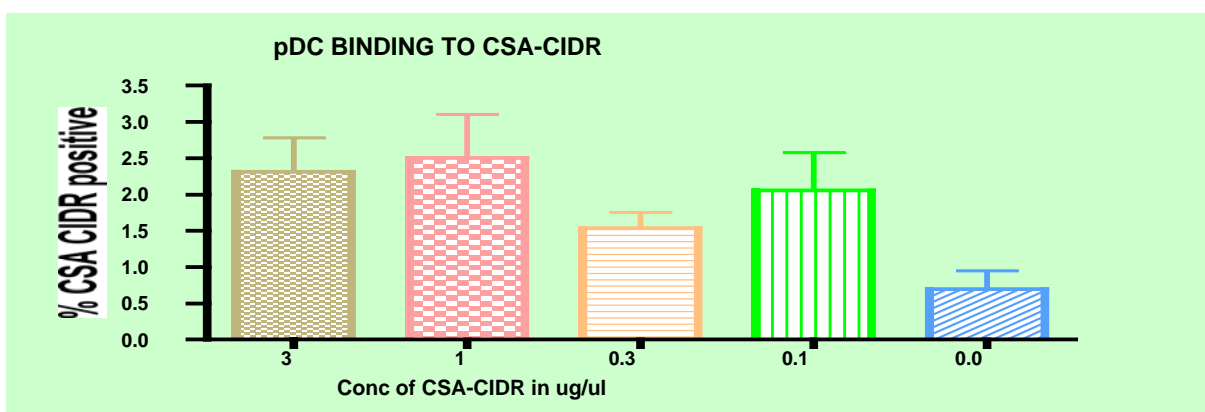
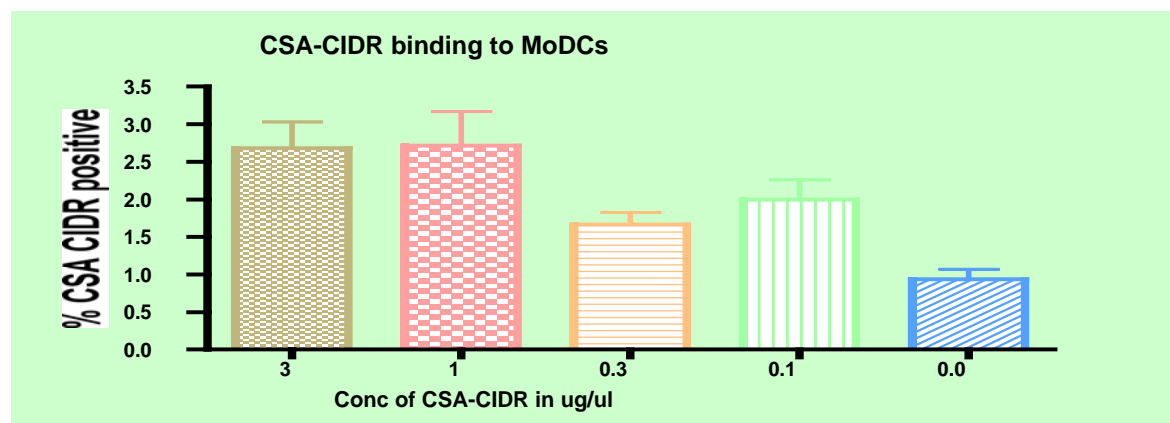


Figure 3.8: Binding of CSA CIDR-1 to plasmacytoid dendritic cells



100,000 monocytes (fig 3.7) and plasmacytoid dendritic cells (fig 3.8) were incubated with an increasing concentration of biotinylated CSA CIDR (0,0.1, 0.3, 1 and 3ug/ml). The cells were stained with FITC-conjugated anti-biotin antibody before analyzed on flow cytometry. Shown are proportions of monocytes and plasmacytoid DCs CSA CIDR-1 positive cells at different concentrations of the protein. n=4. The bars indicate the standard error mean for 4 independent experiments.

Fig 3.9 Binding of CSA CIDR-1 to monocyte-derived dendritic cells



100,000 monocyte-derived DCs were incubated with an increasing concentration of biotinylated CSA CIDR (0,0.1, 0.3, 1 and 3ug/ml). The cells were then stained with FITC-conjugated anti-biotin antibody before analyzed on flow cytometry. The bars indicate the standard error mean for 4 independent experiments.

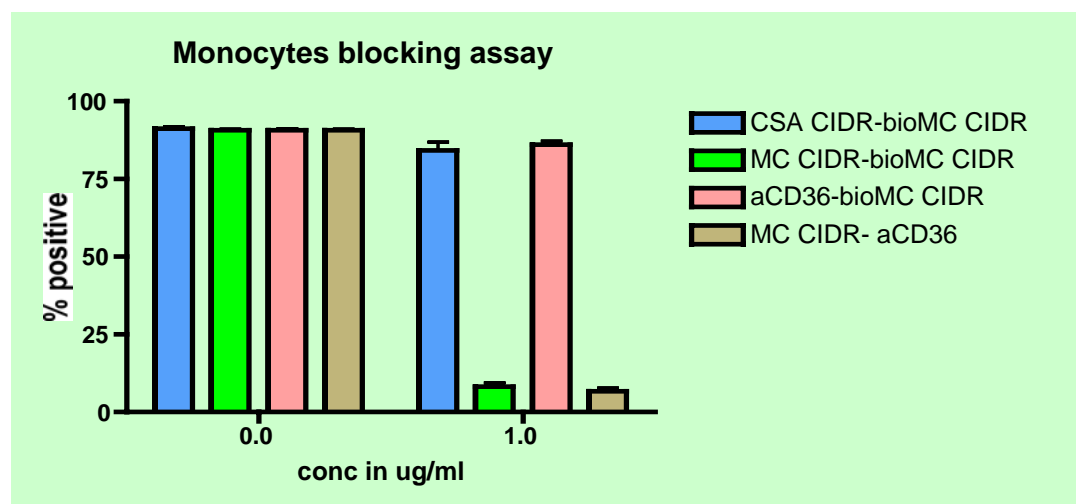
3.3 Specificity of MC CIDR-1 binding to CD36 on monocytes and dendritic cells

Having analysed the proportions of CD36 and MC CIDR-1 positive cells on monocytes and dendritic cells, the next step was to examine the specificity of MC CIDR-1 binding to monocytes and dendritic cells. To achieve this, monocytes, monocyte-derived dendritic cells and peripheral blood plasmacytoid DCs were pre-incubated in 1ug/ml of non-biotinylated MC CIDR-1 or non-biotinylated CSA CIDR-1 molecules or anti-CD36 antibodies or left untreated as negative control. The cells were then incubated with biotinylated MC CIDR-1 before addition of FITC-conjugated anti-biotin antibody.

Monocytes, monocyte-derived dendritic cells and plasmacytoid dendritic cells that were pre-incubated with medium alone or CSA-CIDR-1 molecules and then incubated with biotinylated MC CIDR-1 before addition of the FITC-conjugated anti-biotin antibody showed a high proportion of MC CIDR-1 positive cells at 91%, 65%, and 84% respectively (figures 3.10, 3.11, 3.12). Cells that were pre-incubated with non-biotinylated MC CIDR-1 before addition of biotinylated MC CIDR-1 showed low proportion of MC CIDR-1 positive

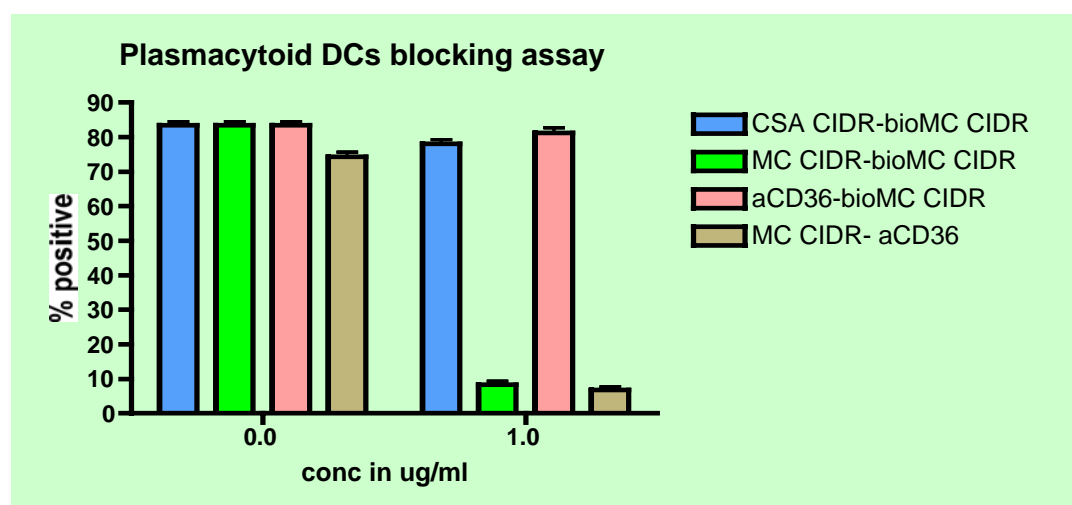
cells as analysed by flow cytometry. The median proportions of MC CIDR-1 positive cells for the monocytes, plasmacytoid dendritic cells and monocyte-derived dendritic cells were 8%, 7% and 6% respectively (table 2). The addition of non-biotinylated MC CIDR-1 before addition of anti-CD36 antibodies to the cells showed a lower proportion of CD36 positive cells after staining with the monocytes, plasmacytoid dendritic cells and monocyte-derived dendritic cells giving a median score of 7%, 6%, and 7% respectively (table 2). These results suggests that binding of MC CIDR-1 to monocytes and dendritic cells was specific and could be blocked by pre-incubation of cells with MC CIDR-1, but not by pre-incubation with CSA CIDR-1, which does not bind to CD36. Additionally, binding of anti-CD36 antibodies to monocytes and dendritic cells was blocked by pre-incubation of dendritic cells with MC CIDR-1. Pre-incubation of dendritic cells with CSA CIDR-1 had no effect on binding of anti-CD36 antibodies to monocytes and dendritic cells. These results show that MC CIDR-1 binds specifically to CD36 expressed on monocytes and dendritic cells.

Figure 3.10 Blockage of MC CIDR-1 binding to monocytes



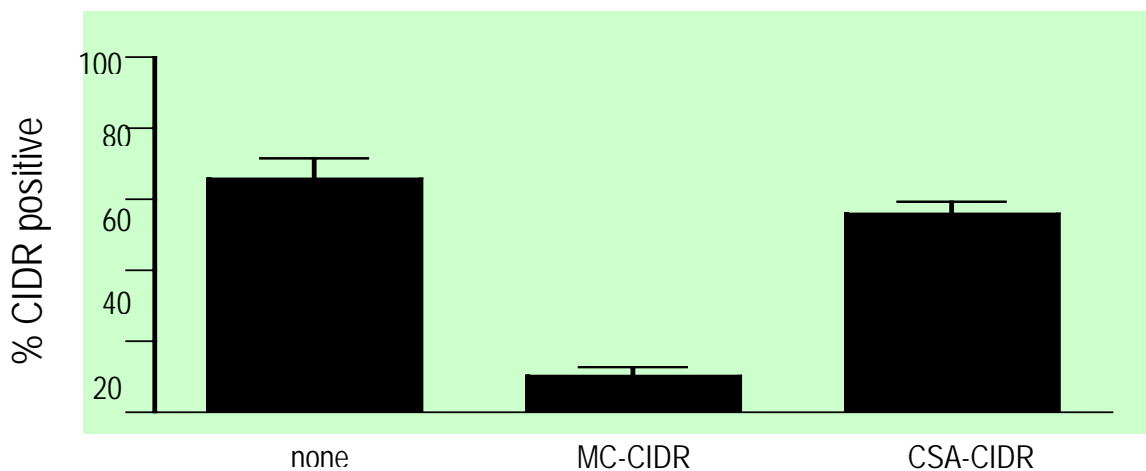
Monocyte were resuspended to a concentration of 1 million cells/ml. 100.000 cells were pre-incubated with medium alone, 1ug/ml of MC CIDR-1 or CSA CIDR-1. After 30 minutes incubation the cells were incubated with 1 ug/ml of biotinylated MC-CIDR-1 before staining with FITC-conjugated anti-biotin antibody as described previously. The bars indicate the standard error mean for 4 independent experiments.

Figure 3.11 Blockage of MC CIDR-1 binding to plasmacytoid DCs



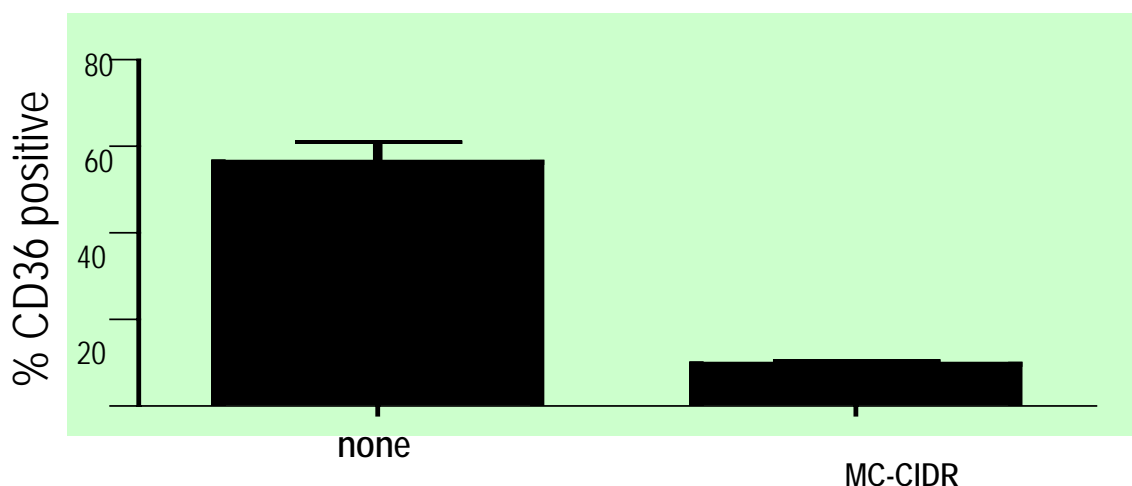
Plasmacytoid DCs were resuspended to a concentration of 1 million cells/ml. 100,000 cells were pre-incubated with medium alone, 1ug/ml of MC CIDR-1 or CSA CIDR-1. After 30 minutes incubation the cells were incubated with 1 ug/ml of biotinylated MC-CIDR-1 before staining with FITC-conjugated anti-biotin antibody as described previously. The bars indicate the standard error mean for 4 independent experiments

Figure 3.12: Blockage of MC CIDR-1 binding to MoDCs



Monocyte-derived DCs were resuspended to a concentration of 1 million cells/ml. 100,000 cells were pre-incubated with medium alone, 1ug/ml of MC CIDR-1 or CSA CIDR-1. After 30 minutes incubation the cells were incubated with 1 ug/ml of biotinylated MC-CIDR-1 before staining with FITC-conjugated anti-biotin antibody as described previously. The bars indicate the standard error mean for 4 independent experiments

Figure 3.13: Specificity of MC CIDR-1 binding to MoDCs



Monocyte-derived DCs were resuspended to a concentration of 1 million cells/ml. 100.000 cells were were pre-incubated with medium alone or 1ug/ml of MC CIDR-1. After 30 minutes incubation the cells were then incubated with 1 ug/ml of anti-CD36 antibody before staining with mouse anti-human FITC-conjugated Ig antibody as described previously. The bars indicate the standard error mean for 4 independent experiments.

Table 2: PROPORTIONS OF CELLS IN BLOCKING ASSAY

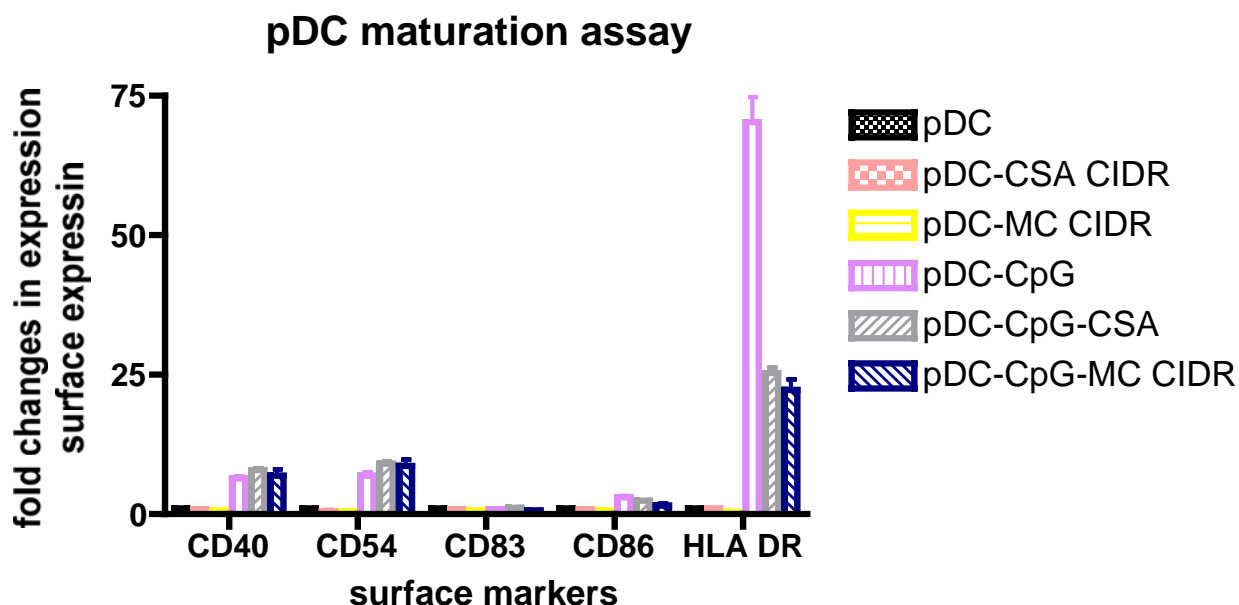
Conc ug/m	Monocytes		Plasmacytoid DC		Mono-derived DC	
	0	1	0	1	0	1
CSA CIDR- bio MC CIDR	91.2	84.3	83.5	78	65	63
MC CIDR- bio MC CIDR	91.2	8.25	83.5	7.9	65	6.5
anti-CD36- bio MC CIDR	91.2	86.1	83.5	81.3	65	64
MC CIDR-						

Proportions of positive cells after incubating monocytes, plasmacytoid DCs and monocyte-derived DCs with non-biotinylated MC CIDR-1 or non-biotinylated CSA CIDR-1 or anti-CD36 antibodies or left alone followed by incubation with biotinylated MC CIDR-1 or anti-CD36 antibodies and analysed for the percentage of cells positive for biotinylated MC CIDR-1 or CD36 by flow cytometry. The figures indicate the median proportions of positive cells.

3.4 Plasmacytoid DCs maturation by CpG Oligonucleotides

We were keen to investigate the effect of a known dendritic cell stimulant CpG oligonucleotides on maturation of plasmacytoid DCs. We therefore exposed plasmacytoid DCs of IL-3 to MC CIDR-1 or CSA CIDR-1 molecules or to control medium alone and analyze their maturation in response to CpG oligonucleotides. CpG oligonucleotides up-regulated expression of most surface markers analyzed. CD40 and CD54 were up-regulated by 6 folds as compared to the control with CD86 having a 3-fold increase in expression. HLA DR surface expression was increased by a massive 75-folds. Surprisingly the expression of CD83 was comparable to the control cells (table 3). Plasmacytoid DCs incubated with CSA CIDR-1 showed similar expression profile like control cells except there was a reduction in expression of CD54 (figure 3.14). Incubation of plasmacytoid DCs with MC CIDR-1 resulted in lower expression of all surface markers analysed as compared to control. HLA DR was reduced by up to 50% of the control expression (table 3). Incubation of plasmacytoid DCs with CSA and MC CIDR-1 followed by stimulation with CpG oligonucleotides gave almost similar results in surface expression of markers analysed. In both sets the expression of HLA DR was decreased as compared to CpG oligonucleotide stimulated plasmacytoid DCs. CSA and MC CIDR-1 treated plasmacytoid DCs which were then stimulated with CpG oligonucleotides expressed a third of HLA DR expressed in CpG oligonucleotide stimulated plasmacytoid DCs (table 3).

Figure 3.14 Maturation of plasmacytoid dendritic cells by CpG oligonucleotides



Plasmacytoid DCs (500,000 cells) were transferred into wells of a 12 well plate with or without CpG to induce maturation. Alternatively plasmacytoid DCs were pre-incubated in duplicate with different concentrations of MC-CIDR-1 and CSA-CIDR-1. After two hours incubation, one well was treated with CpG to induce maturation whereas the other well left untreated. 36-48 hours after induction of maturation, dendritic cells were harvested transferred into individual wells of the 96-round bottom well plate (Nunclon) and mixed with 30ul of buffer (negative control) or 30ul of the primary antibodies against HLA DR, CD40, CD54, CD83 or CD86. After incubation FITC-conjugated anti-mouse Ig antibody (1:25 DAKO) was added to each well before analyzed on flow cytometry.

Table 3: Plasmacytoid DCs maturation with CpG

Fold changes in surface markers expression

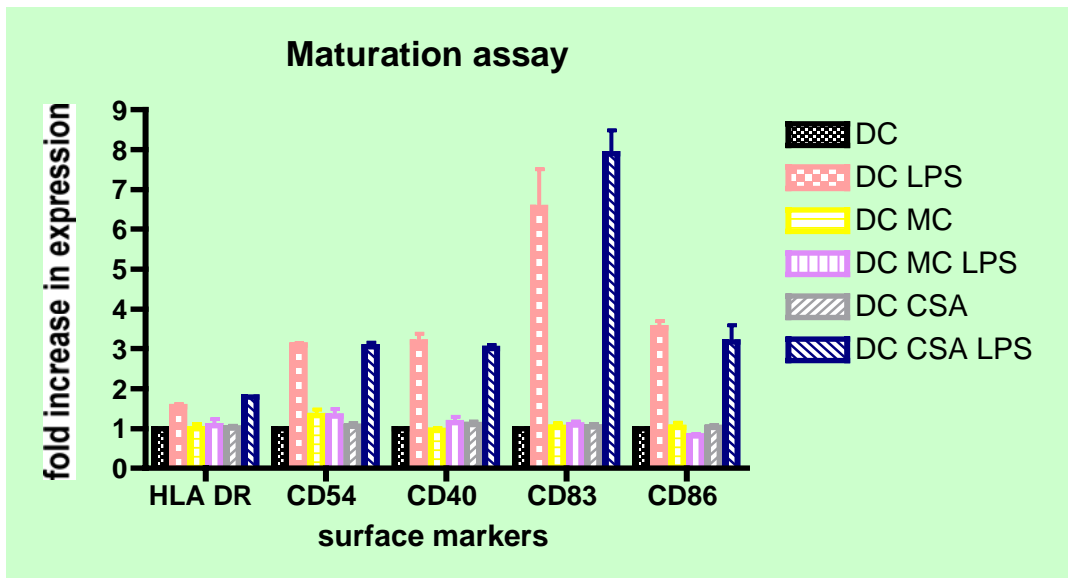
	pDC alone	pDC-CSA CIDR	pDC-MC CIDR	pDC-CpG	pDC-CpG- CSA CIDR	pDC-CpG- MC CIDR
CD40	1.0	0.8	0.7	6.0	8.3	8.1
CD54	1.0	0.4	0.6	6.4	9.6	9.8
CD83	1.0	0.9	0.7	0.9	1.0	0.8
CD86	1.0	0.8	0.7	2.9	2.3	2.0
HLA DR	1.0	1.1	0.5	74.9	26.4	24.2

The relative fold increase in surface marker expression was calculated by dividing the mean fluorescence intensity (MFI) of CpG-exposed plasmacytoid DCs by the MFI of immature DCs after subtracting values obtained with isotype-control antibody.

3.5 MC CIDR-1 inhibited LPS-induced dendritic cells maturation

We wished to establish whether ligation of CD36 expressed on the surface of DCs by MC CIDR-1 could account for their modulation. We therefore exposed immature DCs obtained by culturing monocytes for 5 days in the presence of IL-4 and GM-CSF to MC CIDR-1 or CSA CIDR-1 or to control medium alone and analyze their maturation in response to inflammatory stimulus, LPS. Immature DCs exposed to medium alone or to CSA CIDR-1 molecules and then matured with LPS increased their surface expression of HLA DR-1 molecules, CD54, CD40, CD86 and CD83. By contrast, DCs exposed to MC CIDR-1 consistently failed to mature despite stimulation with LPS, showing no significant increase in any of the surface markers analysed; phenotypically they closely resembled immature DCs (Figure 3.15). The difference in MFI (mean of at least three independent experiments) compared with LPS- matured DCs alone were significant for most the surface markers (Table 4). Surface expression of some markers sometimes appeared to be even below that of immature DCs, but this difference was not statistically significant. In LPS stimulated DCs, CD40, CD54 and CD86 were increased by 3-folds compared to control cells. CD83 expression in this group was increased by about 7-folds (table 4). CSA CIDR-1 treated DCs which were then stimulated with LPS showed surface expression up-regulation similar to LPS treated DCs.

Figure 3.15: MC CIDR-1 inhibits maturation of monocyte-derived DCs.



Between day 5 and day 7 of culture, monocyte-derived dendritic cells were harvested and one million cells were transferred into wells of a 12 well plate with or without LPS to induce maturation. The plates were incubated at 37°C with 5% CO₂ for between 36-48 hours. Alternatively MoDCs were pre-incubated in duplicate with different concentrations of MC-CIDR-1 and CSA-CIDR-1. After two hours incubation, one well was treated with LPS to induce maturation whereas the other well left untreated. 36-48 hours after induction of maturation, dendritic cells were harvested from individual wells and 30ul of the cell suspension were transferred into individual wells of the 96-round bottom well plate (Nunclon) and mixed with 30ul of buffer (negative control) or 30ul of the primary antibodies against HLA DR, CD40, CD54, CD83 or CD86. After incubating for 30 minutes FITC-conjugated anti-mouse Ig antibody (1:25 DAKO) was added to each well before analyzed on flow cytometry.

Table 4. FOLD CHANGES IN SURFACE MARKERS ON DENTRITIC CELLS

	MoDCs	MoDC-LPS	MoDC-MC CIDR	MoDC-CSA CIDR	MoDC-MC CIDR-LPS	MoDC-CSA CIDR-LPS
HLA DR	1	1.5	1	1	0.9	1.8
CD40	1	3	1	1	1	3
CD54	1	3	1	1	1	3
CD83	1	6.5	1	1	1	7.5
CD86	1	3	1	1	1	3

The relative fold increase in surface marker expression was calculated by dividing the mean fluorescence intensity (MFI) of LPS-exposed DCs by the MFI of immature DCs after subtracting values obtained with isotype-control antibody.

CHAPTER 4

DISCUSSION

It has been shown in human *in-vitro* studies that infected red blood cells (IRBCs) binds to monocyte-derived dendritic cells using the glycoprotein CD36 that is expressed on dendritic cells. This attachment of the IRBCs to dendritic cells is thought to lead to modulation of dendritic cells by inhibiting the expression of maturation markers CD83 and CD86 (Urban, 1999 and Urban *et al.*, 2001). This modulation of maturation on dendritic cells by malaria parasites was shown in a similar study to be dependent on the attachment of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) expressed on IRBCs to the CD36 receptor on dendritic cells. In a field study looking at the effect of malaria infection on dendritic cells phenotypic expression, Urban and others found that the expression of HLA DR was dramatically reduced in children with different forms of malaria whether mild or severe malaria as compared to the control group with no parasites (Urban *et al.*, 2001). The surface expression of HLA DR on dendritic cells was negatively correlated with parasitemia. Taken together, these findings suggest that the IRBCs binding to dendritic cells may modulate the maturation of DCs as evidenced by their lack of activation of T cells and reduced expression of HLA DR in children with malaria. We sort to establish whether other molecules could mimic the effect of IRBCs on dendritic cells. We subjected monocytes, plasmacytoid dendritic cells and monocyte-derived dendritic cells to different CIDR-1 molecules; MC CIDR-1 that binds to CD36 and CSA CIDR-1 that do not bind to CD36 but binds to CSA receptor and looked at the proportions of CD36 positive cells and relate the presence of CD36 to binding of different CIDR-1 molecules. The specificity of this binding was also analysed together with the effect of the two CIDR-1 molecules on monocyte-derived dendritic cells maturation. In this report, we show that monocytes and dendritic cells, both peripheral blood plasmacytoid and monocyte-derived dendritic cells had a high

proportion of CD36 positive cells. There was high proportion of CD36 positive cells in the monocytes with a median of >90%, plasmacytoid DCs 89% and finally the monocyte-derived dendritic cells had a median of 60% of CD36 positive cells (table 1). CD36 is an important receptor for *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1), a parasite derived ligand thought to have a significant contribution towards ability of the parasite to cytoadhere to endothelial cells to avoid destruction in the spleen. Irrespective of the dilutions of anti-CD36 antibodies used, all preparations gave a high proportion of CD36 positive cells as analysed by flow cytometry. The proportion of CD36 positive cells on monocytes and DCs was important for binding of these cells to MC CIDR-1 molecules (figure 3.3, 3.4, 3.5). The proportion of MC CIDR-1 positive cells in monocytes was at a median of 78%, in plasmacytoid DCs at 74% and in the monocyte-derived dendritic cells at 60%. In both monocytes and dendritic cells the proportion of CD36 positive cells gave a reflection on the binding ability to MC CIDR-1 molecules as seen by the proportions of MC CIDR-1 positive cells in the cells. We have also shown in this study that monocytes, plasmacytoid DCs and monocyte-derived dendritic cells specifically bound to MC CIDR-1 molecule via CD36 receptor (figure 3.13). Pre-incubation of monocytes and dendritic cells with non-biotinylated MC CIDR-1 before addition of biotinylated MC CIDR resulted in a lower proportion of MC CIDR-1 positive cells in this group. Monocytes, plasmacytoid DCs and monocyte-derived dendritic cells pre-incubated with non-biotinylated MC CIDR-1 showed a median of 8%, 7% and 6% of MC CIDR-1 positive cells respectively (Table 2). These results show that the non-biotinylated MC CIDR-1 was able to block the binding of biotinylated MC CIDR-1 to monocytes and dendritic cells. In the same group the proportion of CD36 positive cells was reduced with a median of 6%, 5% and 7% of positive cells in the monocytes, plasmacytoid DCs and monocyte-derived dendritic cells respectively (table 2). Taken together, these results show that, MC CIDR-1 binds to the CD36 expressed on

monocytes and dendritic cells. Preincubation of the cells with CSA-CIDR-1 did not block the binding of biotinylated MC CIDR-1 to monocytes and dendritic cells as shown by the proportions of MC CIDR-1 positive cells in cells pre-incubated with CSA CIDR-1. The proportion of MC CIDR-1 positive cells gave a median of 84%, 78% and 65% in the monocytes, plasmacytoid DCs and monocyte-derived dendritic cells respectively (Table 2). These findings show that the CSA CIDR-1 does not bind to CD36 expressed on monocytes and dendritic cells. We were interested to establish the effect of a known dendritic cells stimulant CpG oligonucleotide on maturation of plasmacytoid DCs. Plasmacytoid DCs were pre- incubated with CSA CIDR-1 or MC CIDR-1 or left untreated and afterward stimulated with CpG oligonucleotides. Plasmacytoid DCs that were incubated with CSA CIDR-1 alone gave similar expression profile as untreated DCs. However, there was a decreased expression of CD54 in this group by 60% as compared to the controls (table 3). Incubation of plasmacytoid DCs with MC CIDR-1 led to overall reduction of surface expression in all markers analysed. In this group expression of HLA DR was reduced by 50% (table 3). The expression of all markers analysed were even lower than the controls. CpG oligonucleotides stimulated plasmacytoid DCs showed an increase in expression of all surface markers analysed. CD40 and CD54 were increased by 6-folds and CD86 by 3-folds as compared to control. There was massive up-regulation of HLA DR expression to up to 75-folds. Surprisingly, CpG oligonucleotide treatment had no effect on expression of CD83 (table 3). Taken together these results show that CpG oligonucleotides can induce upregulation of plasmacytoid DCs surface markers except for CD83. Stimulation of plasmacytoid DCs with CpG oligonucleotides after pre-incubation with CSA or MC CIDR-1 showed up-regulation of surface markers analysed. In both groups the HLA DR expression was low as compared to CpG treated plasmacytoid DCs but over 24-folds higher than the control group. Having analysed the proportions of CD36 and MC CIDR-1 positive cells in

the monocytes and dendritic cells, we finally examined the effect of CSA CIDR-1 or MC CIDR-1 on maturation of monocyte-derived dendritic cells. Immature DCs were pre-incubated with medium alone, CSA CIDR-1 or MC CIDR-1 and stimulated with LPS or left untreated. The expression of DCs surface markers was then analysed with antibodies directed against HLA DR, CD40, CD54, CD83 and CD86. DCs pre-incubated with medium alone or CSA CIDR-1 and then stimulated with LPS showed an increase in the surface expression of HLA DR, CD40, CD54, CD83 and CD86 as compared to unstimulated DCs (figure 3.15). There was 7-fold increase in surface expression of CD83 in the LPS stimulated DCs as compared to the unstimulated group. CD40, CD54 and CD86 showed a 3 fold increase in surface expression in DCs stimulated with LPS as compared to the control group (table 4). There was marginal changes in the surface expression of HLA DR at 1.8 fold increase in the LPS treated DCs as compared to unstimulated cells. Pre-incubation of DCs with MC CIDR-1 showed an absence of up-regulation of HLA DR, CD40, CD54, CD83 and CD86. There was no up-regulation of expression of surface molecules HLA DR, CD40, CD54, CD83 and CD86 even after stimulating the DCs with LPS. The stimulated DCs phenotypically resembled immature DCs with surface expression equal or even below that of immature DCs (Table 4).

In earlier work using intact IRBCs *in vitro*, it was shown that IRBCs were able to bind to CD36 expressed on DCs (Urban *et al.*, 2001). The interaction between CD36 expressed on DCs and PfEMP-1 has been shown to modulate DCs functions by inhibiting DC maturation process. The IRBCs modulated DCs failed to activate the proliferation of naive T cells during lymphocyte proliferation assays (Urban *et al.*, 2001). The intact IRBCs were able to modulate maturation of DCs *in-vitro* as shown by the absence of up- regulation of expression of DCs surface molecules HLA DR, CD40, CD54, CD80, CD83 and CD86. In this study, MC-CIDR-1 molecules were able to mimic the inhibition of maturation of DCs

by IRBCs *in-vitro*. The MC CIDR-1 treated DCs showed no up-regulation of expression of surface markers even after stimulating with on MC CIDR-1. This modulation of DCs by MC CIDR-1 may affect the capacity of DCs to mature and thus interact effectively with naive T cells. Interaction of mature DCs with naïve T cells or memory T cells is important for activation of T cells responses. Failure of DCs to up-regulate expression of costimulatory molecules and the major histocompatibility complex may induce state of T cell unresponsiveness in the presence of an antigen. This can have the effect of the pathogen exist harmoniously within the host. Modulation of DC maturation by MC-CIDR-1 may shed some light on the mechanisms used by other immunomodulatory agents that inhibit maturation of dendritic cells. Other agents that have been reported to induce changes in maturation of dendritic cells include the malaria parasite pigment haemozoin and intact IRBCs haemozoin, intact IRBC (Schwarzer *et al.*, 1996, Urban *et al.*, 1999). Here we demonstrate that MC-CIDR-1 molecules interfere with maturation differentiation of DCs *in-vitro* as shown by the absence of up-regulation of HLA-DR, CD40, CD54, CD83 and CD86. DCs are known to be the only antigen presenting cells that can prime naïve and activate memory T cells. They achieve this important immunologic process by up-regulating the surface molecules upon encountering an antigen or microbe. The production of the cytokine IL-12 plays a crucial role for activation of T cells. In order to circumvent this important process, pathogens have evolved ways of dampening the expression of costimulatory molecules on activated DCs. The targets for down-regulation included CD80, CD40, CD86 and production of IL-10 instead of IL-12. This modulation may lead to inefficiency of DC-T cells interactions, reducing the signaling through the TCR. This results in T cell anergy with no activation of T cells even after DC activation (Banchereau *et al.*, 2000). From our data we showed that MC-CIDR-1 affected the expression of costimulatory molecules on DCs. The expression of CD80, CD86 and CD40 was lower in DCs incubated with MC-CIDR-

1 even after stimulation with LPS. It will be interesting to investigate how this down-regulation of costimulatory molecules expression as a result of MC-CIDR-1 impacts on DCs signaling events.

There are many stimuli that can initiate DCs maturation process *in vitro*. These include cytokines IL-12, TNF and bacterial products such as lipopolysaccharide (LPS). Ligation of CD40 by CD40L and the engagement of Fc receptor complexes have also been shown to stimulate maturation, as have CpG DNA motifs found in prokaryotic DNA and viral double stranded RNA. LPS has also been shown to lead to the maturation of DCs *in vivo*. Our observations on DC maturation modulation by MC CIDR-1 molecules may illuminate some observations on field isolates of IRBCs. Although almost all field and laboratory isolates bind to CD36 and or TSP (Baruch *et al.*, 1997), those with higher affinity for CD36 are more frequently isolated from children with mild than with severe malaria (Newbold *et al.*, 1997). Furthermore, a nonsense mutation in CD36 is common in African populations. Although one study reported that the frequency of this mutation is increased in patients with cerebral malaria, another data suggest that it is reduced in patients suffering from respiratory distress, severe malarial anemia, or hypoglycemia (Aitman *et al.*, 2000, Pain *et al.*, 2001). Without further functional studies, the consequences of this mutation for the immune response to malaria cannot be deduced. However, modulation of DCs by IRBCs is a contact-dependent process; if it occurred *in vivo*, it most likely would affect DCs in the circulation as well as in the liver and in the marginal zone of the spleen. Modulation should depend on a critical level of IRBC and on their affinity for CD36. Cumulative modulation of DCs might result in a progressive polarization or inhibition of T-cell priming and so may dampen anti-parasite immune responses; at the same time, it might reduce associated immunopathology. Indeed, some studies suggest that induction of primary immune responses is impaired during acute phases of malarial disease (Greenwood *et al.*, 1980). Nevertheless, T-cell-dependent

humoral immune responses are clearly induced during acute malaria, and adults living in endemic areas often show high levels of anti-malarial IgG, although antibodies against individual parasite antigens frequently seem to be short-lived (Cavanagh *et al.*, 1998). Possibly the secretion of IL-10, at least in part by IRBC-modulated DCs, may promote the induction of Th2 responses, whereas the progressive impairment of DC function could interfere with the induction of memory in T and B cells. Clearly, the immunological consequences of parasite mediated modulation of DCs are not yet fully understood, but they may contribute significantly to mechanisms of immune evasion by the asexual blood stages of *P. falciparum*. Unraveling these mechanisms may provide therapeutic clues for the treatment of malaria. Conversely, investigating IRBC-mediated modulation of DCs may lead us to new approaches for regulating the pathological immune responses in autoimmune diseases and transplantation.

Even though analysis of cytokine production in dendritic cells as a result of culturing DCs with CIDR-1 molecules was not investigated in this study, the production of cytokines has been shown to have an effect on the maturation of DCs and subsequent interaction with naive T cells. Induction of cytokine production by DC in malaria is not limited to *P. falciparum* IRBCs. In animal models, DCs, being the most important antigen presenting cells (APCs) interact with other plasmodia species and indeed other pathogens. *P. chabaudi* infected red cells have also been reported to induce up-regulation of costimulatory and the production of pro-inflammatory cytokines including IL-12, IL-6 and TNF by bone marrow derived DCs in mice (Langhorne *et al.*, 2004). In addition, Myd88 knock out (KO) mice infected with *P. berghei* were shown to have a decreased production of endogenous IL-12 and less severe pathology than wild type mice (Adachi *et al.*, 2001). This study suggested that IL-12 induced via the Toll/IL-1 receptor (TIR) signaling pathway (which contains Myd88 as a cytoplasmic adaptor common to TLR signaling) is involved in the pathogenesis

of *P. berghei* malaria in murine models. Various microbial organisms have also been reported to induce expression of surface molecules and production of cytokines IL-2 and IL-12 in DC (Granucci *et al.*, 2004) and similar results have been reported with protozoan parasites including *Leishmania major* (Gorak *et al.*, 1998) and *Toxoplasma gondii* (Aliberti *et al.*, 2004). The detection of IL-10 in response to IRBC as well as CIDR-1 α suggests that IRBC might interact with DCs via CIDR-1 α . That CIDR-1 α might interact with DCs via the scavenger receptor, CD36, has been suggested previously (Urban *et al.*, 1999; Urban *et al.*, 2001). However, whether IRBC or CIDR-1 α stimulates plasmacytoid DCs to make cytokines by binding to CD36 is not known. Stimulation of monocyte-derived DCs with anti-CD36 monoclonal antibodies had the same effect as apoptotic cells, IRBCs and CIDR-1 α , all of which are known to interact with CD36 (Urban *et al.*, 2001). Alternatively, CIDR-1 α and IRBCs may interact with DCs via TLRs. While major advances have been made in the assignment of individual TLRs to defined roles in bacterial infection (Takeda and Akira, 2004), such identification has only begun to emerge in protozoan parasites (Teixeira *et al.*, 2002). A recent study demonstrated that *P. falciparum* schizonts or schizont extracts could activate human plasmacytoid DCs by inducing CD86 expression and IFN- γ synthesis (Pichyangkul *et al.*, 2004). The *P. falciparum* stimulated pDCs elicited poor T cell response but promoted T cell proliferation and IFN- γ production, and these stimulatory effects could be reproduced with murine DC and required the TLR-9-myD88 signaling pathway. In addition, the study by Adachi and colleagues on MyD88 KO mice demonstrated that signaling via the TIR pathway was critical for production of *P. berghei*-induced IL-12 (Adachi *et al.*, 2001). These two studies demonstrated that Plasmodia species could interact with DCs via TLR resulting in immune regulation and immunopathology in malaria infections.

SUMMARY

Though there is more behind severe malaria than adhesion, sequestration remains an important feature involved in the pathogenesis of *falciparum* malaria leading to severe disease. We have shown here for the first time monocytes, plasmacytoid dendritic cells and monocyte-derived dendritic cells from same donor have high expression of CD36 on their surface. The CD36 expression was important for binding of malaria parasite MC CIDR-1. We have also shown that binding to CD36 can be inhibited by pre-incubating the cells with CIDR-1 from a CD36 binding parasite phenotype. The binding of malaria parasite to CD36 on dendritic cells has been thought to inhibit the maturation of dendritic cells as shown by down regulation of surface markers. Our findings are in agreement with this suggestion as seen by reduced expression of surface markers on dendritic cells after pre-incubating the cells with MC CIDR-1. Even stimulation with LPS did not reverse the expression of surface markers. Plasmacytoid DCs treated with CpG oligonucleotides maturation process as shown by increased expression of surface markers. MC CIDR-1 incubated plasmacytoid DCs had low expression of surface markers analysed than plasmacytoid DCs that were left untreated. MC CIDR-1 molecules inhibited the maturation of these cells.

Previous studies have shown that the avidity of IRBCs for CD36 is higher in children with uncomplicated malaria than in children with severe disease. In addition, children with severe malaria and heterozygous for a null-mutation in CD36 are more likely to be infected with parasites expressing PfEMP-1 with a high frequency of recognition by heterologous immune serum indicating that adhesion to CD36 selects for parasites with a low frequency of recognition. So, what could be the relationship between adhesion to CD36, the immune response to PfEMP-1 and immune-selection of the expressed PfEMP-1 in non-immune and semi-immune individuals? One explanation would be that parasites with no or low binding to CD36 are more immunogenic because they induce the production of IL-12 rather than IL-

10 by DCs and possibly monocytes resulting in better T-cell activation and helper function. High or low frequency of recognition of a parasite isolate by heterologous immune serum could then be due to differences in immunogenicity of the expressed PfEMP-1. Likewise, the observation that responses to some vaccines are reduced in children with acute malaria might be explained, at least in part, by cytoadhesion the properties of the acute parasite isolate that may alter DC function or influence the cytokine environment. Longitudinal studies addressing the interaction between the phenotype and duration of immune responses to *P. falciparum* infection and the parasites genetic and phenotypic make-up may be able to answer these questions.

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APPENDICES

AP 1.0 SOURCE OF REAGENTS AND CONSUMABLES

Below is a list of reagents and consumables used on the study described in this thesis.

PROCEDURE	REAGENT	SUPPLIER
Blood collection	Heparin	LEO LABS LTD, Bucks, UK
Cell Cultures	Malaria non-immune sera	Blood Transfusion Services Southmead Road, Bristol, UK
	Lymphoprep	NYCOMED PHARMA AS, Oslo, Norway
	Hypoxanthine	Sigma
	RPMI 1640	Sigma
	HEPES Buffer	Sigma
	Kanamycin	Sigma
	LPS	GIBCO/invitrogen
	IL-4	PeptoTech, Rocky Hill, USA
	GM-CSF	Schering Ploigh, UK
	IL-3	GIBCO/invitrogen
	L-glutamine	GIBCO/invitrogen
	Glucose	Sigma
	Sodium hydroxide	Sigma
	Giensa	Sigma
	Immersion oil	Sigma
	Culture gas mixture	BOC
	Magnetic microbeads	Mitenyl biotec, Germany
Flow cytometry	CD36	DAKO, UK
	CD54	DAKO, UK
	CD80	DAKO, UK
	CD83	DAKO, UK
	CD86	DAKO, UK
	HLA DR	DAKO, UK
	MHC I	DAKO, UK
	Bovine serum albumin	SIGMA
	Phosphate buffer saline	SIGMA
	FITC-conjugate	DAKO
Binding Assay	MC-CIDR	Damien
	CSA-CIDR	Damien
	Biotin	Sigma
	IgG FITC	DAKO

CONSUMABLES

ITEM	SUPPLIER
Nunclon 48, 24 and 12 well plates	Nunclon
Syringes	Becton Dickinson, France
Needle	Becton Dickinson, France
Aspirating pipettes 2 ml	Fahrenheit, Milton Keynes, UK
Pipets plastic disposable 1ml, 5ml, 10ml, 25ml, 50ml	Fahrenheit, Milton Keynes, UK
Falcon centrifuge tubes	Becton Dickinson, France
Pasteur pipette plastic sterile	Alpha, Hampshire, UK
PIPETTE MICRO-VOLUME TIPS (0.5-10ul)	Fisher Scientific, UK
(Eppendorf)	
Pipette tips 200ul maximum (Eppendorf)	Fisher Scientific, UK
Pipette tips 1000ul maximum (Eppendorf)	Fisher Scientific, UK
Eppendorf tubes	Anderman and Co., Surrey, UK
LD columns	Miltenyi Biotec, Germany
LS columns	Miltenyi Biotec, Germany
Film	Amersham life sciences Ltd., UK
Haemocytometer	Sigma, UK
Falcon 96 well plate	Becton Dickinson, France
Falcon tubes	Becton Dickinson, France