Characterisation of macrophage migration inhibitory factor homologues in plasmodium species

Thesis

Characterisation of Macrophage Migration Inhibitory Factor Homologues in Plasmodium Species

Damien Vaughan Cordery,

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DECLARATION

I hereby declare that this submission is my own work and to the best of my knowledge it contains no material previously written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at an educational institution, except where due acknowledgment is made in the thesis. Any contribution made to this research by others, with whom I have worked is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Signed

Damien Cordery

ABSTRACT

Malaria is responsible for 1-3 million deaths per year worldwide, with half the global population at risk of infection. *Plasmodium falciparum* is the parasite responsible for severe malaria and accounts for almost all fatal cases. The *Plasmodium falciparum* genome sequencing project identified a putative protein that shares sequence homology with the cytokine macrophage migration inhibitory factor (MIF). MIF has been shown to have a wide range of functions including the modulation of inflammatory responses. The major objective of this project was to characterise the potential MIF homologue in *P. falciparum* (PfMIF) and to test the hypothesis that this protein may influence the host immune system during the course of *P. falciparum* infection.

Sequence analysis and modelling techniques were used to show that PfMIF shares important structural similarities to other MIF species. Studies of parasites in culture demonstrated that PfMIF mRNA and protein are expressed during ring and trophozoite stages of the parasite life cycle. Furthermore, PfMIF was found to be exported into the cytosol of the infected erythrocyte, and released upon schizont rupture, thus providing an opportunity for PfMIF to interact directly with the host immune system. Recombinant PfMIF protein was generated and used to treat monocytes *in vitro*. These experiments showed that PfMIF inhibits the random migration and chemotaxis of monocytes and influences surface molecule expression, as evidenced by a decrease in TLR2, TLR4 and CD86. Access to a cohort of children in Kenya allowed examination of patient antibody responses to PfMIF, which showed a pattern of expression similar to antibody responses to other malaria antigens previously examined.

In conclusion, these studies suggest that PfMIF could be an important molecule involved in the interaction between the parasite and the host immune system during the course of *P. falciparum* infection.

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PUBLICATIONS

Manuscripts arising directly from this thesis

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Abstracts

Cordery DV, Kishore U and Urban BU. Further characterization of *Plasmodium falciparum* homologue of MIF. Oxford Tropical Network Meeting, 19-22nd March 2006, Oxford, UK.

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Publications arising in conjunction with this thesis

Cordery DV and Urban BC. Recognition of *Plasmodium*-infected red blood cells by leukocytes. Target Pattern Recognition in Innate Immunity. *In press* (Springer/Landes Biosciences).

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ABBREVIATIONS

AP-1	activator protein-1
BmMIF	*Brugia malayi* macrophage migration inhibitory factor
CIDR	cysteine-rich interdomain region
CM	cerebral malaria
CR-1	complement receptor 1
CSA	chondroitin sulphate A
DAPI	4′,6-diamidino-2-phenylindole
DBL	Duffy binding like
DC	dendritic cells
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
GIF	glycosylation inhibiting factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
huMIF	human macrophage migration inhibitory factor
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon γ
Ig	immunoglobulin

IL	interleukin
IPTG	isopropyl β-D-thiogalactopyranoside
iRBC	infected red blood cells
JAB1	JUN-activation domain-binding protein 1
JNK	JUN N-terminal kinase
KO	knockout
LAL	Limulus Amoebocyte lysate
LPS	lipopolysaccharide
MAP	mitogen activated protein
MCP-1	Monocyte Chemotactic Protein 1
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinases
MyD88	myeloid differentiation factor 88
NFkB	nuclear factor κB
NK	natural killer
NKT	natural killer T
NO	nitric oxide
OPD	o-phenylenediamine dihydrochloride
PBMC	peripheral blood mononuclear cells
PbMIF	*Plasmodium berghei* macrophage migration inhibitory factor
PBS	phosphate buffer saline
PfEMP1	*Plasmodium falciparum* erythrocyte binding protein-1

PfMIF	*Plasmodium falciparum* macrophage migration inhibitory factor
PfSBP1	*Plasmodium falciparum* skeleton binding protein 1
PGE_2	prostaglandin-E_2
PGF_{2a}	prostaglandin-F_{2a}
PGN	peptidoglycan
PI3	phosphoinositide 3 kinase
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RBC	red blood cells
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
TNF	tumour necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VSA	variant surface antigen

CHAPTER 1

Introduction

Malaria has historically been, and still remains, a major global health burden. Investigation into the development of immunity to malaria, although greatly advanced in recent years, is still far from complete. One aspect of the immune response to malaria that requires particular attention is direct interactions between the parasite and host. A major advance in malaria research in recent years has been the sequencing of the *Plasmodium falciparum* genome. This project identified a gene that shares significant sequence homology with the mammalian cytokine macrophage migration inhibitory factor (MIF). Identification of a cytokine homologue expressed by the malaria parasite suggests the possibility of interaction between parasite and host that could contribute to the development of immunity to malaria. This hypothesis forms the basis of this thesis.

The following introductory chapter provides an overview of malaria including global burden, transmission, symptoms and immunity to the disease. Additionally, the activity of macrophage migration inhibitory factor, including current knowledge of its role in immune responses to malaria and other infectious and immune mediated diseases, is also reviewed.

1.1 MALARIA – BURDEN AND DISTRIBUTION

Prior to intervention, malaria was much more widespread than it is today. In 1900, the time of maximal distribution of malaria, areas of *Plasmodium* transmission spread to the latitudes of 64° north and 32° south (i.e. extending from Moscow to Durban). This distribution was estimated to cover 53% of the Earth's land surface, thereby exposing 77% of the world's population to *Plasmodium* infection (Hay *et al.*, 2004). Since 1900, malaria controls have restricted this distribution dramatically; however as of 2002, half

the planet's population remains exposed to the risk of malaria (figure 1.1). Firm data on the global morbidity and mortality caused by malaria is not available, but it has recently been estimated that there are around 500 million malaria cases per year worldwide (Snow *et al.*, 2005). Estimates of the number of deaths caused by malaria are equally vague but generally fall between 700,000 and 2.7 million per year (Breman, 2001). The majority of the malaria burden is borne by sub-Saharan Africa, accounting for approximately 70% of global infections. It has also been reported that the risk of death after *Plasmodium* infection is higher in Africa than in South East Asia or the Western Pacific (Snow *et al.*, 2005).

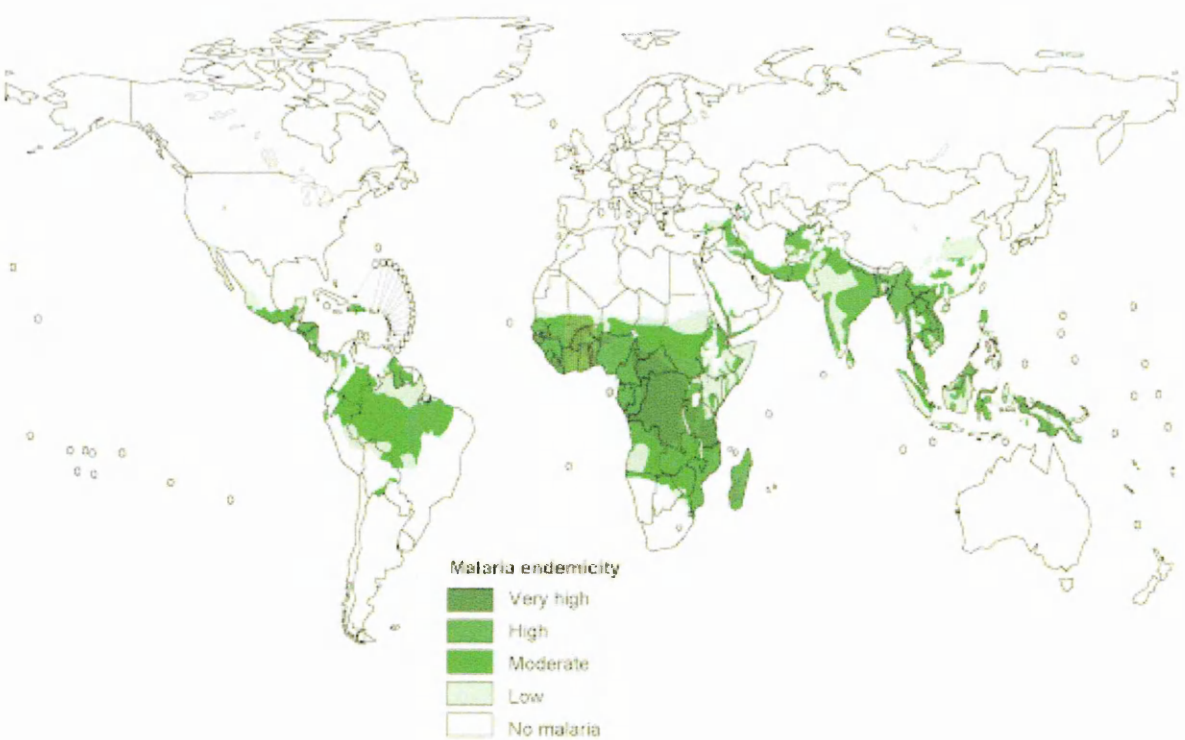


Figure 1.1: Global malaria transmission risk, 2003. From World Health Organization, World Malaria Report 2005. http://www.rbm.who.int/wmr2005 (WHO, 2005).

The burden of disease depends on the nature of malaria transmission in a given region. In hyperendemic and holoendemic areas, the burden of severe disease and malaria mortality is borne by the under-5 age group. This is a common situation in sub-Saharan Africa. In regions with more seasonal transmission, such as South East Asia, more malaria cases are seen in adults that have not developed immunity. The burden of malaria comprises not just morbidity and mortality, but also the financial cost to malarial countries. Estimates of malaria's economic impact are difficult to calculate but the gross domestic product of malarial nations is five times lower than the global average (Gallup and Sachs, 2001). It remains unclear whether malaria is a major contributor to poverty or simply an effect of poverty.

Malaria is transmitted to vertebrate hosts by the bite of female *Anopheles* mosquitoes infected with a protozoan parasite of the genus *Plasmodium*. There are four species of *Plasmodium* responsible for malaria in humans. *Plasmodium ovale* and *Plasmodium malariae* are infrequent causes of clinical malaria. In Asia, South America and Oceania *Plasmodium vivax* is a major cause of clinical malaria, but is rarely fatal. *Plasmodium falciparum* is responsible for the most severe disease and almost all malaria deaths, with the greatest burden of mortality in sub-Saharan Africa.

1.2 *P.FALCIPARUM* LIFE CYCLE

The lifecycle of *Plasmodium falciparum* parasites is complex and involves stages in mosquito and mammalian hosts (figure 1.2). During the ingestion of a blood meal by an *Anopheles* mosquito, sporozoites are injected into the bloodstream of the mammalian

host. Sporozoites make their way to the liver within minutes of inoculation and infect hepatocytes where they undergo a period of intracellular replication. In the subsequent 5 to 10 days, parasites differentiate and multiply within the hepatocytes and finally 20,000 to 40,000 merozoites are released into the bloodstream where they invade erythrocytes. The intraerythrocytic phase of infection lasts approximately 48 hours, during which time parasites develop and multiply. When the infected red blood cell (iRBC) bursts, 6-32 merozoites per erythrocyte are released into the bloodstream to invade uninfected erythrocytes and begin a new cycle. Parasite multiplication continues until it is controlled by the immune response or drug treatment and it is during this repeated intraerythrocytic cycle that symptoms of disease develop. A small proportion of the invading merozoites undergo differentiation into either male or female gametocytes, which are subsequently taken up in a mosquito blood meal. In the mosquito mid-gut, the male and female gametes are released and fuse to form a zygote, which then undergoes a series of complicated differentiation and growth stages that results in the production of infective sporozoites in the salivary glands of the mosquito.

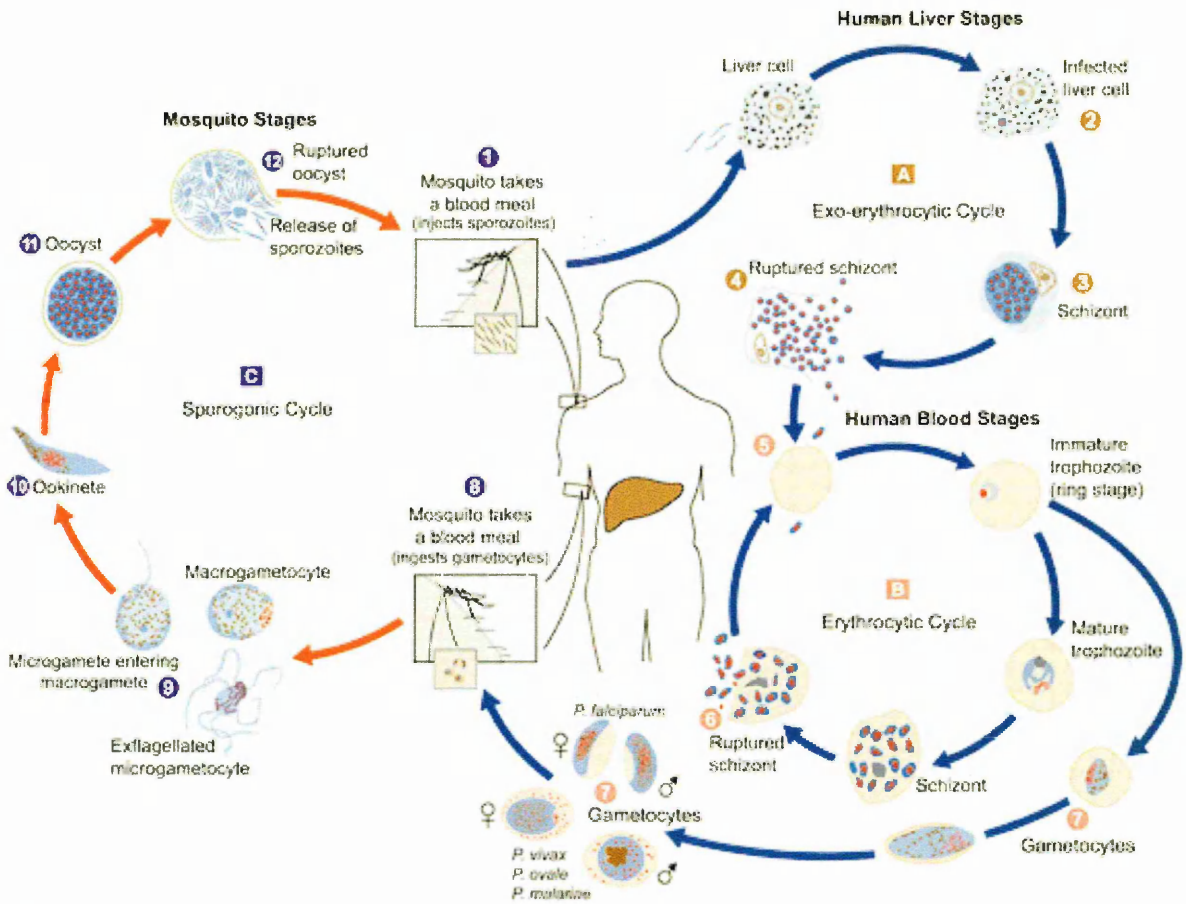


Figure 1.2: *Plasmodium* lifecycle. The stage of the lifecycle that is the primary focus in the course of this thesis is the erythrocytic cycle (B). Modified from CDC, http://www.cdc.gov/malaria/biology/life_cycle.htm (CDC, 2006).

1.3 CYTOADHERENCE OF INFECTED ERYTHROCYTES

One major characteristic of *P. falciparum*, unique to *Plasmodium* parasites that infect humans, is the phenomenon of cytoadherence. As *P. falciparum* parasites mature during the 48hr replicative blood stage cycle they express variant surface antigens, including *Plasmodium falciparum* erythrocyte binding protein-1 (PfEMP1), on the surface of the infected erythrocyte. PfEMP1 proteins have been shown to bind to multiple receptors on the vascular endothelium including CD36, intercellular adhesion molecule (ICAM)-1, complement receptor 1 (CR-1), E-selectin, CSA, vascular cell adhesion molecule

(VCAM)-1, CD31, E-selectin and hyaluronic acid. This process is primarily thought to be a mechanism of avoiding circulation to, and subsequent destruction by, the spleen. This process of avoiding the spleen also leads to concentration of parasites in various other organs, depending on the specific PfEMP1 being expressed and the endothelial receptor expression profile. This sequestration of iRBC in organs and blood vessels has pathological consequences for the progress of malarial disease severity. Parasitised RBC also bind to thrombospondin, but this adherence precedes the expression of PfEMP1 on the surface of iRBC implicating another binding partner, possibly another parasite variant antigen (Gardner *et al.*, 1996).

1.3.1 Rosetting

Infected red blood cells (iRBC) from some *P. falciparum* isolates bind to CR-1, which is expressed on uninfected erythrocytes, to form rosettes. Blood group A and immunoglobulins have also been shown to play a role as ligands in the rosetting phenotype (Fernandez *et al.*, 1998; Rowe *et al.*, 1995). Although rosetting is an *in vitro* observation, it has been suggested that if it does occur *in vivo* it may enhance merozoite invasion of uninfected erythrocytes upon schizont rupture or help shield the iRBC from the immune system (Rowe *et al.*, 2002). A rosetting phenotype has been linked with severe malaria in some studies (Rowe *et al.*, 1995) but the impact of a rosetting phenotype remains to be clarified.

1.3.2 Agglutination

Some laboratory lines of *P. falciparum* are also capable of forming clumps of iRBC. This agglutination is mediated by platelets and is expressed by some but not all CD36-binding parasite lines (Pain *et al.*, 2001). This is a further mechanism whereby iRBC may accumulate or be retained in the microvasculature and thereby avoid destruction.

This particular phenotype may also contribute to vascular obstruction and more severe pathology.

1.4 SYMPTOMS

Infection with *Plasmodium falciparum* can result in anything from asymptomatic infection, through a range of disease severities: from mild febrile illness through to life-threatening illness characterised by one or more syndromes including severe anaemia, acidosis, cerebral malaria and placental malaria.

1.4.1 Severe malarial anaemia

Severe malarial anaemia is the most common complication of malaria. Malarial anaemia results not only from the lysis of iRBC, but also involves the destruction of unparasitised red blood cells (RBC) as well as suppression of RBC production (Angus *et al.*, 1997; Jakeman *et al.*, 1999).

Destruction of non-parasitised RBC is not generally via haemolysis, which would be characterised by haemoglobin in the urine (Casals-Pascual and Roberts, 2006). Instead, it is thought to be mediated by macrophages in the red pulp of the spleen. This is consistent with observations that onset of anaemia is simultaneous with a distended spleen.

Blackwater fever is the relatively uncommon pathological state that is the exception to the intravascular haemolysis rule (Bruneel *et al.*, 2001). It is characterised by dark red to almost black urine due to the release of haemoglobin and pathogenesis remains unclear. Blackwater fever is primarily associated with quinine use to treat severe malaria.

The number of reticulocytes in circulation, a direct indicator of recent erythropoietic activity, has been shown to be reduced during acute *P. falciparum* infection (Kurtzhals *et al.*, 1997). Malaria has been shown to suppress erythropoiesis during malaria in murine models. Lysates from the murine parasites *P. berghei* and *P. chabaudi* have been shown to cause erythropoietic suppression *in vivo* implicating bioactive parasite products in the process (Rudin *et al.*, 1997). It has subsequently been shown that the malarial pigment haemozoin contributes to abnormal erythropoiesis *in vitro* (Casals-Pascual *et al.*, 2006). Additionally, in malaria patients the proportion of monocytes containing haemozoin and the levels of plasma haemozoin are associated with both anaemia and reticulocyte suppression (Casals-Pascual *et al.*, 2006).

1.4.2 Metabolic acidosis

Metabolic acidosis is characterised by respiratory distress, deep breathing and hypovolaemia. It has been suggested that inadequate oxygen supply, due to a combination of severe anaemia and vascular obstruction via cytoadherence (see section 1.3), leads to a shift to anaerobic glucose metabolism and increased lactic acid production (Dondorp *et al.*, 1997; Vander Jagt *et al.*, 1990). Metabolic acidosis is strongly associated with disease severity and a predictor of poor outcome (Marsh *et al.*, 1995).

1.4.3 Cerebral malaria

Cerebral malaria (CM) is described as a syndrome consisting of unrousable coma not attributable to hypoglycaemia, convulsions or meningitis in a patient with *P. falciparum* parasitaemia. It is generally believed that cerebral malaria is primarily caused by the sequestration of iRBC in the microvasculature of the brain. However, some studies suggest that cerebral malaria is mediated by another mechanism, possibly aberrant

cytokine responses (Hunt and Grau, 2003). This latter hypothesis is supported by the fact that patients who recover from cerebral malaria have relatively low rates of subsequent neurological impairment, which would be expected to result from the vascular obstruction caused by parasite sequestration (Brewster *et al.*, 1990). In a study by Taylor et al., 23% of fatalities attributed to CM actually resulted from other causes, including severe anaemia, pneumonia and meningitis (Taylor *et al.*, 2004). Accurate diagnosis of CM is clearly difficult and studies involving CM patients need to be cautious of this fact.

1.4.4 Placental malaria

In areas of high malaria transmission, women who reach childbearing age have already developed considerable immunity to malaria. However during their first or second pregnancy, women are at risk of developing placental malaria. Placental malaria is mediated by the binding of iRBC to chondroitin sulphate A (CSA) and hyaluronic acid that are preferentially expressed on placental endothelial cells (Beeson *et al.*, 1999; Beeson *et al.*, 2000). This leads to accumulation of iRBC in the placenta and results in low birth weight.

1.5 IMMUNITY TO *PLASMODIUM FALCIPARUM*

Immunity to malaria is never sterile and develops with increasing exposure and age. Mathematical models suggest that protection against severe malaria is acquired after only one or two successful infectious bites (Gupta *et al.*, 1999). Clinical immunity to mild malaria, resulting in asymptomatic infection, takes much longer to develop.

1.5.1 Cellular immune responses

A broad range of cell types discussed briefly below have been shown to be involved in responses to *P. falciparum* infection.

1.5.1.1 Monocytes and Macrophages

Mononuclear phagocytes play an important role in innate immune responses to malaria due to their ability to phagocytose infected erythrocytes in the absence of antibodies that opsonise, (i.e. effectively mark for destruction) iRBC (Serghides *et al.*, 2003). Opsonin-independent phagocytosis is mediated by the binding of infected erythrocytes to CD36, most likely mediated by PfEMP-1. This opsonin-independent phagocytosis of iRBC by macrophages leads to an accumulation of haemozoin and subsequent cellular dysfunction (see section 1.5.3.3).

1.5.1.2 Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells that provide a vital link between the innate and adaptive arms of the immune system. Two major DC subsets can be detected *in vivo* that have distinct but overlapping functions. Myeloid DC are the main producers of interleukin (IL)-12, while plasmacytoid DC are the main producers of interferon (IFN)-α. *In vitro* studies have shown that RBC infected with *P. falciparum* are capable of binding to monocyte-derived DC in a CD36-dependent manner and modulate subsequent DC function (Urban *et al.*, 1999). DC modulated by parasites are capable of secreting IL-10 and TNF-α but fail to up-regulate adhesion, co-stimulatory and major histocompatibility complex (MHC) molecules on their surface and are unable to activate naïve T cells. Modulation of DC function by *P. falciparum* may be a primary method of immune evasion utilised by the parasite. DC maturation has also been shown to be modulated by the malarial pigment haemozoin. Monocytes that have taken up

haemozoin have been shown to express fewer surface molecules such as MHC class II and ICAM-1, indicating an impairment of immune responses (Schwarzer *et al.*, 1998). These haemozoin loaded monocytes also failed to mature into dendritic cells *in vitro* implicating a role for haemozoin in impairing DC maturation.

1.5.1.3 Natural Killer cells

Natural killer (NK) cells are primarily associated with the destruction of virus-infected cells. NK and natural killer T (NKT) cells are the first cells to produce IFN-γ after exposure to iRBC (Artavanis-Tsakonas and Riley, 2002; Artavanis-Tsakonas *et al.*, 2003). Parasitised RBC interact directly with NK cells, resulting in the production of IFN-γ. The nature of this interaction remains to be determined. The *P. chabaudi* mouse malaria model supports the importance of NK cells in innate immune responses during *Plasmodium* infection. This model showed that NK cell-depleted mice infected with *P. chabaudi* exhibit more severe disease (Artavanis-Tsakonas and Riley, 2002).

1.5.1.4 γδ T cells

γδ T cells are another bridge between the innate and adaptive immune responses. There is a large increase in circulating γδ T cells during acute *P. falciparum* infection. Although the clinical relevance of this expansion is not fully understood, γδ T cells have been shown to be a source of large amounts of IFN-γ (Hviid *et al.*, 2001; Pichyangkul *et al.*, 1997).

1.5.1.5 Regulatory T Cells

$CD4^+CD25^+$ regulatory T cells are thought to suppress $CD4^+$ and $CD8^+$ T cell activation thereby contributing to chronic infections. This has been demonstrated in mouse malaria models where the depletion of regulatory T cells protected mice from lethal *P. yoelii*

infection (Hisaeda *et al.*, 2004). In experimental sporozoite challenge of human volunteers the presence of regulatory T cells is associated with increased parasite growth rates (Walther *et al.*, 2005).

1.5.1.6 B cells

Chronic *P. falciparum* infection leads to overactivation of B cells, which results in the secretion of a broad range of autoantibodies (Adu *et al.*, 1982), hyperglobulinaemia and frequent occurrence of Burkitt's lymphoma (Greenwood *et al.*, 1970). The cysteine-rich interdomain region (CIDR)-1α domain of PfEMP-1 has been implicated in the polyclonal activation of B cells via the binding of surface immunoglobulin molecules (Donati *et al.*, 2004).

1.5.1.7 Eosinophils

Eosinophils are present at low levels in the circulation and primarily responsible for the extracellular killing of multicellular parasites. The role of eosinophils during *P. falciparum* infection has not been extensively studied. It has been shown that during acute illness in children the relative number of eosinophils on blood films is decreased compared to controls (Kurtzhals *et al.*, 1998b). Markers of eosinophil activity were increased during acute malaria. Meanwhile, children with asymptomatic infection had a relative increase in eosinophil frequency compared to controls (Kurtzhals *et al.*, 1998b). It was also shown that eosinophil activity was higher during cerebral malaria compared to uncomplicated malaria. Further work is required to elucidate the function and relevance of eosinophils during malaria.

1.5.2 Pattern Recognition Receptors

Innate immune recognition relies on pattern recognition receptors (PRR) to recognise pathogen molecules and initiate an immune response. A growing number of PRR have

been examined in recent years including Toll-like receptors, scavenger receptors and complement receptors.

1.5.2.1 Toll-like receptors

Toll-like receptors (TLR) recognise conserved molecular motifs predominantly found in microorganisms that do not occur in vertebrates. To date ten TLR members have been identified in mammals.

The activation of TLR pathways by iRBC or their products has been intensely investigated in recent years. The study of rodent models of malaria, using mice deficient in specific receptors or adaptor molecules, has considerably advanced the field. However, when comparing events in rodent models of malaria with the human disease, the differences in TLR expression on monocytes, macrophages and DC subsets have to be taken into consideration. In mice, TLR2, TLR4 and TLR9 are expressed on both myeloid and plasmacytoid DC. In humans, myeloid DC express TLR2 and TLR4 but not TLR9 and plasmacytoid DC express TLR7 and TLR9 (Shortman and Liu, 2002).

One of the first reports of the involvement of TLR in the immune response to *Plasmodium* infection indicated that myeloid differentiation factor 88 (MyD88), part of the downstream signalling cascade of TLR, was essential for responses to infection (Adachi *et al.*, 2001). It was demonstrated that MyD88-deficient mice failed to produce IL-12 in response to infection with *P. berghei*, preventing subsequent liver damage that is associated with IL-12 production during infection in this model. The specific TLR mediating this response was not identified but TLR2, TLR4 and TLR6 were ruled out because knock out mice for these receptors showed normal increases of IL-12 in response to *P. berghei* infection (Adachi *et al.*, 2001).

In vitro experiments using *P. falciparum*-iRBC demonstrated that intact iRBC, lysates, or the soluble fractions of lysates all activate human plasmacytoid DC. Although the viability of plasmacytoid DC was maintained, they never fully matured in response to subsequent stimulation and induced only poor proliferation in allogeneic $CD4^+$ T cells (Pichyangkul *et al.*, 2004). The plasmacytoid DC did however efficiently activate γδT cells in the presence of lysate, consistent with a marked increase in circulating γδT cells observed during acute *P. falciparum* infection (see section 1.5.1.4) (Behr and Dubois, 1992; Goodier *et al.*, 1993). Following these observations further work was undertaken to characterise the molecule or molecules responsible for these effects. While the factors inducing activation of plasmacytoid DC had characteristics of a protein, factor(s) activating γδT cell appeared to be lipids as had been reported before (Farouk *et al.*, 2004). Further experiments indicated that the effect of *P. falciparum* lysate on mouse plasmacytoid DC *in vitro* was dependent on TLR9 (Farouk *et al.*, 2004).

Studies by Coban *et al.* addressed activation of murine DC by *P. falciparum* haemozoin (Coban *et al.*, 2005). They observed that pro-inflammatory cytokine production was not evident in MyD88 knock out mice indicating involvement of TLR pathways. Myeloid and plasmacytoid DC derived from wild type mice or TLR2, TLR4 and TLR7 knock out mice were activated by haemozoin, as indicated by an increase in CD40 and CD86 expression. However, neither activation nor cytokine production was observed in DC derived from TLR9 knock out mice. Cytokine responses were the same for parasite-derived or synthetic haemozoin, indicating that in this model the effects were due to haemozoin itself rather than contaminating factors such as lipids, proteins or DNA.

Plasmodium glycosylphosphatidylinositol (GPI) has long been suspected to induce inflammatory signals (see section 1.5.3.2). Recent studies have demonstrated that GPI

binds to TLR2 and, to a lesser extent, to TLR4 expressed by mouse and human macrophages and induces TNF-α secretion (Krishnegowda *et al.*, 2005; Zhu *et al.*, 2005). Mouse macrophages were also shown to produce IL-12, IL-6 and nitric oxide in response to GPI when they were first primed with IFN-γ. It is therefore possible that GPI will also activate other myeloid cells such as myeloid DC via TLR2 and TLR4 in humans. Of note, free GPI has been shown to be quickly inactivated *in vivo* by phospholipases in serum and on cell surfaces. This may explain why activation of myeloid cells by *Plasmodium* GPI has long been suspected but very difficult to prove.

A recent study has examined TLR polymorphisms in African children in relation to malaria susceptibility (Mockenhaupt *et al.*, 2006). It was shown that there was no difference in the distribution of known TLR9 polymorphisms in children with severe malaria compared to controls. In the case of TLR2, two polymorphisms found in Caucasians, Asians and North Africans were screened for, but none of the cases or controls had either of these polymorphisms in the study population. One child with severe malaria had a previously unidentified TLR2 single nucleotide polymorphism (SNP). When this SNP was characterised *in vitro* it was shown to be unresponsive to the TLR2 ligand Pam_3Cys. Two TLR4 polymorphisms were shown to be significantly more common in patients with severe malaria compared to controls. Although these polymorphisms have not been fully characterised, this study does provide further evidence that TLR2 and TLR4 are important for the innate immune responses to *Plasmodium* infection.

Together, the above studies suggest that both haemozoin and as yet unidentified protein(s) can activate DC by binding to TLR9, while GPI can activate DC by binding to TLR2 and TLR4. In mice, TLR9-mediated signalling occurs in both myeloid and

plasmacytoid DC. In this case, TLR9-mediated signalling can induce tolerance to TLR4-mediated signalling (Perry *et al.*, 2005). In human malaria however, only plasmacytoid DC will respond to TLR9 ligands. The activation of plasmacytoid DC and their production of IFN-α can then induce the maturation of myeloid DC. Whether or not myeloid DC will be more or less responsive to TLR2 and TLR4-mediated signals, however, remains to be investigated for *P. falciparum* infection.

1.5.2.2 Scavenger receptors

Scavenger receptors consist of a group of receptors that bind to chemically modified lipoproteins and mediate endocytosis. These receptors are broadly expressed on macrophages, DC and some endothelial cells. The class B scavenger receptor, CD36, is the most important scavenger receptor studied so far in *P. falciparum* malaria. Studies suggest that CD36 is the crucial receptor for the phagocytosis of iRBC that have not been opsonised by either complement proteins or antibodies. Macrophages from CD36-deficient mice ingest *P. falciparum*-iRBC at a much lower rate than macrophages expressing normal levels of CD36 (McGilvray *et al.*, 2000; Patel *et al.*, 2004). Neither human nor rodent macrophages produce TNF-α in response to phagocytosis of iRBC suggesting that non-opsonic phagocytosis does not result in the activation of myeloid cells. This is similar to the *in vitro* modulation of DC function mentioned above (see section 1.5.1.2). Similar modulation of DC function was observed in response to antibodies against CD36 suggesting that adhesion of iRBC to CD36 expressed on DC is sufficient to cause modulation of activity (Urban *et al.*, 2001). Interestingly, it has recently been reported that TLR2 and CD36 cooperate in the recognition of microbial diacylglycerides (Hoebe *et al.*, 2005; Stuart *et al.*, 2005).

The role of scavenger receptors other than CD36 in *Plasmodium* blood stage infection is

less clear. Mice resistant to *P. chabaudi* infection show some increase in phagocytic activity compared to susceptible mice. Subsequent blocking of scavenger receptors with polyinosinic acid inhibited phagocytosis of iRBC and merozoites *in vitro* and led to increased parasitaemia *in vivo* in resistant mice compared to susceptible mice. However, blocking of scavenger receptors did not affect the development of protective immune responses (Su *et al.*, 2002). Furthermore, type I and II class A scavenger receptor knock out mice showed a similar course of *P. chabaudi* infection to wild type mice. The involvement of mannose receptors was tested by inhibition with mannan. Again, a decrease in phagocytosis was seen without altering the course of infection *in vivo* (Su *et al.*, 2002). Together, these results suggest that scavenger receptors are involved in innate responses to *Plasmodium* infection but not critical for the control of parasitaemia or indeed survival in rodent models of malaria.

1.5.2.3 Complement receptors

Complement receptor 1 (CR-1) is an immune regulatory molecule expressed on the surface of a range of cell types including erythrocytes. CR-1 is responsible for binding activated complement components C3b and C4b, leading to the clearance of immune complexes, increased phagocytosis and the regulation of complement activation (Ahearn and Fearon, 1989). CR-1 is also required for rosetting, a process associated with severe malaria where iRBC bind uninfected RBC to form aggregates (see section 1.4.1)(Rowe *et al.*, 1995). CR-1 on uninfected RBC has been shown to be required for the formation of rosettes by interacting with PfEMP1 expressed on the surface of iRBC (Rowe *et al.*, 1997). Several studies have investigated the effect of CR-1 polymorphisms on malarial disease but the role of CR-1 remains to be clarified. CR-1 polymorphisms in West Africans have been identified in association with malaria

(Moulds *et al.*, 2000; Thomas *et al.*, 2005). Subsequent work in Papua New Guinea indicated that a CR-1 polymorphism that reduces CR-1 expression confers protection against severe malaria (Cockburn *et al.*, 2004). This was, however, associated with the heterozygotes not the homozygotes for the CR-1 low-expression allele. It is still to be determined whether effects mediated by CR-1 are due to rosetting or its ability to bind immune complexes and C3b. Either hypothesis for its method of protection from severe malaria is possible depending on which of the two theories of cerebral malaria pathogenesis is preferred; occlusion of microvasculature due to rosetting or the stimulation of inflammation via the binding of immune complexes. A further study indicated that Thai adults homozygous for low CR-1 expression were in fact more susceptible to severe malaria (Nagayasu *et al.*, 2001). However there was no significant difference in CR-1 levels between the homozygous controls and the heterozygotes, leading to questions of whether the effects are due to CR-1 expression or another factor.

1.5.3 Pattern recognition receptor ligands in *Plasmodium*

Several ligands that are thought to interact with pattern recognition receptors have been examined in *Plasmodium* parasites. The major PRR ligand candidates identified so far include *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), glycosylphosphatidylinositol (GPI) and haemozoin.

1.5.3.1 P. falciparum *erythrocyte membrane protein-1 (PfEMP-1)*

PfEMP-1 mediates sequestration of infected red blood cells (iRBC) during *P. falciparum* infection and undergoes clonal antigenic variation. PfEMP-1 is encoded by approximately 60 *var* genes per genome, only one of which is transcribed at any time. The extracellular portion of PfEMP-1 is composed of variable numbers of two main domains: the Duffy binding like (DBL) domain and the cysteine-rich interdomain

region (CIDR) (Gardner *et al.*, 2002). All PfEMP-1 molecules contain at least one CIDR domain and two DBL domains. Although domain structures can be identified, these domains show considerable sequence heterogeneity. Adhesion of specific PfEMP-1 variants to host receptors has been located within different domains (Baruch *et al.*, 1997; Rowe *et al.*, 1997; Smith *et al.*, 2000). Almost two thirds of CIDR domains of PfEMP-1 encoded by *var* genes in the genome of the laboratory parasite line 3D7 bind to the scavenger receptor CD36, whereas some DBL domains can bind to ICAM-1 or complement receptor-1 (CR-1) (Robinson *et al.*, 2003). Binding to CR-1 expressed on erythrocytes mediates rosetting of erythrocytes by iRBC, as discussed above (see section 1.4.1) (Rowe *et al.*, 1997). Whether iRBC that bind to CR-1 on erythrocytes also adhere to myeloid cells (i.e. macrophages in the spleen), remains to be determined.

1.5.3.2 Glycosylphosphatidylinositol (GPI)

Many proteins expressed on the surface of merozoites are anchored by GPI (Sanders *et al.*, 2005). GPI has long been associated with potent activation of innate immune cells resulting in the production of TNF-α, which makes it a good candidate PRR ligand (Schofield and Hackett, 1993). Individuals living in endemic areas readily make antibodies against *P. falciparum* GPI. The expression of GPI antibodies has been associated with protection from severe disease in some studies (Boutlis *et al.*, 2002; de Souza *et al.*, 2002). In addition, GPI has been proposed to be a ligand for CD1d, a non-classical MHC molecule that binds glycolipids. Recognition of CD1d by invariant T cell receptors expressed on NKT cells has been shown to regulate susceptibility to severe disease in rodent models of malaria (Hansen *et al.*, 2003).

1.5.3.3 Haemozoin

The malarial pigment haemozoin is a detoxification product of haem that is usually

found in the food vacuoles of the *Plasmodium* parasite. Haemozoin, together with other debris, is released when mature iRBC rupture and is rapidly taken up by neutrophils, monocyte/macrophages and DC. Haemozoin is not biochemically inert: it reacts with membrane phospholipids which are transformed into hydroxy-polyunsaturated fatty acids, causing membrane peroxidation (Schwarzer *et al.*, 1992; Schwarzer *et al.*, 2003). In addition, haemozoin catalysis induces the formation of prostaglandin-E_2 (PGE_2) and PGF_{2a}. While hydroxy-polyunsaturated fatty acids inhibit monocyte function such as phagocytosis, activation by inflammatory cytokines and generation of an oxidative burst, the release of PGE_2 and PGF_{2a} could alter T- and B-cell functions. Furthermore, monocyte differentiation into DC was impaired in the presence of haemozoin (Skorokhod *et al.*, 2004) and DC co-cultured with haemozoin showed an altered response to maturation signals. These impairments were accompanied by increased expression of the peroxisome proliferator-activated receptor-γ, up-regulation of which is known to interfere with DC maturation (Angeli *et al.*, 2003; Nencioni *et al.*, 2002).

1.5.4 Cytokines in *P. falciparum* infection

Malarial disease and its severity are largely dependent upon the balance of cytokines released during the course of infection (Malaguarnera and Musumeci, 2002). Several different cytokines have been deemed to be required for the development of immunity to *Plasmodium* infection or the development of severe pathology. Early pro-inflammatory (T_H1) cytokine responses seem to be responsible for protective immunity whereas late responses contribute to pathology and may lead to severe disease. In mild malaria it would seem that early inflammatory responses are downregulated by subsequent anti-inflammatory (T_H2) cytokine production.

1.5.4.1 Tumour Necrosis Factor-α

Tumour Necrosis Factor-α (TNF-α) was the first cytokine described as being induced by parasite infection. TNF-α is involved in the regulation of IL-12 and is an important co-factor for IL-12-induced production of IFN-γ by NK cells. Elevated TNF-α levels are associated with parasite clearance and resolution of fever (Tripp *et al.*, 1993).

1.5.4.2 Interferon-γ

NK cells produce the pro-inflammatory cytokine interferon-γ (IFN-γ) during early *Plasmodium* infection and T lymphocytes are the primary source during the specific immune responses that follow. There is evidence that children with uncomplicated malaria are more likely to have higher concentrations of IFN-γ-secreting $CD4^+$ T-cells than children with hyperparasitaemia (Beutler and Grau, 1993).

1.5.4.3 Interleukin-12

Interleukin-12 (IL-12) has been shown to be effective in conferring protection against a broad range of infections (Trinchieri, 1995). IL-12 induces antibody isotype-switching through IFN-γ-dependent and independent mechanisms as well as increasing cell-mediated immune responses. Some but not all studies show that levels of IL-12 are lower in severe falciparum malaria (Luty *et al.*, 2000; Musumeci *et al.*, 2003; Perkins *et al.*, 2000); however, there is a subsequent study that shows the opposite, with a small but significant increase in IL-12 during severe malaria (Lyke *et al.*, 2004).

1.5.4.4 Interleukin-18

Interleukin-18 (IL-18) is an immunoregulatory cytokine that induces TNF-α, IFN-γ and IL-1β secretion by macrophages, induces T_H1 differentiation and NK cell cytotoxicity. IL-18 acts synergistically with IL-12 to induce IFN-γ release from macrophages.

It has been shown that during *P. falciparum* infection there is an increase in IL-18 during acute and convalescent phases of uncomplicated malaria (Torre *et al.*, 2001).

1.5.4.5 Interleukin-4

Interleukin-4 (IL-4) is an anti-inflammatory cytokine that stimulates a T_H2 response whilst inhibiting T_H1 responses by suppressing IFN-γ production.

An association between IL-4 production by T-cells to specific malaria antigens *in vitro* and increasing serum antibodies to the same antigens *in vivo* has been demonstrated (Troye-Blomberg *et al.*, 1990).

1.5.4.6 Interleukin-10

Interleukin-10 (IL-10) induces B cell proliferation, which is essential for the development of malarial antibodies. IL-10 is secreted by macrophages, T_H2 cells and B cells and inhibits cytokine production by T_H1 cells. Low levels of IL-10 have been shown to be associated with severe malarial anaemia compared to cerebral and uncomplicated disease (Kurtzhals *et al.*, 1998a; Othoro *et al.*, 1999).

1.5.4.7 Interleukin-8

IL-8 is a chemotactic pro-inflammatory chemokine that is involved in a range of inflammatory and immune responses (Mukaida *et al.*, 1998).

The role of IL-8 in the course of malaria infection has been examined in a small number of studies. Volunteers experimentally infected with *P. falciparum* show an increase in IL-8 early in the course of infection (Hermsen *et al.*, 2003). Similarly, a study in patients in Mali has shown that there is a small but significant increase in IL-8 levels during severe malaria compared to controls (Lyke *et al.*, 2004).

1.5.4.8 Transforming Growth Factor-β

Transforming Growth Factor-β (TGF-β) plays a role in the transition between T_H1 and T_H2 responses in malaria. TGF-β inhibits the production of IFN-γ and TNF-α, upregulates IL-10 and downregulates surface adhesion molecules (Maeda and Shiraishi, 1996; Nakabayashi *et al.*, 1997). Recently, TGF-β has attracted considerable attention in relation to its role during malaria. Lower levels of TGF-β were detected in acute malaria patients than healthy controls; however there was no difference between acute and severe malaria (Wenisch *et al.*, 1995). A study involving *P. falciparum* sporozoite challenge of human volunteers has suggested that the production of TGF-β is associated with higher growth rates of parasites *in vivo* (Walther *et al.*, 2005).

1.5.5 Nitric oxide

Nitric oxide (NO) has long been suggested to play a role in malarial disease (Clark *et al.*, 1992). The role of nitric oxide has been described as both detrimental in patients in Papua New Guinea (Al Yaman *et al.*, 1996) and protective in patients in Tanzania (Anstey *et al.*, 1996). The inability to measure NO directly, due to its labile nature, leads to a requirement to use its oxidation products as indirect measures. When testing patient samples this makes interpretation of results difficult.

1.5.6 Antibody responses

The importance of antibody responses in malarial immunity is demonstrated by a reduction of parasitaemia by passive transfer of immunoglobulins (Ig). This is supported by the fact that children of less than six months of age, in high transmission areas, are protected from malaria, this being attributed to the transfer of maternal IgG (Riley *et al.*, 2001). Studies of the antibody responses of both convalescent children and immune adults have demonstrated that they have circulating iRBC-agglutinating

antibodies (Marsh and Howard, 1986). These agglutinating antibodies are specific to parasite variant surface antigens (VSA), such as PfEMP-1, exposed on the surface of iRBC. Bull *et al.* showed that Kenyan children are unlikely to be infected with parasites expressing VSA against which they have specific antibodies (Bull *et al.*, 1998). This demonstrates the importance of VSA-recognising antibodies in the protection against subsequent *P. falciparum* infection. It is thought that a component of immunity to malaria is developed through the gradual acquisition of a repertoire of antibodies to the different parasite variants in a human population (Bull and Marsh, 2002). Antibody responses alone however are not thought to be sufficient to provide protection from malaria episodes.

It has been shown that IgE levels are higher in people living in malaria endemic areas and that IgE levels are elevated during malaria, with levels significantly higher in cases of severe and cerebral malaria compared to uncomplicated malaria (Perlmann *et al.*, 1999). Experimentally induced malaria in mice also results in elevated IgE providing indirect evidence that this is induced by the *Plasmodium* parasite itself and not a concomitant infection.

1.5.7 Role of the spleen during malaria

The spleen plays a significant role during *Plasmodium* infection, however its importance has been neglected in recent years. The spleen is responsible for removing damaged and iRBC from the circulation. Splenectomised individuals with malaria have reduced clearance of parasites from the circulation (Chotivanich *et al.*, 2002). Interestingly, splenectomy in mice has been shown to protect against cerebral malaria upon *P. berghei* infection. Similarly, depletion of $CD4^+$ and $CD8^+$ T cells has the same effect, confirming the importance of the spleen as a site for the development of

protective and pathological immune responses following *Plasmodium* infection (Hermsen *et al.*, 1997). Specific T and B cell responses are generated within the spleen. The spleen is also an important site for haematopoiesis and erythropoiesis (Engwerda *et al.*, 2005). Recent evidence suggests that fatal malaria is associated with disorganisation of splenic architecture, including the inhibition of DC migration (Urban *et al.*, 2005). Additionally, immune cells in the spleen can 'pit' iRBC in order to remove and destroy the parasite but leave the erythrocyte intact and able to reenter the circulation (Angus *et al.*, 1997; Chotivanich *et al.*, 2002).

1.5.8 Co-infection with *Plasmodium* and helminth parasites

In areas of malaria transmission *P. falciparum* is not likely to be the exclusive pathogen that a patient carries. Co-infection with different pathogens can influence the generation of immunity to these infectious agents. Parasitic worms have high prevalence rates in regions with malaria transmission and have generated interest in relation to their effect on malaria progression and immunity. Studies from the late 1970's showed that infection with *Ascaris lumbricoides* led to suppression of malaria symptoms and treatment for ascariasis was followed by subsequent malaria recrudescence (Nacher *et al.*, 2002). Ascariasis has subsequently been shown to protect against cerebral malaria (Nacher *et al.*, 2000). In any study involving malaria patients, concomitant infection with worms should be taken into consideration.

In general, it has not been clearly established whether clinical immunity to *P. falciparum* infection is due to regulation of the balance of T_H1 and T_H2 cytokines, development of an appropriate repertoire of antibodies, adaptive immune responses to bioactive parasite products or a combination of mechanisms.

1.6 MACROPHAGE MIGRATION INHIBITORY FACTOR

Macrophage migration inhibitory factor (MIF) was one of the first cytokines described about 40 years ago. It was identified as a T-cell derived factor that inhibited the random migration of monocytes in an *in vitro* model for delayed-type hypersensitivity reaction (David, 1966). The biological activities of MIF were not further elucidated until the cloning of MIF complementary DNA was achieved in 1989 (Weiser *et al.*, 1989). A single human MIF gene is present on chromosome 22 that encodes a 12.5 kDa protein. MIF is highly evolutionarily conserved with homologues identified in birds, jawless fishes (Sato *et al.*, 2003) and nematodes, as well as plants and cyanobacteria (Calandra and Roger, 2003). Mammalian MIF has been assigned cytokine, hormone-like and thioredoxin-like functions. The only other gene with which MIF shares marked homology is D-dopachrome tautomerase. Although MIF has been studied extensively over the last 15 years, clarification of its activities and exact modes of action remains elusive and at times contradictory.

Initial studies identified a role for mouse MIF as a mediator of endotoxic shock (Bernhagen *et al.*, 1993). Subsequently, MIF has been linked to an expanding range of activities in a wide variety of systems.

1.6.1 MIF expression

MIF is expressed in a wide range of tissues and cell types and is not specific to the immune system. The primary source of MIF was initially thought to be T cells; however, it has since been shown that many other cells of the immune system produce MIF, including monocytes, macrophages, eosinophils, dendritic cells, B cells, mast cells, basophils and neutrophils (Calandra *et al.*, 2003). Outside the immune system MIF is expressed in kidney, liver, fibroblasts, Leydig cells, adipocytes and vascular

endothelial cells (Abe *et al.*, 2000; Calandra *et al.*, 1994; Calandra and Roger, 2003; Meinhardt *et al.*, 1996; Nishihira *et al.*, 1998; Rossi *et al.*, 1998; Skurk *et al.*, 2005).

1.6.2 MIF secretion

MIF is released from macrophages in response to LPS, exotoxins, haemozoin, and exposure to gram-negative and gram-positive bacteria, mycobacteria, and pro-inflammatory cytokines including TNF-α and IFN-γ (Calandra *et al.*, 2003). Many of these stimuli elicit a bell-shaped release of MIF (Calandra *et al.*, 1998).

Preformed MIF exists in several cell types indicating that a good deal of MIF is secreted from preformed stores. This is unlike most cytokines for which expression is upregulated by stimulation. The majority of cytokines are secreted via an endoplasmic reticulum (ER) mediated mechanism; MIF however has no discernable signal sequence for translocation to the ER. Furthermore, studies of cell localisation have shown MIF is located in the cytosol, in small vesicles, the nucleus and vesicles outside the cell (Burger-Kentischer *et al.*, 2002; Nishino *et al.*, 1995). This suggests that MIF secretion occurs via a non-classical pathway and subsequent investigation has shown that MIF is secreted by an ABCA1 transporter-dependent pathway (Flieger *et al.*, 2003).

1.6.3 Structure

In 1996, Sugimoto *et al.* published crystallography studies, which suggested that MIF forms a homotrimer (Sugimoto *et al.*, 1996). Subsequent cross-linking experiments found that MIF in fact consists of a mixture of monomeric, dimeric and trimeric forms (Mischke *et al.*, 1998). Estimates of relative human MIF protein densities have shown that 44% of protein exists in the form of a monomer, 33% dimer and 23% formed trimers. Substitution of the cysteine at position 57 results in less trimer formation, which may affect enzymatic activity (see section 1.6.4.1).

1.6.4 MIF mechanisms of action

The molecular mechanisms attributed to MIF are varied and often contradictory. MIF activity has been attributed to its documented enzymatic activities, activation of signalling pathways, regulation of surface molecule expression, induction of expression and secretion of several factors, the binding of intracellular regulatory molecules and a putative cell surface receptor. The stability and hydrophobicity of MIF led to the suggestion that the protein may be a molecular chaperone (Cherepkova *et al.*, 2006). *In vitro* assays have shown that MIF is indeed capable of preventing protein aggregation in a chaperone-like manner (Cherepkova *et al.*, 2006).

1.6.4.1 MIF enzymatic activities

Uniquely for a cytokine, MIF has two distinct enzymatic activities. Initially a phenylpyruvate tautomerase activity was attributed to MIF (Rosengren *et al.*, 1997). This was soon followed by the identification of a thiol-protein oxidoreductase activity (Kleemann *et al.*, 1998). Although both catalytic sites are well described, the physiological substrates and possible roles for these enzymatic activities are still to be determined.

Several groups have examined mutant MIF proteins in order to dissect the role of its different enzymatic activities in various *in vitro* immunological assays. Mutational analysis has determined that the initial N-terminal proline is essential for tautomerase activity. Several separate studies have confirmed that the removal or substitution of the proline at position 1 with another amino acid completely ablates tautomerase activity (Bendrat *et al.*, 1997; Hermanowski-Vosatka *et al.*, 1999; Stamps *et al.*, 1998; Swope *et al.*, 1998). However, MIF proteins with a truncated C-terminus are also incapable of tautomerase activity. This is most likely due to the importance of the C-terminus in

correct protein folding and not to direct involvement at the catalytic site.

The oxidoreductase activity has been shown to be centred on the CXXC motif of MIF (Kleemann *et al.*, 1998). Substitution of either cysteine in this motif reduces oxidoreductase activity. This motif also appears to be required for macrophage activation. Substitution of the cysteines at position 57 or 60 as well as the downstream cysteine at position 81 leads to a reduction in glucocorticoid overriding activity (Kleemann *et al.*, 1999). *In vitro* experiments have shown that a 16-residue MIF peptide that straddles the CXXC motif has oxidoreductase activity, overrides glucocorticoid activity and also increases extracellular signal-regulated kinases (ERK)1/2 phosphorylation (see section 1.6.4.3) (Nguyen *et al.*, 2003).

Although the enzymatic activities of MIF are well established, their contribution to the physiological roles of MIF remains to be determined. N-terminal mutants, which lack tautomerase activity, are still capable of inhibiting chemotaxis and random migration of monocytes *in vitro* (Hermanowski-Vosatka *et al.*, 1999), as well as counter-regulating glucocorticoid inhibition of TNF-α production in monocytes (Lubetsky *et al.*, 2002). This indicates that another mechanism of action, aside from tautomerase activity, is responsible for the migration phenotype of MIF and its effect on glucocorticoid counter-regulation. Numerous studies of MIF mutants form no consensus to clearly link either enzymatic activity to any of MIF's attributed biological actions. The lack of a consistent test for MIF activity across these studies makes this issue more difficult to clarify.

1.6.4.2 Counter regulation of glucocorticoid action

Glucocorticoids have the ability to inhibit the expression of a wide range of pro-inflammatory cytokines. This is exploited during the treatment of chronic inflammatory conditions such as arthritis, Crohn's disease and asthma (Van Molle and Libert, 2005). The initial link between anti-inflammatory glucocorticoids and pro-inflammatory MIF was the surprising finding that dexamethasone (a glucocorticoid analogue) induced MIF expression (Calandra *et al.*, 1995). MIF was subsequently shown to override the dexamethasone mediated inhibition of LPS-induced pro-inflammatory cytokines (figure 1.3). Further studies have demonstrated that there is a balance between pro- and anti-inflammatory responses mediated by glucocorticoids and MIF (Van Molle and Libert, 2005).

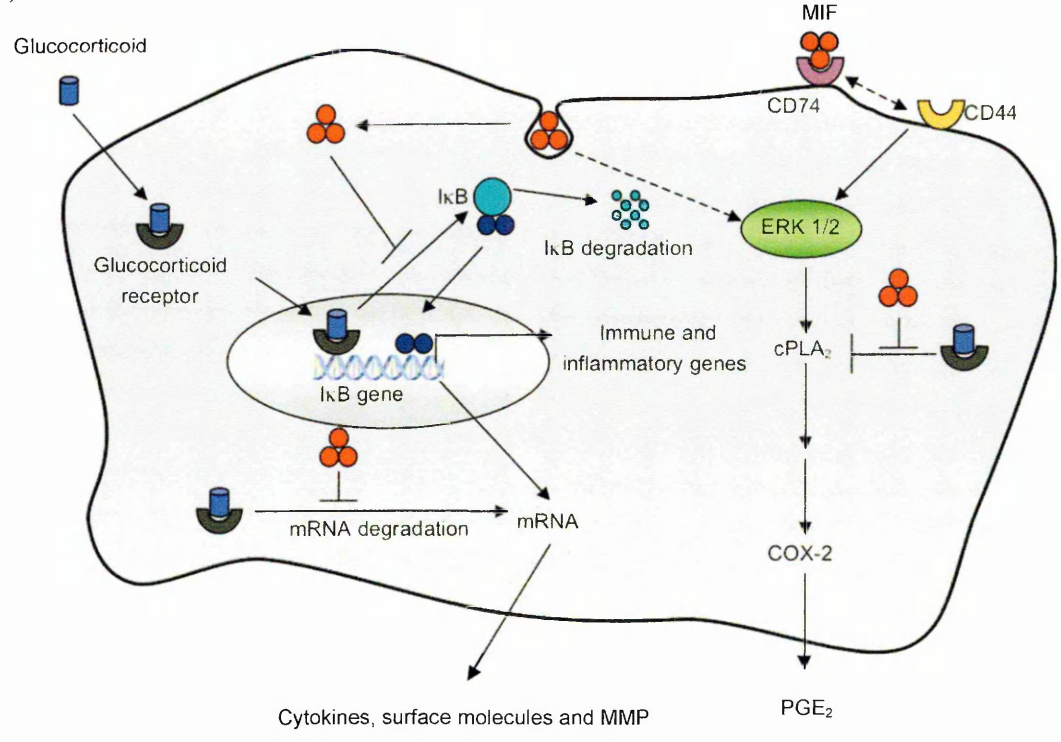


Figure 1.3: MIF counter-regulation of glucocorticoid activity. MIF counter-regulates the immunosuppressive effects of glucocorticoids at the transcriptional and post-transcriptional levels. Modified from Calandra and Roger (2003) (Calandra and Roger, 2003) and Renner *et al.* (2005) (Renner *et al.*, 2005).

1.6.4.3 Activation of signalling pathways

In 1999, Mitchell et al. reported dose-dependent activation of ERK1/2 by recombinant MIF (figure 1.4) (Mitchell *et al.*, 1999). This was the first regulatory affect attributed to MIF and has subsequently been confirmed by several groups (Fukuzawa *et al.*, 2002; Leng *et al.*, 2003; Liao *et al.*, 2003; Lue *et al.*, 2005; Nguyen *et al.*, 2003; Onodera *et al.*, 2002). MIF has also been shown to alter the expression of monocyte surface molecules (see section 1.6.4.6). Inhibition of src kinases, phosphoinositide 3 (PI3) kinase and nuclear factor κB (NFκB) led to reduced expression of surface molecules in response to MIF (Amin *et al.*, 2006). The role of MIF in some other cell signalling pathways is less well defined. For example, Santos and colleagues identified p38 mitogen activated protein (MAP) kinase activation by MIF in synovial cells (Santos *et al.*, 2004). However, this is contradicted by studies in both osteoblasts (Onodera *et al.*, 2002) and endothelial cells (Amin *et al.*, 2003), which report that p38 signalling pathways are not activated by MIF. Many of the contradictory results seen in this area may be due to differing mechanisms of action of MIF in the wide variety of different cell types the protein is known to influence.

1.6.4.4 Inhibition of p53 activity

p53 is a protein involved in mediating growth arrest and apoptosis. Studies performed by Hudson *et al.* identified MIF as a negative regulator of p53 activity (Hudson *et al.*, 1999). An effect of MIF to down-regulate p53 activity, including NO-induced apoptosis, thereby implicates MIF in inflammation and potentially in tumorigenesis. Inhibition of p53 activity by MIF has subsequently been shown to reduce *in vitro* apoptosis in macrophages, thereby demonstrating a potential pro-inflammatory regulatory role for MIF in innate immunity (figure 1.4) (Mitchell *et al.*, 2002).

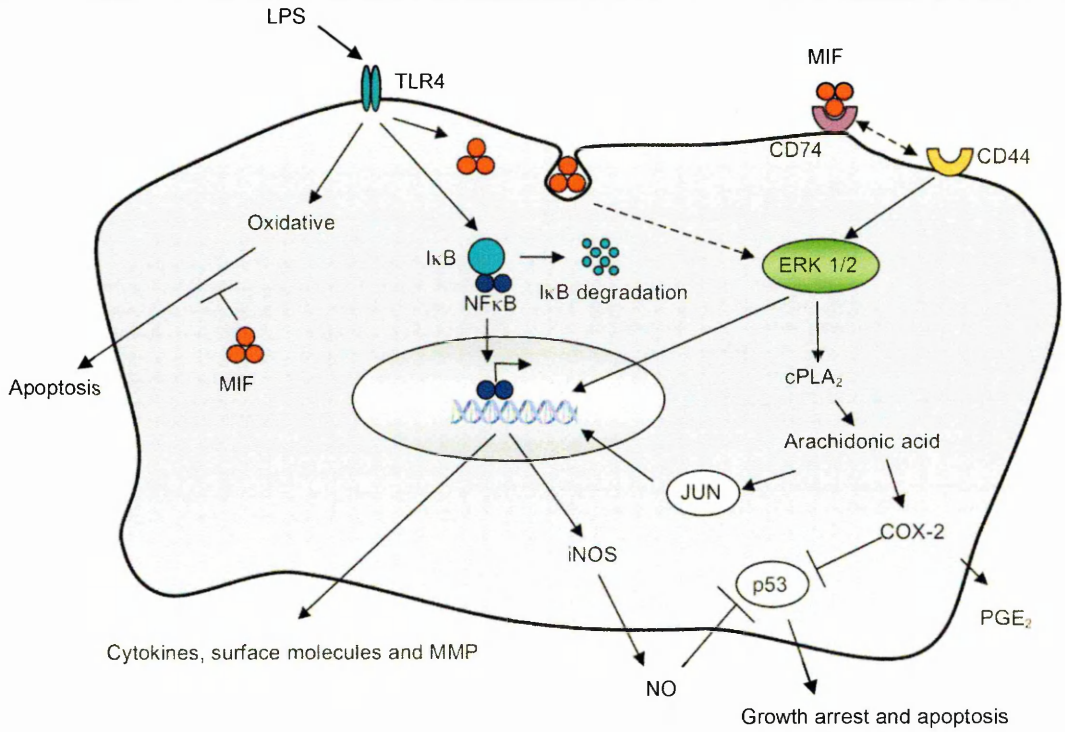


Figure 1.4: Induction and regulation of inflammatory responses by MIF. MIF can activate a cascade of events including ERK1/2 activation, PLA_2 induction, JNK activity and PGE_2 production. Additionally MIF has been shown to be important for TLR4 expression on macrophages, thereby increasing recognition of endotoxin, which promotes the production of cytokines, nitric oxide and other mediators. Modified from Calandra and Roger (2003) (Calandra and Roger, 2003) and Renner *et al.* (2005) (Renner *et al.*, 2005).

The role of MIF in the regulation of p53 during the course of disease progression has so far been examined for a couple of conditions. The inflammation seen during rheumatoid arthritis is underpinned by an imbalance between proliferation and apoptosis. Synoviocytes treated *in vitro* with MIF express less p53 than untreated cells (Leech *et al.*, 2003). Similarly, MIF-deficient mice express more p53 in spleen cells and fibroblasts than wild-type animals and also showed greater rates of sodium

nitroprusside induced apoptosis (Leech *et al.*, 2003). Experimentally induced arthritis was found to be less severe in MIF deficient mice, indicating an important role for MIF in the progression of this disease and also providing further evidence for MIF as a pro-inflammatory signal (Ichiyama *et al.*, 2004). Indirect evidence has also suggested that MIF is responsible for *Helicobacter pylori*-mediated inhibition of apoptosis and development of gastric cancers via the inhibition of p53 activity (Beswick *et al.*, 2006).

1.6.4.5 Binding to JAB1

MIF was shown to bind to JUN-activation domain-binding protein 1 (JAB1) in a yeast two-hybrid system (Kleemann *et al.*, 2000). MIF and JAB1 were subsequently shown to co-localise in the cytoplasm (Kleemann *et al.*, 2000). JAB1 is involved in activating JUN N-terminal kinase (JNK), which phosphorylates JUN and thereby activates the transcription factor activator protein-1 (AP-1). AP-1 is implicated in cell death, transformation and growth. MIF inhibits JAB1 mediated activation of AP-1 while also antagonising JAB1-dependent cell cycle regulation by stabilising p27Kip1 protein leading to cell cycle arrest and apoptosis (figure 1.5).

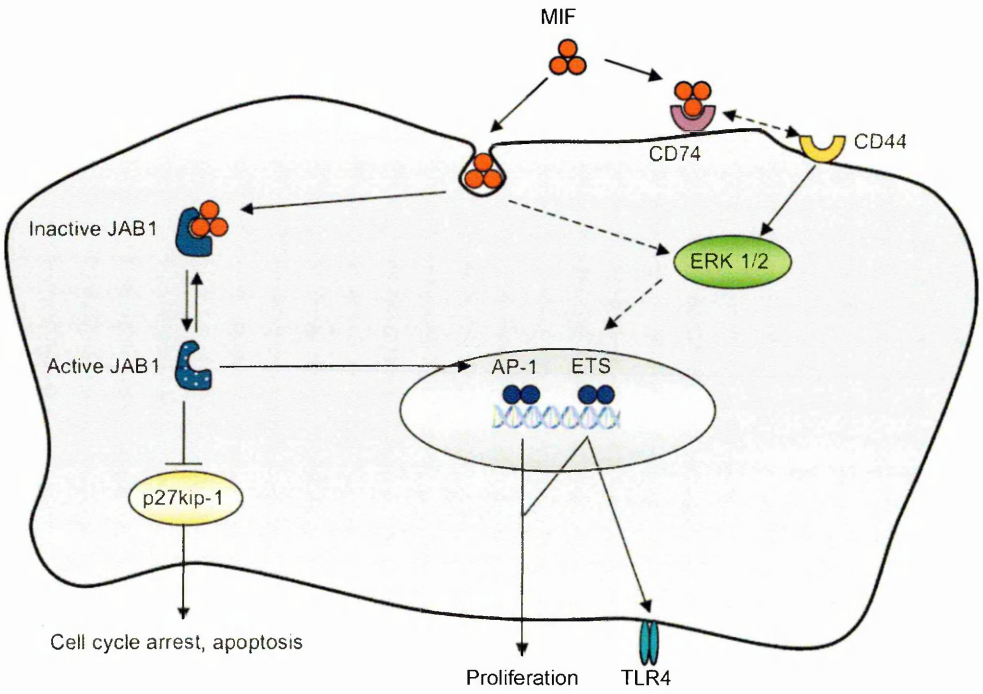


Figure 1.5: MIF influence on JAB-1 activity. MIF binding to JAB1, preventing JAB1-induced degradation of the p27kip1, results in cell cycle arrest and apoptosis. Modified from Calandra and Roger (2003) (Calandra and Roger, 2003) and Renner *et al.* (2005) (Renner *et al.*, 2005).

1.6.4.6 Regulation of surface molecule expression

MIF has been shown to regulate the expression a number of molecules expressed on the surface of cells. An important example is TLR4. RAW 264.7 mouse macrophage cells were transfected with antisense MIF mRNA, thereby lowering endogenous MIF levels. This led to a reduction in expression of TLR4 (Roger *et al.*, 2001). This downregulation was shown to be dependent on reduced TLR4 promoter activity, which is linked to reduced DNA-binding of the transcription factor PU.1 in the absence of MIF. Reduced MIF expression was also shown to reduce LPS induced TNF-α production. A subsequent study in macrophages isolated from MIF KO mice demonstrated that MIF-

deficient macrophages express lower levels of TLR4 and also produce less TNF-α in response to LPS (Roger *et al.*, 2003). These studies primarily address endogenous MIF, however, addition of anti-MIF antibodies to RAW 264.7 macrophage cells also reduced TLR4 expression and LPS-induced TNF-α production (Roger *et al.*, 2003). This suggests that MIF also exerts an autocrine effect on macrophage function and that cells may therefore be responsive to exogenous MIF. The effect of exogenous MIF on TLR4 expression has not yet been examined.

The regulation of cell surface molecules other than TLR4 has been shown to be influenced by exogenous MIF. Monocytes exposed to recombinant MIF *in vitro* significantly upregulated vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) (Amin *et al.*, 2006). This increase in surface molecule expression is dose-dependent but exhibits a bell-shaped dose response with ICAM-1 and VCAM-1 expression peaking with 10nM recombinant MIF and decreasing at higher MIF concentrations. Functional significance of this increase in expression was confirmed by an increase in monocyte adhesion after MIF treatment (Amin *et al.*, 2006). This has significant potential for malaria pathology when considering that parasite cytoadhesion can be mediated by the binding of PfEMP-1 to both ICAM-1 and VCAM-1 (see section 1.4).

Evidence for the potential importance of MIF induced surface molecule expression *in vivo* is provided by studies in MIF KO mice. MIF deficient mice were shown to exhibit reduced interactions between leukocytes and endothelial cells when treated with LPS (Gregory *et al.*, 2004). This reduction in leukocyte adhesion and rolling in MIF KO mice could well be mediated by a reduction in adhesion molecules on endothelial cells due to MIF deficiency.

1.6.4.7 Receptor-mediated actions

CD74 (also known as MHC class II-associated invariant chain) is involved in the transport of MHC class II from the ER to the Golgi apparatus. CD74 has been identified as a putative MIF receptor (Leng *et al.*, 2003). An estimated 5% of cellular CD74 is expressed on the cell surface. The intracellular domain of CD74 has no motifs that interact with known signal-transducing molecules so its role in down-stream MIF cellular activation is questionable. Despite this, several groups have described the importance of CD74 in downstream signalling upon binding of MIF. It has been shown that activation of the ERK signalling pathway by MIF is dependent upon the involvement of CD74 (Leng *et al.*, 2003). CD74 bound with either MIF or anti-CD74 antibody has also been shown to lead to NFκB activation (Beswick *et al.*, 2005; Matza *et al.*, 2001). Recently, it has been shown that downstream ERK 1/2 signalling initiated by MIF binding to CD74 requires the involvement of CD44 (Shi *et al.*, 2006). This indicates that CD74 is essential but not sufficient as a MIF receptor. CD74 appears to be an anchoring molecule that upon binding MIF, interacts with CD44 leading to downstream signal transduction.

1.6.4.8 MIF induction of cellular factors

The treatment of different cells with MIF induces the release of several factors. Treatment of macrophages with MIF induces the release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-12, IL-6 and IL-8 (Calandra *et al.*, 1995; Donnelly and Bucala, 1997). MIF treatment of T cells also promotes IL-2 expression and memory T-cell development (Bacher *et al.*, 1996; Rodriguez-Sosa *et al.*, 2003). Other factors are also induced in response to MIF, including nitric oxide (Bozza *et al.*, 1999), cyclooxygenase 2 (Mitchell *et al.*, 2002) and products of arachidonic acid metabolism

(Mitchell *et al.*, 1999). Studies into arthritis have shown that MIF can also induce the expression of matrix metalloproteinases (see section 1.7.2) (Onodera *et al.*, 2000; Onodera *et al.*, 2002).

1.6.4.9 Antibody regulation

Research published 20 years ago demonstrated that glycosylation inhibiting factor (GIF), a pseudonym for MIF, purified from T cell hybridomas could suppress IgE and IgG1 antibody responses (Akasaki *et al.*, 1986). This effect has been subsequently confirmed using recombinant GIF to treat B cells activated by LPS and IL-4 (Sugie *et al.*, 1999). Modulation of antibody responses is another potential mechanism that MIF may influence during the course of an infection.

In summary, MIF has several potential modes of action. Aside from the two distinct enzymatic activities there is also evidence for receptor-mediated mechanisms of MIF action and direct interaction of MIF with intracellular signalling molecules. The mechanisms of action of MIF in its wide variety of target cell types still requires considerable clarification in order to reveal a clear mode of action of MIF in relation to different diseases.

1.7 MIF AND DISEASE

A role for MIF has been proposed in a multitude of immune-mediated and infectious diseases. Particular attention has been given to the role of MIF during bacterial sepsis (see section 1.7.1) (Calandra *et al.*, 2003) and the onset and progression of rheumatoid arthritis (see section 1.7.2) (Morand *et al.*, 2006). MIF has also been linked to artherosclerosis (Burger-Kentischer *et al.*, 2005), diabetes (Cvetkovic *et al.*, 2005), asthma (Mizue *et al.*, 2005), autoimmune encephalomyelitis (Denkinger *et al.*, 2003) and ulcerative colitis (de Jong *et al.*, 2001) among many others. The involvement in such a wide range of diseases indicates the importance of MIF in the immune system.

1.7.1 The role of MIF in sepsis

The pro-inflammatory activities of MIF in innate immune responses led to the examination of the protein's role in sepsis. Initial studies showed that administration of MIF to mice in conjunction with LPS led to increased mortality compared to LPS alone (Bernhagen *et al.*, 1993). Neutralisation of MIF with anti-MIF antibodies resulted in less mortality and this was reflected in experiments in MIF-deficient mice (Bozza *et al.*, 1999). In experimental sepsis in mice, induced by live bacteria, MIF concentrations were increased in peritoneal fluid and in systemic circulation (Calandra *et al.*, 2003). High MIF levels have also been detected in the circulation of patients with sepsis (Calandra *et al.*, 2003). Survivors of sepsis had significantly lower levels of circulating MIF than those that did not survive. The critical role of MIF in sepsis demonstrates the importance of MIF in inflammatory conditions.

1.7.2 The role of MIF in arthritis

One of the best studied links between MIF and disease is in relation to arthritis. MIF has a described role in both systemic-onset juvenile arthritis and rheumatoid arthritis (RA). It has been shown that MIF levels are increased in the synovial tissues and circulation of animals with experimentally-induced arthritis. Neutralising antibodies against MIF lead to delayed onset and a lower frequency of arthritis (Leech *et al.*, 2003; Mikulowska *et al.*, 1997). Removal of the suppressive effects of glucocorticoids via adrenalectomy exacerbated experimental arthritis providing further indirect evidence of the role of MIF in this disease (Leech *et al.*, 2003). Subsequent studies using MIF KO mice has confirmed the role of MIF in rheumatoid arthritis models. Arthritis induced in MIF KO was less severe with evidence of reduced cartilage damage (Leech *et al.*, 2003).

One aspect of MIF activity, that so far appears to be unique to the progress of arthritic disease, is the induction of metalloproteinase (MMP) expression. MMP are proteolytic enzymes including collagenases, gelatinases and stromelysins. MIF has been shown to stimulate MMP-1 and MMP-3 mRNA transcription in synovial fibroblasts from RA patients, as well as MMP-9 and MMP-13 in rat osteoblasts (Onodera *et al.*, 2000; Onodera *et al.*, 2002). MIF has therefore been suggested to play a role in joint destruction during autoimmune disease and bone remodelling (Onodera *et al.*, 2002). Due to its role in the induction of MMP, MIF has been suggested as a therapeutic target molecule for the treatment of osteoporosis and RA. Upregulation of MMP by MIF is interesting in relation to malaria because MMP-9 activity has been shown to be increased in response to haemozoin, thereby implicating MMP in the pathogenesis of malaria (Prato *et al.*, 2005).

1.7.3 MIF and infectious diseases

MIF has been shown to be responsible for mediating protection against several infectious organisms. In relation to bacterial pathogens, MIF reduces the growth of *Mycobacterium tuberculosis* in macrophages *in vitro* and is also a mediator of immunity to *Salmonella typhimurium* (Koebernick *et al.*, 2002; Oddo *et al.*, 2005). Immune responses to parasitic infection have been more extensively studied. It has been demonstrated that MIF induces macrophages to kill *Leishmania major* parasites in mice; this manifests as a reduced number of infected cells and lower parasitaemia (Juttner *et al.*, 1998). This killing is dependent on TNF-α and reactive nitrogen intermediates. Following from these findings it has been shown that administration of MIF to mice is protective against subsequent *L. major* infection (Xu *et al.*, 1998). Similarly, MIF KO mice are more susceptible to *L. major* infection than wild type mice, with larger legions and higher parasite loads (Satoskar *et al.*, 2001). MIF KO mice also produce less NO and superoxide in response to *L. major* infection.

Evidence of the role of MIF in adaptive as well as innate immunity is provided by a study into *Trypanosoma cruzi* infection. Compared to wild type mice, MIF KO mice have higher parasitaemia and mortality rates upon *T. cruzi* infection (Reyes *et al.*, 2006). This is characterised by reduced cytokine responses in MIF KO mice including TNF-α, IL-12, IFN-γ and IL-4. Adaptive immune responses in the form of protective antibody responses to *T. cruzi* were also reduced in MIF KO mice. These findings are consistent with findings in helminth infections. *Taenia crassiceps* infection in MIF KO mice results in higher parasite loads, with lower circulating IL-12 and IgG2a responses (Rodriguez-Sosa *et al.*, 2003).

1.7.3.1 MIF and malaria

The first report linking MIF to *Plasmodium* infection was indirectly related to malarial anaemia. It was demonstrated that the addition of MIF to bone marrow cultures reduced the development of erythroid and myeloid progenitors (Martiney *et al.*, 2000; McDevitt *et al.*, 2006). This could be reversed by the addition of anti-MIF antibodies. *In vitro* assays also demonstrated that RBC infected with *P. chabaudi* induced MIF release from macrophages (Martiney *et al.*, 2000). It was subsequently shown that, during the course of *P. chabaudi* infection, circulating MIF levels increased, with the peak in concentration coinciding with peak parasitaemia. Further examination of the expression of MIF during *P. chabaudi* infection indicated that MIF was expressed in the spleen, liver and bone marrow. This study suggested that host-derived MIF may be involved in the pathophysiology of malarial anaemia but no direct evidence was provided (Martiney *et al.*, 2000). A subsequent study in *P. chabaudi*-infected MIF KO mice showed that they had significantly less severe anaemia and better survival than control animals (McDevitt *et al.*, 2006). It was also demonstrated that, during the course of infection, MIF KO mice displayed greater erythroid progenitor maturation than in control mice. This is, however, contradicted by a recent study that identified an association between decreased MIF levels in children with acute malaria and severity of anaemia (Awandare *et al.*, 2006c).

Initial studies of MIF during human *Plasmodium* infection addressed malaria during pregnancy. MIF levels are seen to increase during *P. falciparum* infection in pregnant women, with levels higher in primigravid than multigravid women (Chaisavaneeyakorn *et al.*, 2002; Chaiyaroj *et al.*, 2004). Two subsequent studies measuring circulating MIF levels in *P. falciparum*-infected children produced conflicting results. The first study

performed in Gabon showed a significant decrease in circulating MIF in patients with malaria compared to aged-matched controls (Awandare *et al.*, 2006a). By contrast, a similar study in Zambia showed a significant increase in plasma MIF in *P. falciparum*-infected children compared to age-matched controls (McDevitt *et al.*, 2006). A further study, with larger numbers of subjects will need to be carried out to clarify this discrepancy.

The only other study investigating the role of MIF during malaria infection addressed the tissue distribution of MIF in samples from fatal malaria cases (Clark *et al.*, 2003). The primary finding was that MIF was present in the chest wall blood vessels but not in those of the brain. All of these studies fail to address the possible influence of *P. falciparum*-derived MIF, or another parasite-derived MIF homologue, as a confounder of these results. All the studies utilise anti-MIF antibodies that could potentially cross-react with MIF homologues from other species. This may explain, at least in part, the conflicting results.

1.8 MIF GENE POLYMORPHISMS

Two *MIF* gene polymorphisms that alter the transcription levels of the gene have been identified. These polymorphisms have been examined in several conditions, the severity of which may be influenced by MIF expression levels.

Studies into the pathogenesis of arthritis first identified two polymorphisms of the human MIF gene that predispose to the condition. The first identified polymorphism was a single nucleotide polymorphism, G-to-C transition, at position -173 in the 5' promoter region that conferred a 2-fold increased risk of developing juvenile idiopathic

arthritis (Donn *et al.*, 2001). Characterisation of this polymorphism showed that carrying the G/C or C/C combination of alleles resulted in significantly higher circulating MIF compared to individuals with the G/G alleles (Donn *et al.*, 2002). This would suggest that higher circulating MIF predisposes to juvenile idiopathic arthritis. The second polymorphism is a CATT tetranucleotide repeat beginning at position -794 and ranging between 5 and 8 repeats (Baugh *et al.*, 2002). The 5-CATT allele was shown to have the lowest transcription activity and correlated with reduced disease severity in rheumatoid arthritis. A subsequent study demonstrated an association between 5-CATT allele and reduced susceptibility to RA, but no relationship with severity (Barton *et al.*, 2003). Recently, an association of 5-CATT polymorphism with both sarcoidosis and mild asthma has been described in Caucasians (Plant *et al.*, 2006)(Mizue, 2005). *MIF* gene polymorphisms may also be important in a range of other disease states where the levels of MIF expression seem to play an important part.

Studies into MIF gene polymorphisms and their relation to malaria susceptibility have recently been carried out. A limited survey of MIF polymorphisms in Zambians showed a higher proportion of 5-CATT and -173G polymorphisms compared to European samples, both of which are associated with low expression of MIF (Zhong *et al.*, 2005). Analysis of the genotype of malaria patients reported a correlation between 5/X CATT repeat genotype (where X represents 6-, 7- or 8-CATT) and lower parasitaemia. However, much larger studies are required to confirm these and any other possible effect of this polymorphism in malaria. Another study, examining the role of the -173 G/C polymorphism in Kenyan children, found that MIF -173 C/C was associated with high parasitaemia compared to MIF -173 G/G (Awandare *et al.*, 2006b). No association was seen between -173 alleles and severe malarial anaemia. This study also confirmed

that homozygous G alleles were associated with lower circulating basal MIF compared to G/C alleles, which was previously seen in arthritis studies. MIF secretion from peripheral blood mononuclear cells in response to haemozoin have shown that cells with the 5-CATT polymorphism produce less MIF compared to 6- or 7-CATT (McDevitt *et al.*, 2006). Interestingly, mononuclear cells with the -173 G/G genotype, which is associated with low basal MIF levels, produce increased MIF in response to haemozoin, whereas cells carrying the -173GC genotype produce less MIF in response to haemozoin (Awandare *et al.*, 2006b).

These preliminary studies suggest an important role for *MIF* gene polymorphisms in the progression of diseases for which MIF has been implicated.

1.9 MIF HOMOLOGUES IN PARASITIC SPECIES

Several parasitic species express homologues of MIF. The first indication of a potential MIF homologue in parasites was the detection of dopachrome tautomerase activity attributed to MIF in soluble extracts from several species of parasite worms (Pennock *et al.*, 1998). In 1998, a MIF homologue was identified in the filarial nematode *Brugia malayi* (Pastrana *et al.*, 1998). *B. malayi* MIF (BmMIF) was detected in all stages of the parasite lifecycle as well as in the excretory/secretory products of all stages. Recombinant BmMIF protein was found to inhibit human macrophage migration in a similar fashion to huMIF. It was subsequently shown that *B. malayi* expresses a second copy of BmMIF (Falcone *et al.*, 2001). Although the two homologues share only 27% identity, both MIF homologues are capable of inhibiting monocyte migration and demonstrate tautomerase activity. The extent of enzymatic activity of the different

homologues differs depending on the substrate tested. These two MIF homologues have also been shown to induce IL-8 and TNF-α expression in monocytes (Zang *et al.*, 2002). A single study investigating the role of MIF homologues during nematode infections has so far been described (Falcone *et al.*, 2001). Helminth infection is associated with eosinophilia, characterised experimentally by recruitment of eosinophils to the abdominal cavity during *B. malayi* infection. Injection of recombinant BmMIF into the peritoneal cavity of mice led to an increase of eosinophil recruitment, whereas tautomerase inactive mutants of BmMIF did not, suggesting this phenomenon is dependent on the enzymatic activity of BmMIF (Falcone *et al.*, 2001). The inhibition of BmMIF during infection, with a neutralising antibody for example, would be required to determine if BmMIF was exclusively responsible for eosinophil recruitment. MIF homologues have since been identified in several more species of parasitic nematodes (Tan *et al.*, 2001; Zang *et al.*, 2002), as well as two tick species *Amblyomma americanum* (Jaworski *et al.*, 2001) and *Haemaphysalis longicornis* (Umemiya *et al.*, 2006).

A putative protein identified during the sequencing of the *P. falciparum* genome showed sequence homology to the pro-inflammatory cytokine macrophage migration inhibitory factor (PlasmoDB entry for PfMIF: PFL1420w) (Gardner *et al.*, 2002). The *PfMIF* gene is located on chromosome 12 of the parasite genome. Microarray data indicate that it is primarily expressed in ring and trophozoite stages of the asexual cycle of the parasite (Bozdech *et al.*, 2003; Le Roch *et al.*, 2004). Clearly, the presence of a cytokine homologue in parasite species raises a question as to how these proteins may be influencing the immune system of the host during the course of infection.

Although several MIF homologues have been identified, the role of parasitic MIF

during infection is yet to be clearly defined. As previously mentioned, the two BmMIF proteins, the best characterised of the homologues, have very similar structures to mammalian MIF protein, exhibit tautomerase activity and inhibit monocyte migration. However, the role of MIF homologues during infection is poorly understood. Aside from one study examining eosinophil recruitment, there has been no clear determination of the possible role of MIF homologues during infection. One hypothesis put forward is that continuous secretion of MIF homologues may induce a counter-inflammatory response, either by desensitisation or by stimulating macrophages beyond the short-term acute phase usually examined (Maizels *et al.*, 2001). It is also possible that an as yet unidentified difference in parasite MIF homologues may confer a counter-inflammatory response, possibly by blocking the binding of MIF to receptors or substrates.

The discovery of a MIF homologue in *P. falciparum* raises interesting questions as to why a parasite would express an apparently pro-inflammatory cytokine homologue and whether this protein is capable of influencing the host immune system during the course of malarial disease. As mentioned above, these issues remain to be addressed in other parasites that express MIF homologues as well as during the course of *P. falciparum* infection.

1.10 THESIS AIMS

The potential role of PfMIF during the course of malaria is yet to be investigated. The mRNA expression profile of PfMIF during the blood stages of the parasite lifecycle has been described; however, further characterisation of PfMIF remains to be carried out. The initial aim of this thesis is to characterise the potential properties of PfMIF by comparison to the known sequence and structure of human MIF. Following from this, the expression of PfMIF protein during the course of parasite blood stages requires confirmation. Characterisation of PfMIF also requires the investigation of the localisation of PfMIF within the infected erythrocyte.

A wide range of activities have been attributed to mammalian MIF. So far however, there has been no characterisation of the activity of the *P. falciparum* homologue. The second aim of this thesis is to determine some of the *in vitro* activities of PfMIF. Elucidation of the activity of PfMIF could provide an insight into the actions of PfMIF during the course of malarial disease.

Finally, the potential role of PfMIF during the course of *Plasmodium* infection remains to be investigated. In order to do this, a third aim of this thesis is to determine PfMIF protein levels in the circulation of infected patients as well as anti-PfMIF antibody responses in those patients. These will then be compared to known demographic and immunological data also collected from the patients in order to determine potential influences that PfMIF may have on the course and severity of infection.

CHAPTER 2

Methods, Materials and Patients

2.1 SEQUENCE, PHYLOGENETIC AND STRUCTURE ANALYSIS

Sequence comparisons were carried out using the Bioedit program available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html. The phylogenetic tree was drawn by the neighbour-joining method using amino-acid sequence *p*-distances, utilising Geneious software available at http://www.geneious.com. MIF sequences from GenBank and PlasmoDB (http://www.plasmoDB.org) were used for phylogenetic analysis. The three dimensional structural prediction of PfMIF was generated using a program available at http://swissmodel.expasy.org. The instructions available on this website were followed using the known structure of huMIF as a template for the PfMIF prediction. The protein structure generated was viewed using Swisspdb viewer which is freely available on the website mentioned above.

2.2 RECOMBINANT PfMIF PROTEIN PRODUCTION

2.2.1 PfMIF cloning

PfMIF sequence was amplified by reverse transcriptase-polymerase chain reaction from *P. falciparum* total RNA (kindly provided by Dr Sue Kyes, Molecular Parasitology Group, Weatherall Institute of Molecular Medicine, Oxford) using oligo (dT) primers and SuperScript II reverse transcriptase (Invitrogen, USA). The two terminal primers used were: 5'-GAATTCCATATGCCTTGCTGTGAAGTAATAACAAACG-3' and 5'-CGCCCTAGGCTAGCCGAAAAGAGAACCAC-3'. The amplified DNA fragment was sub-cloned into T7/NT-TOPO expression vector that contained an in-frame N-terminal histidine-tag following the standard protocol (Invitrogen, USA). Briefly, 1µl of amplified DNA, 1µl of salt solution, 2µl of sterile water and 1µl of TOPO vector were mixed and incubated at room temperature for 5 minutes followed by chilling on ice and

transforming into TOP10F' competent *Escherichia coli* cells. Recombinant plasmids with the correct sized insert determined by restriction digest were sequenced to confirm the correct orientation and frame.

2.2.2 PfMIF protein expression

The construct containing the complete PfMIF sequence in correct orientation was transformed into *Escherichia coli* BL21(DE3) pLysS strain (Invitrogen, USA). The transformed cells were cultured for approximately 3 hours using a saturated overnight culture as an inoculum, and then induced at 0.6 OD_{600} with 0.5mM isopropyl β-D-thiogalactopyranoside (IPTG) for another 4 hours. Cells were then pelleted and either used for PfMIF protein purification or stored at -20°C for later use.

2.2.3 Protein purification

The pelleted cells were lysed using Bugbuster reagent (Novagen, USA) according to manufacturer's instructions and the crude bacterial extract was purified through Ni-NTA column (Invitrogen, USA). The crude bacterial lysate was diluted with binding buffer (50mM NaH_2PO_4, pH 8.0 and 0.5M NaCl) and run through the nickel column. The histidine-tagged protein bound to the column was eluted using 200mM imidazole in the binding buffer and 2ml fractions collected. Levels of PfMIF were assessed in each fraction by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; see section 2.5.2.2). The peak fractions were subjected to ion exchange chromatography using DEAE Sepharose (Amersham Biosciences, UK). The PfMIF protein eluted between 150mM and 250mM NaCl. Isolated PfMIF protein required refolding in order to be active. Purified protein was denatured in 6M urea containing 10mM β-mercaptoethanol for 20 minutes. The protein was then dialysed against a buffer consisting of 20mM Tris-HCl, 50mM NaCl, pH 7.5 with sequential 2 hour incubations

in decreasing concentrations of urea (4M, 2M and 1M) until final dialysis with no urea overnight. The activity of PfMIF on random migration of monocytes was found to be unstable and lost approximately 10 days after refolding. Therefore, after each batch of PfMIF protein purification and refolding the activity of the protein was assessed by inhibition of random migration of monocytes (see section 2.6.3). If the protein inhibited random monocyte migration by 60-70% compared to controls then it was used for subsequent experiments within 7 days of refolding.

2.2.4 Endotoxin removal

Lipopolysaccharide (LPS) was removed using EndoTrap (Profos, Germany) according to the manufacturer's instructions. The protein preparation was applied to an EndoTrap resin column, which acts to retain the endotoxin on the column, allowing decontaminated protein to run through. LPS removal was confirmed using QCL-1000 Limulus Ameobocyte Lysate (LAL) assay (Cambrex, USA). The assay was performed using the manufacturer's "microplate" method. A 50µl volume of standard or sample was added to glass tubes pre-warmed to 37°C along with 50µl of LAL solution. After 10min, 100µl of substrate solution was added followed by a further 6min incubation. The reaction was stopped by the addition of 50µl of 25% acetic acid. Samples were transferred to a microplate and absorbance read at 405nm. LPS concentration in recombinant protein solutions was routinely found to be <2pg per µg protein.

2.2.5 Determination of protein concentration

Protein concentrations were determined using a BCA (bicinchoninic acid) Protein Assay kit (Pierce Biotechnology, USA). The kit was used according to the manufacturer's protocol. Briefly, each assay was performed in a microplate with 25µl of sample or protein standard added to each well followed by 200µl of a 50:1 mix of

Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) and Reagent B (4% cupric sulphate). The plate was then incubated at 37°C for 30 minutes followed by the measure of absorbance at 570nm.

2.2.6 Cloning of Human MIF

Attempts were made to generate human MIF (huMIF) protein following the same methods that were used for PfMIF cloning (see section 2.2.1). The human RNA used was isolated from human peripheral blood mononuclear cells (PBMC) using TRIzol (Invitrogen, USA). The two terminal primers used to amplify huMIF DNA were: 5'-GGGTCTCCTGGTCCTTCTGCCATC-3' and 5'-TTAGGCGAAGGTGGAGTTGTTCCAGCC-3'. Several attempts were made to generate amplified huMIF DNA fragments and to ligate these into the T7/NT-TOPO expression vector. Plasmids with an insert of the correct size were sequenced to determine correct orientation and frame. Unfortunately, no plasmid capable of expressing huMIF protein was identified.

2.3 PRODUCTION OF ANTI-PfMIF ANTIBODIES

2.3.1 Antibody generation

Polyclonal anti-PfMIF serum was generated by immunization of New Zealand white rabbits with four subcutaneous injections of 50μg recombinant PfMIF over 35 days (Charles River, USA). The initial injection was emulsified with Freund's Complete Adjuvant with the following 3 injections emulsified with Freund's Incomplete Adjuvant. Serum was harvested at day 49 after the initial injection.

An anti-peptide antibody was also generated in rabbits against a PfMIF specific peptide sequence (NRSNNSALADQITKC) (Sigma-Genosys, USA). This sequence of PfMIF was chosen because there is relatively little homology with huMIF in this region. Additionally, structural modelling predicted this sequence to be on the outer surface of the protein and therefore easily accessible for antibody binding.

2.3.2 Determination of antibody specificity

Antibody specificity was tested by immunoblotting (following the procedure in section 2.5.2.3) membranes bound with 130ng each of huMIF (R&D Systems, USA) and recombinant PfMIF proteins with different dilutions of the two antisera (ranging from 1:20 to 1:2000).

2.3.3 Purification of IgG

Antisera and/or purified IgG were used in subsequent experiments. Rabbit IgG was purified from anti-PfMIF and anti-peptide sera using Protein G Sepharose (Amersham, UK). Rabbit sera were diluted with 20mM sodium phosphate binding buffer, pH 7.0 and run through a Protein G Sepharose column. The column was then washed with 5 column volumes of phosphate buffer. Bound IgG was eluted using 0.1M glycine, pH 3.0 and 200µl fractions were collected into 1M Tris-HCl, pH 9.0 to neutralise fractions. The presence of antibodies in each fraction was confirmed using SDS-PAGE.

2.4 *P. falciparum* PARASITE CULTURE

2.4.1 Culturing conditions

Intraerythrocytic stage *Plasmodium falciparum* parasites derived from the ITG/A4 clone were cultured *in vitro* following the protocol described previously (Roberts *et al.*,

1992). Parasites were cultured in RPMI 1640 medium (Sigma, USA) supplemented with 10% pooled human serum (National Blood Service, UK), 37.5mM HEPES buffer (Sigma, USA), 0.18% glucose (Sigma, USA), 6mM NaOH (Sigma, USA), 25μg/ml gentamicin sulphate (Sigma, USA), 2mM glutamine (Sigma, USA) and 0.1μM hypoxanthine. Cultures were maintained in human Type O RBC (National Blood Service, UK) at 1% haematocrit, gassed with a mixture containing 1% oxygen, 3% carbon dioxide and 96% nitrogen and incubated at 37°C.

2.4.2 Synchronisation of parasites

Parasite cultures were synchronised using a sorbitol lysis method (Lambros and Vanderberg, 1979). Briefly, parasite cultures were pelleted and resuspended with 10 times volume of pre-warmed 5% sorbitol (w/v in H_2O) and stood upright at 37°C for 20 minutes. Cells were then washed with serum free medium and returned to culture with fresh RBC. Tightly synchronised parasites were sampled throughout the asexual blood stages in order to perform immunoblotting and immunofluorescence microscopy (see sections 2.5.2.3 and 2.5.3). At each time point a blood film was made to monitor parasite development during the time course.

2.5 DETECTION OF PfMIF EXPRESSION

2.5.1 PfMIF mRNA expression

2.5.1.1 Sample preparation

Briefly, parasites were sorbitol-synchronized, cultures were at 3–10% parasitaemia and 200–500 μl of packed iRBCs were processed for each RNA sample. Cells were spun directly from culture and packed cells resuspended in the appropriate volume of TRIzol (Invitrogen, USA).

2.5.1.2 Northern blot

Northern blots were kindly carried out by Dr Sue Kyes. Prepared samples were processed as previously described (Kyes *et al.*, 2000).

2.5.2 Detection of PfMIF protein expression

2.5.2.1 Sample preparation

Tightly synchronised parasites were sampled during ring and trophozoite stages of the erythrocytic cycle. A small aliquot was removed for immunofluorescence microscopy and the remaining iRBC were lysed with 0.01% saponin in PBS (Sigma, USA) and the parasites thoroughly washed with fresh 0.01% saponin/PBS to remove haemoglobin and other RBC proteins (Hsiao *et al.*, 1991). For the detection of PfMIF in the culture supernatant, a 4% haematocrit culture of 20% trophozoites was cultured overnight to allow schizont development and rupture. Culture supernatant was then collected and spun at 10,000rpm to remove any remaining RBC and used in immunoblotting (see section 2.5.2.3).

2.5.2.2 SDS-PAGE

Purified parasite lysates were diluted in loading buffer and run on SDS-PAGE gels. All SDS-PAGE gels were 12% acrylamide (Protogel, USA) run at 30 amps per minigel in running buffer consisting of 25mM Tris-base, 192mM glycine and 0.1% (w/v) SDS. Proteins were visualised by staining with coomassie blue followed by destaining with 10% methanol and 20% acetic acid.

2.5.2.3 Immunoblotting

For immunoblotting, proteins were transferred from SDS/PAGE gels to nitrocellulose membranes (Schleicher and Schuell, Germany) at 100 volts for 1hr in a buffer

consisting of 25mM Tris-base, 192mM glycine and 20% methanol. Membranes were then blocked with 1-2% milk powder and immunoblotted with a 1:500 dilution anti-PfMIF rabbit sera for 1 hour at room temperature. This was followed by washing in TTBS (0.5M NaCl, 20mM Tris-HCL and 0.1% Tween-20). Membranes were then immunoblotted with alkaline phosphatase-conjugated swine anti-rabbit IgG secondary antibody (Dako, Denmark) for 1 hour. After washing, membranes were developed using BCIP/NBT substrate solution (Invitrogen, USA).

2.5.3 PfMIF immunofluorescence microscopy

2.5.3.1 Sample preparation

Parasites were cultured as described in section 2.4. Tightly synchronised parasites at 10% parasitaemia were sampled at appropriate intervals throughout the asexual blood stages in order to perform immunofluorescence microscopy. Microscope slides were prepared by washing with 70% ethanol followed by flooding with 0.1% poly-l-lysine solution for 5 minutes and allowing to air dry. Parasite cultures were pelleted and washed in warm PBS and resuspended in PBS to give a volume 10 times the original sample volume. To each section of the slide 100µl of washed RBC was allowed to attach for 10 minutes. Cultures were then removed and the adhered cells fixed by the addition of 4% paraformaldehyde in 0.25M HEPES buffer for 15 minutes in the dark. Fixed cells were washed twice with room temperature PBS. Slides were then stained immediately or stored at -20°C for later staining.

2.5.3.2 Staining procedure and visualisation

Cells were permeabilised with 0.2% Triton-X in PBS for 10 minutes followed by 3 washes with PBS. Slides were blocked with 10% fetal calf serum in PBS for 30 minutes. Primary and secondary antibody solutions were prepared in the blocking

solution. The primary antibodies used were purified rabbit anti-PfMIF peptide IgG and mouse anti-*P. falciparum* skeleton binding protein 1 (PfSBP1) sera (kindly provided by Prof. Catherine Braun-Breton, Dynamique Moléculaire des Interactions Membranaires, University Montpellier 2, France), which has been shown to localise to the Maurer's cleft (Blisnick *et al.*, 2000).

Fixed and permeabilised iRBCs were blocked with fetal bovine serum and stained with primary antibody (anti-PfMIF peptide: 1:100 dilution; anti-PfSBP1 1:400 dilution) for 30 minutes at room temperature. Slides were then washed 3 times with PBS. FITC conjugated anti-rabbit IgG (Dako, Denmark) and Alexa-Fluor® 546 conjugated anti-mouse IgG (Molecular Probes, USA) were used as secondary antibodies (1:40 and 1:400 dilutions respectively). Slides were incubated with secondary antibodies for 30 minutes at room temperature. Slides washed 3 times with PBS at room temperature. Antibodies were cross-linked by incubating in 4% paraformaldehyde solution for 10 minutes, followed by 3 washes in PBS. The final PBS wash contained 4'-6-diamidino-2-phenylindole (DAPI) in order to stain the parasite nuclei (Sigma, USA). Slides were visualised on a Nikon Eclipse 50i microscope and the images captured using Nikon ACT-2U.

2.6 PfMIF FUNCTIONAL ASSAYS

2.6.1 Monocyte isolation

Monocytes were isolated from buffy coats (National Blood Service, UK) using anti-CD14 magnetic beads (Miltenyi Biotec, Germany). Buffy coats were diluted with 50ml of room temperature PBS and the PBMC were separated by sedimentation through

Lymphoprep by spinning at 1800rpm for 30min. PBMC were washed 3-4 times with cold RPMI. After the final spin, PBMC were resuspended in 2.5ml of wash buffer consisting of PBS supplemented with 2mM EDTA and 2% human serum. Anti-CD14 microbeads (Miltenyi Biotec, Germany) were added at 300µl per buffy coat and incubated at 4°C for 30min. Cells were then washed with 20ml of wash buffer and run through a prewashed LS column attached to a Miltenyi magnet. The column was washed three times with wash buffer followed by the removal of the column from the magnet and the CD14 positive cells eluted with 4ml of RPMI medium. Monocytes were then washed to remove EDTA, counted and used as required. All culturing and subsequent assays of monocytes were carried out in RPMI 1640 medium (Sigma, USA) supplemented with 2mM glutamine, 50µM kanamycin (Sigma, USA) and 2% pooled human serum (NBS, UK).

2.6.2 Dendritic cell maturation

Dendritic cells (DC) were generated from monocytes isolated using the protocol outlined above (section 2.6.1). Monocytes were cultured at 5 x 10^5 cells/ml of RPMI 1640 medium (detailed in section 2.6.1) supplemented with 50ng/ml IL-4 (Peprotec) and 50ng/ml GM-CSF (Leucomax) to promote maturation into DC. Medium was changed every 2-3 days and after 4 days in culture onwards, cells were used as immature dendritic cells.

2.6.3 Migration assays

Migration assays were performed using 24 well, 6.5mm Transwell membranes with 5.0µm pore size (Corning, USA). Briefly, 5x10^4 purified monocytes or dendritic cells in 100µl medium were added to the upper chamber with or without 100ng/ml recombinant PfMIF or 100pg/ml lipopolysaccharide (LPS). Medium (600µl) with or without 100

ng/ml Monocyte Chemotactic Protein 1 (MCP-1) added to the lower chamber (R&D Systems, UK). Plates were incubated at 37°C (5% CO_2) for 2hr. After incubation, the base of the membrane was rinsed twice with 200µl medium and cells that had passed into the lower chamber were counted using a FACScalibur flow cytometer (BD, USA).

2.6.4 Surface molecule expression

Monocytes or dendritic cells were cultured in 24 well plates (Corning, USA) at $5x10^5$ cells per well in 500µl medium. Cells were incubated with PfMIF at different concentrations or LPS at 100pg/ml. After 24hr incubation at 37°C/5% CO_2, supernatant was collected for enzyme-linking immunosorbent assays (ELISA) and NO assays. The cells were harvested, washed and stained for surface molecules. All washes and staining steps were carried out with PBS supplemented with 2% human serum, 5mM EDTA and 0.02% sodium azide. The primary antibodies used were: anti-CD54 (ICAM-1; 1:100 dilution; Dako, Denmark), anti-CD40 (1:25 dilution; Serotec, UK), anti-CD86 (1:50 dilution; Serotec, UK), anti-TLR2 (1:50 dilution; eBioscience, USA), anti-TLR4 (1:100 dilution; eBioscience, USA) and anti-HLA DR (1:100 dilution; Dako, Denmark). Cells were stained with primary antibodies for 30 minutes. Cells were washed again and then incubated with secondary antibody for 30 minutes (FITC conjugated anti-mouse IgG; 1:25 dilution; Dako, Denmark). Cells were then washed again and fixed in wash buffer supplemented with 1% formaldehyde. Staining was assessed by flow cytometry (FACScalibur, USA) and the data analysed using FlowJo (Treestar Inc., USA).

2.6.5 Cytokine enzyme-linked immunosorbent assay

Culture supernatants from the surface expression experiments were stored at -20°C to assay for cytokine secretion. Enzyme-linked immunosorbent assays (ELISA) for IL-8, IL-10, IL-12 and TNF-α were performed according to manufacturer's instructions

(Pharmingen, USA). Briefly, plates were coated with capture antibodies in 0.1M carbonate buffer, pH 9.5, overnight at 4°C. Plates were washed 3 times with PBS containing 0.05% Tween 20, all subsequent incubations were followed with 6 washes with PBS/Tween. Plates were blocked with 1% bovine serum albumin (BSA) in PBS, pH 7.5 for 1hr at room temperature. Standards and samples were diluted appropriately and 100µl was added for 2hr at room temperature. This was followed by a one hour incubation with detection antibody and horseradish peroxidase together. Plates were developed using o-phenylenediamine dihydrochloride (OPD) substrate (Sigma, UK). Colour development was stopped after 20min by the addition of 50µl 2M H_2SO_4 and the optical density (OD) read at 490nm.

2.6.6 Nitric oxide assays

Nitrite levels (an indirect measure of nitric oxide) in the culture medium of cells treated with PfMIF were determined used the Griess method (Hevel and Marletta, 1994). Briefly, culture supernatants from the surface molecule expression experiments were diluted appropriately and 100µl added to a microplate in duplicate along with nitrite standards. To each well was added 50µl of 1% sulphanilamide (w/v) in 5% H_3PO_4, followed by 50µl 0.1% naphthylethylenediamine (w/v) in 5% H_3PO_4. Absorbance was measured at 530nm on an MRX Revelation-TC plate reader.

2.7 PATIENTS

Patient samples were made available to us through a collaboration with the Kenya Medical Research Institute/Wellcome Trust Centre based at the Kilifi District Hospital in coastal Kenya. A large cohort of children living in Kilifi District has been established

over several years to study malaria. Blood samples were collected from children living in the Ngerenya area of Kilifi District, who were under active surveillance for malaria as detailed previously (Nyakeriga *et al.*, 2004). This area has two rainy seasons: the "long" rains in May-July and the "short" rains in November creating distinct malaria transmission seasons. It has been estimated that residents of Ngerenya have, on average, 10 infective bites/person/year (Mbogo *et al.*, 1995). We analyzed plasma from 117 children that were collected during the cross-sectional survey conducted during low transmission season in October 2003. All children were examined clinically, and venous blood samples were collected for whole blood counts and to determine the presence of malaria parasites. Children who were negative for *P. falciparum* blood stage parasites by microscopy were included in the study. In August 2004 and January 2005, blood samples were collected from children attending the outpatient clinic at Kilifi District Hospital with mild, uncomplicated malaria (fever > 37.5°C, associated with a blood film positive for *P. falciparum* parasites and with no alternative explanation on careful clinical examination), and from children admitted to the wards with severe malaria. All 80 subjects included in this study were invited to donate a convalescence blood sample 14 days after discharge from hospital; 35 convalescent samples were collected. The study was approved by the Kenya Medical Research Institute / National Ethical Review Committee and the Oxford Tropical Research Ethical Committee. Written informed consent was obtained from the parents or guardians of the participating children.

2.7.1 PfMIF antibody ELISA

A general ELISA protocol was used to assess the levels of antibodies present in patient samples that recognise recombinant PfMIF. Briefly, plates were coated with 100µl of 0.1M carbonate buffer (pH 9.5; Sigma, USA) containing 15µg/ml recombinant PfMIF

and incubated at 4°C overnight. The following morning plates were blocked with 1% Bovine Serum Albumin (PAA Laboratories, Austria) in PBS for 1 hour. After blocking, patient sera, diluted 1:100 in the block solution, were added and incubated for one hour at room temperature. This was followed by the addition of a 1:4,000 dilution of horse radish peroxidase conjugated rabbit anti-human IgG detection antibody (Dako, Denmark) and incubated at room temperature for 30 minutes. Finally 200µl of o-phenylenediamine dihydrochloride (OPD) substrate was added to each well and allowed to develop for 20-30 minutes. Development was stopped with 50µl 2M H_2SO_4 and absorbance read at 495nm. Between each incubation step the plates were washed 3-6 times with PBS/0.05% Tween 20. Patients' sera were assayed in duplicate and 7 sera from nonimmune European adults were used to control for non-specific binding. Sera that showed binding two standard deviations above the average of the European controls were considered positive.

2.7.2 PfMIF sandwich ELISA

Development of a sandwich ELISA to measure the level of PfMIF in the circulation of malaria patients was attempted using the antibodies that have been generated against PfMIF. Purified IgG from both antisera (see section 2.3) were tested in combination as capture and detection antibodies for a PfMIF protein ELISA. Recombinant PfMIF protein was used to test the affinity of these ELISA assays. Neither combination could successfully detect recombinant PfMIF. When recombinant PfMIF protein was bound to the ELISA plate as a capture antigen it was still not recognised by either IgG.

2.8 STATISTICAL ANALYSIS

All data were analysed using SPSS (SPSS Inc., USA). Comparisons were done using Pearson's Chi-squared, Mann-Whitney U tests and paired t-tests.

CHAPTER 3

PfMIF structure and expression

3.1 INTRODUCTION

In recent years, much attention has been given to examining the immune responses to *Plasmodium* infection and to the interaction between parasite and host. It was therefore of particular interest when a potential cytokine homologue, *Plasmodium falciparum* macrophage migration inhibitory factor (PfMIF), was identified during the sequencing of the *P. falciparum* genome (Gardner *et al.*, 2002). The hypothesis that PfMIF represents a novel immune evasion mechanism that the parasite may utilise to influence the host immune system gave rise to this PhD project.

At the onset of this project, nothing was known regarding the function of PfMIF, and the only evidence of the gene's expression was at the mRNA level, with PfMIF shown to be transcribed during ring and trophozoite stages of the *P. falciparum* lifecycle (Bozdech *et al.*, 2003). In order to establish whether PfMIF could in fact represent an important link between the parasite and the host immune system, a number of basic characteristics of the protein, such as its structure and expression, needed to be investigated.

The first priority of this project was therefore to characterise PfMIF based on its predicted sequence and to assess whether the protein was expressed during the course of human infection. By comparing the sequence of PfMIF with the characterised sequences and tertiary structure of MIF from other species it was aimed to predict whether the *Plasmodium* protein shares structure and enzymatic activities that have been confirmed in other MIF proteins.

A second aim was to assess if PfMIF is expressed during the course of human infection. If expression of PfMIF cannot be demonstrated in parasite blood stages then the protein

could clearly not be involved in modulating the immune system during this stage of *P. falciparum* infection.

Finally, a third aim was to determine the localisation of PfMIF within the infected erythrocyte. This is an important consideration in relation to the influence of PfMIF on the host immune system. If PfMIF does not move from the parasite into the infected erythrocyte during blood stages it would bring into question whether PfMIF could have any meaningful interaction with the host immune system. If however, PfMIF were trafficked into the iRBC it would indicate that the protein could be released upon schizont rupture thereby interacting with host myeloid cells.

3.2 RESULTS

3.2.1 MIF sequence comparison

Comparison of PfMIF with other MIF amino acid sequences was carried out using Bioedit as described in section 2.1. The results from these comparisons showed that PfMIF shares a moderate amino acid sequence identity with huMIF of 28%. Similarity between these sequences was 46%. Importantly, although this similarity is reasonably low, PfMIF shares residues that are highly conserved in MIF proteins across a broad range of species (figure 3.1), indicating a likely functional similarity to these proteins.

The proline residue at position 1, which is essential for tautomerase activity (section 1.6.4.1), is conserved in PfMIF. The residues clustering around the tautomerase active site are also conserved, suggesting that this enzymatic activity would be maintained. In contrast, the second cysteine in the CXXC motif, which is essential for oxidoreductase activity in huMIF (section 1.6.4.1), is not present in PfMIF. This, however, is also the

case with MIF homologues in other parasites. Oxidoreductase activity of these proteins has not been assessed. Thus, whether this second enzymatic activity is extant cannot be definitively determined from sequence analysis alone.

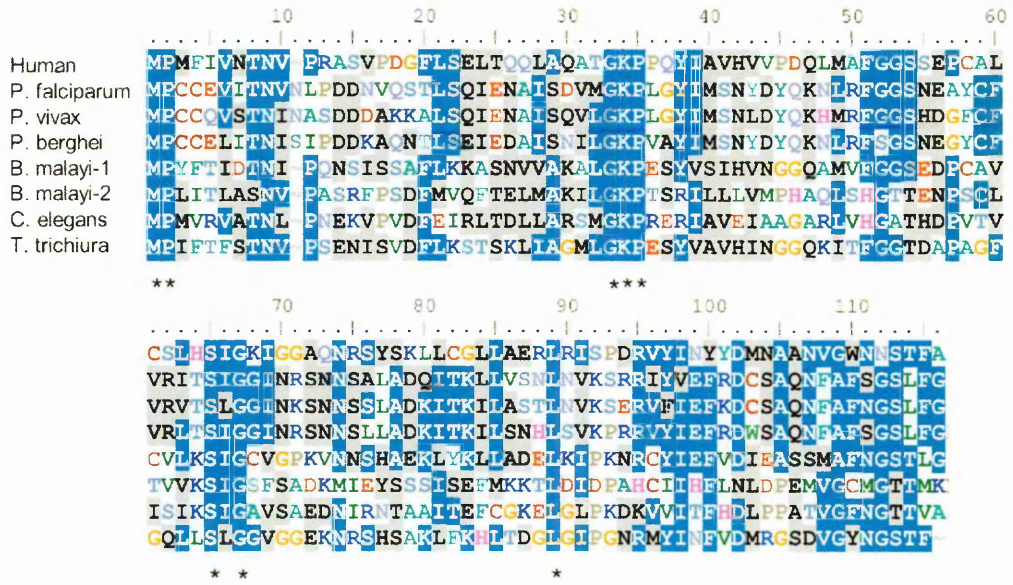


Figure 3.1: Alignment of MIF amino acid sequences from several species. Human, *Plasmodium* and both free living and parasitic nematode MIF proteins are aligned for comparison. The residues in blue are conserved sequences and grey residues share similarity. The marked residues (*) are shared across the species shown.

Like mammalian MIF, PfMIF lacks any known signal sequence (see section 1.6.2). Approximately 8% of all predicted genes in the *P. falciparum* genome carry a motif denoted *Plasmodium* export element or vacuolar transport signal (Pexel/VTS). Pexel/VTS is considered an export motif as it was identified in protein sequences that are linked to protein export from the parasite into the host erythrocyte (Hiller *et al.*, 2004; Marti *et al.*, 2004). Notably however, the Pexel/VTS motif is not essential for this function since it is not present in all proteins exported to the host cell. Sequence

analysis of PfMIF identified no Pexel/VTS motif and therefore no direct indication from the sequence that PfMIF is exported to the host erythrocyte. This however does not mean that PfMIF is not exported into the host cell as evidenced by mammalian MIF, which also lacks a signal sequence and is secreted by a non-classical pathway most likely involving the ABCA1 transporter (Flieger *et al.*, 2003) (section 1.6.2).

Overall, primary sequence comparison has shown that PfMIF shares some sequence similarity to MIF proteins from other species, especially in relation to conserved residues, thereby indicating its possible functional similarity to these proteins.

3.2.2 Phlyogenetic analysis of MIF proteins

To examine the phylogenetic relationship between the MIF proteins from different species the neighbour-joining distance method was used (see section 2.1). Included in this analysis were vertebrate MIF proteins, including those from humans, mice and Rhesus macaques, all hosts for *Plasmodium* species, along with sequences from parasitic and free-living nematodes, protozoan parasites, plants and cyanobacteria. The phylogenetic tree separates into three broad groups (see figure 3.2). The first group consists of cyanobacteria, plants and the protozoan *Leishmania* parasites. The second group includes other protozoan parasite MIF proteins including those from *Plasmodium* species. The remaining group includes the vertebrates as well as nematodes, ticks and jawless fish. The presence of MIF across a broad range of species would suggest that it arose in early eukaryotes as has been suggested previously {Sato, 2003 #78}. This analysis also reveals that *Plasmodium* MIFs are not as closely related to host MIF proteins as those from parasitic nematodes.

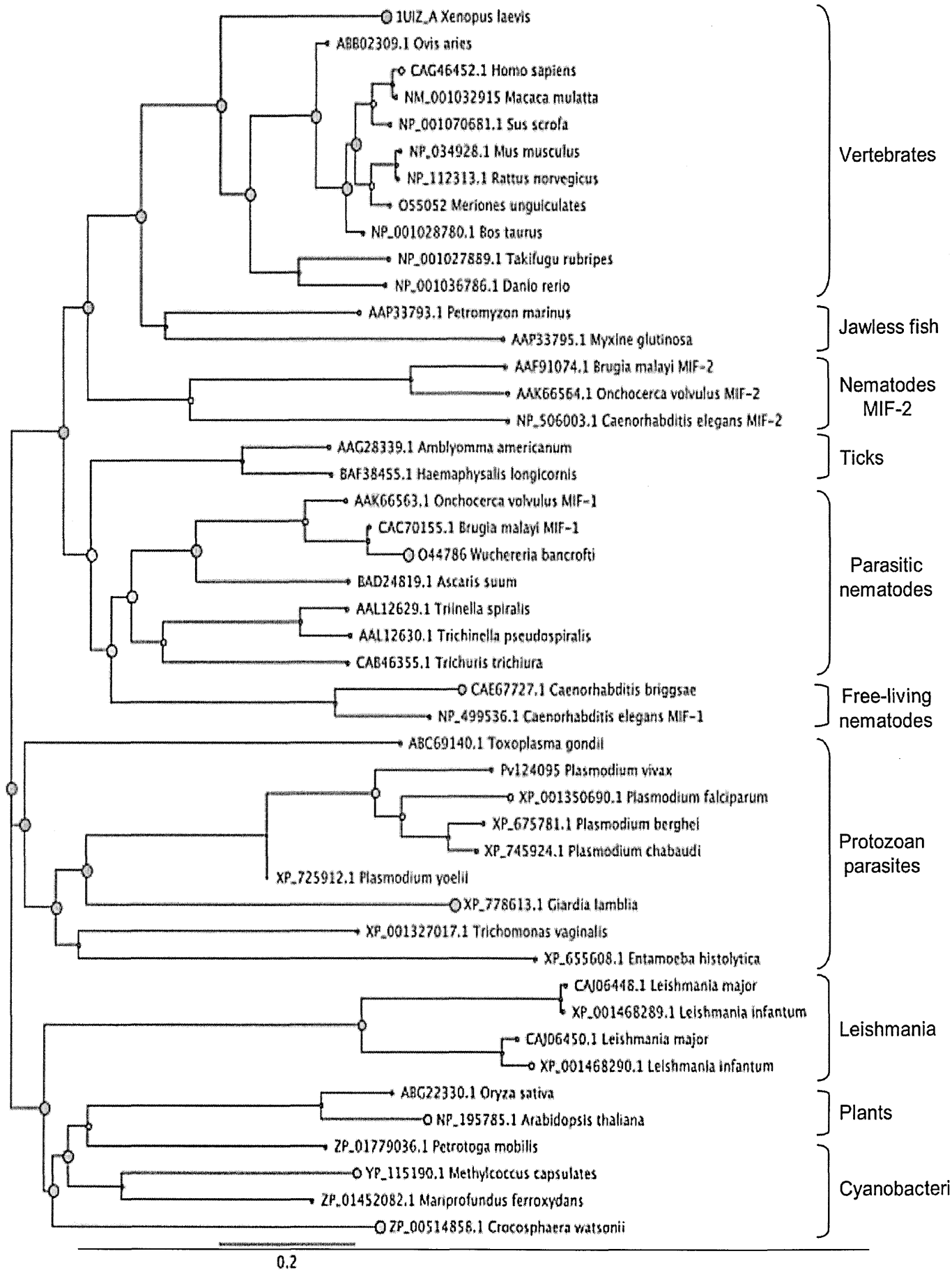

1UIZ_A Xenopus laevis
ABB02309.1 Ovis aries
CAG46452.1 Homo sapiens
NM_001032915 Macaca mulatta
NP_001070681.1 Sus scrofa
NP_034928.1 Mus musculus
NP_112313.1 Rattus norvegicus
O55052 Meriones unguiculates
NP_001028780.1 Bos taurus
NP_001027889.1 Takifugu rubripes
NP_001036786.1 Danio rerio
Vertebrates
AAP33793.1 Petromyzon marinus
AAP33795.1 Myxine glutinosa
Jawless fish
AAF91074.1 Brugia malayi MIF-2
AAK66564.1 Onchocerca volvulus MIF-2
NP_506003.1 Caenorhabditis elegans MIF-2
Nematodes MIF-2
AAG28339.1 Amblyomma americanum
BAF38455.1 Haemaphysalis longicornis
Ticks
AAK66563.1 Onchocerca volvulus MIF-1
CAC70155.1 Brugia malayi MIF-1
O44786 Wuchereria bancrofti
BAD24819.1 Ascaris suum
AAL12629.1 Trilnella spiralis
AAL12630.1 Trichinella pseudospiralis
CAB46355.1 Trichuris trichiura
Parasitic nematodes
CAE67727.1 Caenorhabditis briggsae
NP_499536.1 Caenorhabditis elegans MIF-1
Free-living nematodes
ABC69140.1 Toxoplasma gondii
Pv124095 Plasmodium vivax
XP_001350690.1 Plasmodium falciparum
XP_675781.1 Plasmodium berghei
XP_745924.1 Plasmodium chabaudi
XP_725912.1 Plasmodium yoelii
XP_778613.1 Giardia lamblia
XP_001327017.1 Trichomonas vaginalis
XP_655608.1 Entamoeba histolytica
Protozoan parasites
CAJ06448.1 Leishmania major
XP_001468289.1 Leishmania infantum
CAJ06450.1 Leishmania major
XP_001468290.1 Leishmania infantum
Leishmania
ABG22330.1 Oryza sativa
NP_195785.1 Arabidopsis thaliana
Plants
ZP_01779036.1 Petrotoga mobilis
YP_115190.1 Methylcoccus capsulates
ZP_01452082.1 Mariprofundus ferroxydans
ZP_00514858.1 Crocosphaera watsonii
Cyanobacteri
0.2

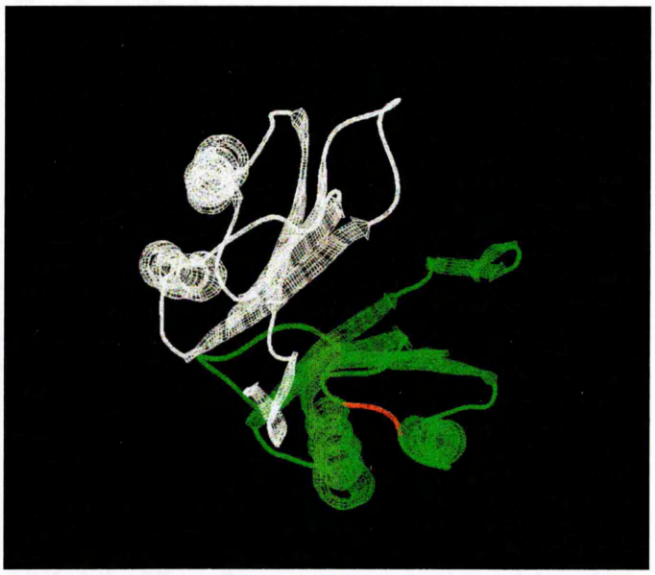
Figure 3.2 (previous page): Phylogenetic tree of MIF sequences from different species. The tree is drawn by the neighbour-joining method using amino acid sequence. Each species name is accompanied by a GenBank accession code with the exception of *P. vivax* which is accompanied by a PlasmoDB code.

3.2.3 Prediction of PfMIF protein structure

To extend the analysis of PfMIF structure beyond the primary sequence comparisons, the three-dimensional structure of PfMIF was then predicted by employing the Swiss-Model protein modelling server, using the known structure of huMIF as a template (section 2.1).

Although, as shown above, the sequence homology between PfMIF and huMIF was relatively modest, the structural predictions for PfMIF show that its amino acid sequence fits the known tertiary structure of huMIF, as illustrated in figure 3.3.

a)



b)

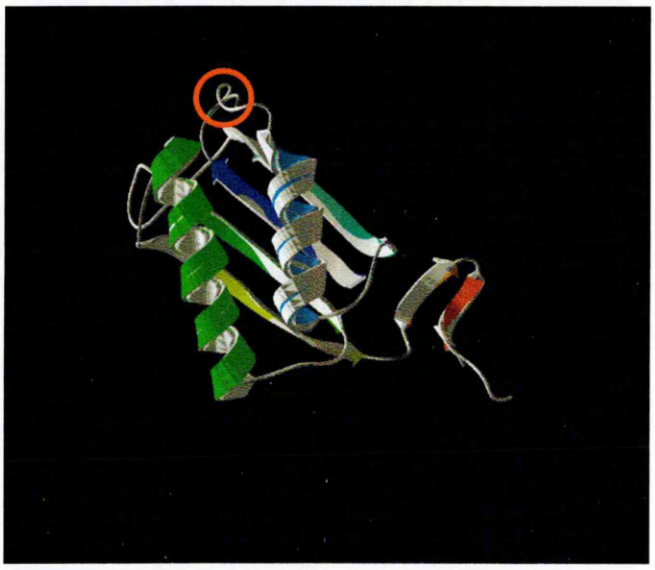


Figure 3.3: Predicted 3 dimensional structure of MIF proteins. a) Predicted dimer, the white subunit shows the known structure of huMIF while the predominantly green subunit is the predicted structure of PfMIF. The green portion of PfMIF represents the sequence that fits the structure of PfMIF, whereas the red portion is the one region that does not fit the template. b) Overlay of huMIF and predicted PfMIF 3D structures. The predicted 3D structure of PfMIF is overlaid with the known structure of huMIF that had been used as a template. The region of PfMIF that does not fit the structure of huMIF is outlined by the red circle.

Figure 3.3a shows a MIF dimer, with the mainly green monomer lying to the right hand side representing the predicted three-dimensional structure of PfMIF. The white subunit on the left side is the known structure of huMIF, provided for comparison. The portions of PfMIF that fit the structure of huMIF are represented in green and the area highlighted in red represents the single amino acid of PfMIF that does not fit the huMIF structure. Figure 3.3b shows an overlay of the known structure of huMIF and the predicted structure of PfMIF, with the single amino acid that does not fit the structure circled. This amino acid is the aspartate at position 15 and has been determined not to thread appropriately onto the known huMIF structure based on high mean force potential energy. This aspartate is in a region of the protein that is not near the active sites for either of the enzymatic activities attributed to MIF, and so is unlikely to affect either of these enzymatic functions. This amino acid is however an external residue and therefore could potentially influence other MIF activities such as receptor binding.

3.2.4 PfMIF expression in blood stage parasites

Having confirmed that the structure of PfMIF is consistent with the functional characteristics of MIF proteins in other species, it was then necessary to characterise PfMIF expression in *P. falciparum* during parasite blood stages.

3.2.4.1 PfMIF mRNA transcription

As an initial investigation into PfMIF expression, Northern blots were carried out using specific probes to detect PfMIF mRNA, as described in section 2.5.1. These experiments confirmed that PfMIF mRNA transcription begins during ring stages and peaks during late ring and trophozoite stages of the *P. falciparum* lifecycle (figure 3.4a). This confirmed the findings from two previous microarray studies using tightly synchronised parasites that demonstrate PfMIF mRNA transcription being initially

detected in ring stage parasites and peaking during trophozoites stages of the *P. falciparum* lifecycle (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003).

3.2.4.2 PfMIF protein expression

Despite the previous studies having reported PfMIF mRNA transcription during the blood stages of parasite development (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003), expression of PfMIF at the protein level has never been demonstrated.

Tightly synchronised parasites were therefore established and sampled at various stages of the blood stage cycle and immunoblotted for PfMIF protein. A representative Western blot is shown in Figure 3.4b. This figure demonstrates that in parallel with its mRNA expression, PfMIF protein is expressed during both ring and trophozoite stages of the parasite life cycle. Uninfected RBC were also included in these experiments and no protein was detected.

The presence of PfMIF protein in blood stage cultures raises the possibility that PfMIF may be released from the iRBC in order to have an affect on the host immune system. In order to investigate this, samples of the supernatant taken from *P. falciparum* cultures during ring or trophozoite stages of development were immunoblotted and no PfMIF was detected (data not shown). This could indicate that either PfMIF was not being trafficked from the iRBC or, alternatively, that this methodology was not sufficiently sensitive to detect the levels of PfMIF, if only small amounts were released. Efforts made to develop a sandwich ELISA for PfMIF, which would provide a more sensitive method to detect protein exported from the intact iRBC were unsuccessful, as discussed in section 5.2.2. Immunoblots of supernatant sampled after schizonts had ruptured the RBC to release merozoites did however detect PfMIF protein (figure 3.4b). Release of PfMIF after rupture indicates that PfMIF would be released into the

circulation of patients at this point and would thereby come into direct contact with the host immune system.

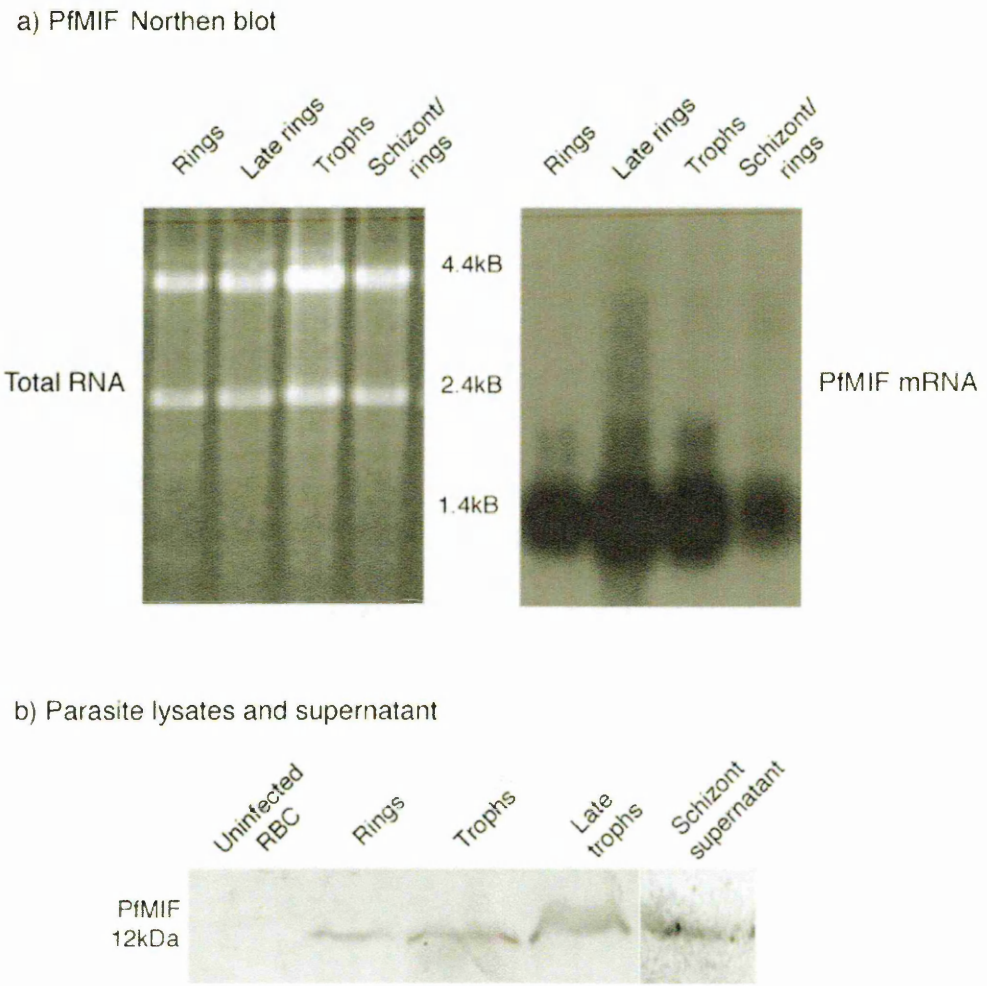


Figure 3.4: Expression profile of PfMIF in erythrocyte stage *P. falciparum* parasites. (A) Northern blot of synchronised blood stage parasite RNA blotted with PfMIF specific probes. (B) Western blots of synchronised blood stage parasite lysates and culture supernatant taken from parasites after schizont rupture respectively, blotted with anti-PfMIF peptide IgG. The parasite lysate blot is representative of several experiments. The schizont rupture supernatant blot represents a single experiment. No protein was detected in uninfected RBC.

3.2.5 PfMIF localisation

The next aim was to confirm that PfMIF is trafficked from the parasite into the cytosol of the iRBC during the course of intra-erythrocytic parasite development. In order to do this indirect immunofluorescence microscopy was used to determine the localisation of PfMIF in iRBC.

During the ring stage of development, PfMIF was exclusively located within the parasite, as indicated in the upper panel of figure 3.5. However, as the parasites developed into trophozoites (figure 3.5, lower panel), PfMIF was clearly detected in both the parasite and the iRBC cytosol.

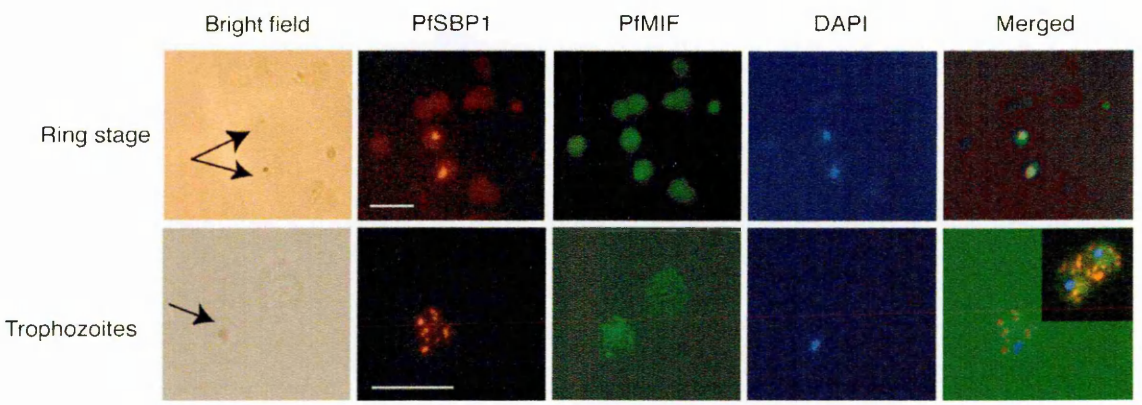


Figure 3.5: Localisation of PfMIF in blood stage parasites. Tightly synchronised parasites at different developmental stages were stained with DAPI to identify parasitised RBC, PfSBP1 to indicate the iRBC cytosol and anti-PfMIF peptide IgG to determine its localisation within the iRBC. The inset shows the co-localisation of PfSBP1 and PfMIF more clearly with the yellow areas representing co-localisation of the two proteins. This experiment was carried out three times, co-localisation was seen in many iRBC during each experiment. Arrows in bright-field micrographs indicate iRBC. The bar represents 10μm.

Anti-PfSBP1 antibody was initially used in these experiments as a control to highlight the infected erythrocyte surrounding the parasite itself. PfSBP1 is a protein that spans the Maurer's cleft membrane. It has been shown to be essential for the delivery of PfEMP-1 (section 1.5.3.1) to the erythrocyte membrane, possibly by mediating the anchoring of the Maurer's cleft to the RBC plasma membrane (Blisnick *et al.*, 2000; Cooke *et al.*, 2006). Although no SBP1 proteins have been identified in other Plasmodium species, PfSBP1-specific antibodies have been shown to react with *P. berghei* and *P. chabaudi* infected erythrocytes suggesting that SBP1 proteins are conserved among malaria species {Blisnick, 2000 #160}. If PfMIF export to the host erythrocyte occurs it would most likely be trafficked via the Maurer's cleft, therefore co-localisation of PfMIF and PfSBP1 would expected. PfMIF in iRBC did appear to be associated with distinct vesicles and co-localised with PfSBP1, suggesting an association with the Maurer's cleft (figure 3.5, lower panel, inset). This showed that PfMIF moved from the parasite into the iRBC during trophozoite stages and that the protein was associated with distinct vesicles in the cytosol.

3.3 DISCUSSION

This chapter demonstrates that PfMIF is similar to other known MIF proteins in both sequence and potentially in structure. It has also been confirmed that PfMIF is expressed during the blood stages of the *P. falciparum* lifecycle and is released upon schizont rupture. Immunofluorescence studies showed that, during the trophozoite stage of parasite development, PfMIF moves from the parasite into the host erythrocyte and is localised in specific vesicles. These vesicles are also positive for PfSBP1, indicating that PfMIF is also located in the Maurer's cleft.

PfMIF was identified as a hypothetical *P. falciparum* protein that shared sequence homology with mammalian MIF. Although overall sequence homology is only moderate, analyses of the amino acid sequence shown in this chapter reveal important similarities between PfMIF and MIF proteins from other species. Comparisons of the amino acid sequence of PfMIF with MIF sequences from diverse species demonstrate that this homologue shares all residues that are conserved across the range of MIF proteins examined. The amino acid sequence of PfMIF shows no obvious differences that may suggest a variation in function. Furthermore, the predicted three-dimensional structure of PfMIF, based on threading the PfMIF sequence into the known structure of huMIF, demonstrated that PfMIF could form an almost identical structure to other, better characterised mammalian MIF proteins.

The results described above clearly show that PfMIF is expressed during the erythrocytic stages of the *P. falciparum* life cycle. Additionally, they demonstrate that PfMIF is transported from the parasite into the erythrocyte, and from there it is released into circulation upon schizont rupture. These results imply that PfMIF enters the circulation of the patient during the course of infection and thereby has the ability to come into direct contact with the host immune system. This means that PfMIF has the potential to modulate host immune responses to *P. falciparum*, particularly when it is considered that local concentrations of PfMIF may actually be quite high in areas of parasite accumulation, such as the spleen or other sites of parasite sequestration.

The localisation of PfMIF in the cytosol of iRBC is interesting in view of recent findings that the Maurer's cleft has an important role in trafficking of parasite proteins to the surface of the erythrocytes, and that mammalian MIF has chaperone-like properties *in vitro* (Cherepkova *et al.*, 2006; Cooke *et al.*, 2006; Potolicchio *et al.*,

2003). Maurer's clefts are parasite-derived vesicular structures that appear in the RBC cytosol during the early trophozoite stage. Cooke and colleagues recently demonstrated that PfSBP1, which is associated with the Maurer's cleft, is responsible for the final translocation step of PfEMP1 to the iRBC plasma membrane. As previously described, PfMIF does not have an identified motif that targets it for export from the parasite (see section 3.2.1). The targeting of PfMIF to the Maurer's cleft may indicate a mechanism of parasite protein release independent of the Pexel/VTS sequence. Alternatively, recent studies examining the peptide binding properties of mammalian MIF *in vitro* as well as its role in heat-induced protein aggregation have highlighted its potential role as a chaperone-like protein (Cherepkova *et al.*, 2006; Potolicchio *et al.*, 2003). Considering this, it may be possible that the apparent localisation of PfMIF to the Maurer's cleft is associated with a role for PfMIF in protein trafficking within the iRBC.

Many of the findings in this chapter have been recently confirmed by another study characterising *Plasmodium* MIF homologues (Augustijn *et al.*, 2006), as discussed in Chapter 6.

In summary, results from this chapter show that PfMIF exhibits similar sequence characteristics and predicted structure to the more extensively studied mammalian and parasite MIF proteins. It has been confirmed that PfMIF protein is expressed in the blood stage replicative cycle of the *P. falciparum* life cycle. Finally, PfMIF is trafficked from the parasite into the iRBC cytosol. PfMIF is associated with the Maurer's cleft, and released upon schizont rupture, and therefore able to interact with the host's immune system.

CHAPTER 4

In vitro activity of PfMIF

4.1 INTRODUCTION

In Chapter 3 it was shown that PfMIF shares sequence and structural similarity with MIF proteins from other species. Whether this structural consistency with other MIF proteins also translates into similar immunological activity remains to be determined. The primary step in the assessment of any potential MIF protein has been to examine *in vitro* activity, and accordingly, this became the next objective of this project. The assessment of whether PfMIF behaves in a similar or different manner to other previously described MIF proteins *in vitro* should give some insight into the possible role of PfMIF during malarial disease.

Inhibition of random cellular migration *in vitro* was the first function attributed to MIF and is generally the first activity to be tested when new MIF homologues are identified. Although the inhibition of migration by MIF proteins has only been demonstrated *in vitro*, it does suggest that MIF proteins can influence the activity of myeloid cells. Hence, an initial aim of this chapter was to determine whether PfMIF affects the migration characteristics of myeloid cells in a similar fashion to other MIF proteins previously studied. This would be achieved by measuring the effect of recombinant PfMIF on the random migration and chemotaxis of myeloid cells in culture.

Other important markers of both malaria and MIF activity include changes in cytokine production, surface molecule expression and NO generation by myeloid cells (see section 1.6.4). For example, increased expression of cytokines IL-10, IL-12, TNF-α and IFN-γ has been associated with the pathogenesis of malaria (Beutler and Grau, 1993; Trinchieri, 1995; Tripp *et al.*, 1993; Troye-Blomberg *et al.*, 1990). IL-8 has been shown to be induced by MIF treatment in monocytes and increased early during *P. falciparum* blood stage infection (Hermsen *et al.*, 2003; Lyke *et al.*, 2004; Murakami *et al.*, 2002).

TLR4 surface expression has been shown to be dependent on endogenous MIF in macrophages (Roger *et al.*, 2001). Furthermore, TLR4 and TLR2 have also been implicated in innate immune responses to *Plasmodium* infection (see section 1.5.2.1). Each of these parameters represents a possible way in which the parasite might influence host immune responses. Thus it was also aimed to measure the expression of a range of cytokines and surface molecules as well as NO generation in myeloid cells exposed to PfMIF.

The investigation of these potential *in vitro* activities requires high concentrations of purified PfMIF protein. Thus, the first objective for these studies involved the development of a system to generate and purify recombinant bacterial PfMIF, which could subsequently be used to treat myeloid cells *in vitro*.

4.2 RESULTS

4.2.1 Expression of recombinant PfMIF

In order to begin to dissect the activities of PfMIF protein itself, high concentrations of purified protein were required. Recombinant PfMIF was therefore expressed using a bacterial expression system and purified using several different methods to remove contaminating proteins and endotoxin, as described in section 2.2.

Figure 4.1a shows the overexpression of PfMIF induced by IPTG in *E. coli trxB* strain. PfMIF was subsequently concentrated and purified using a nickel column (Figure 4.1b). The protein was further purified by running through an ion exchange column to remove any contaminating proteins that remained. Purified recombinant PfMIF was refolded and then contaminating endotoxin removed using an Endotrap column. The final protein

preparation was consistently greater than 500µg per ml PfMIF, with LPS contamination less than 2pg per µg protein.

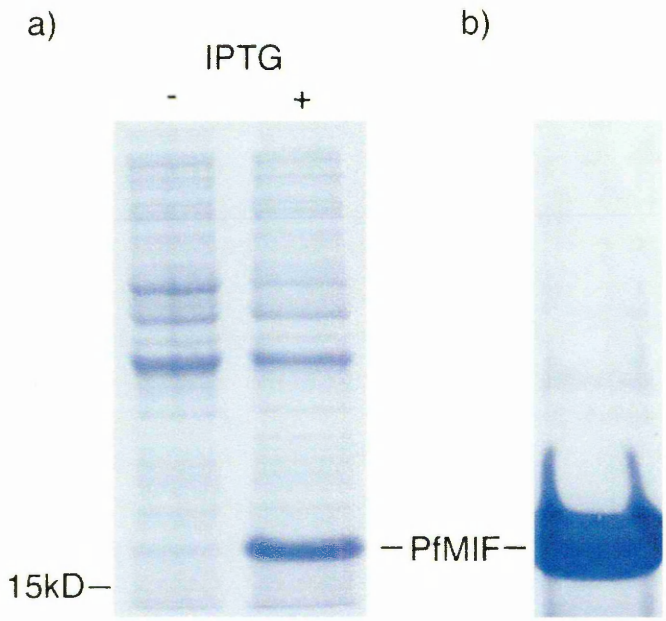


Figure 4.1: Recombinant PfMIF produced in *E.coli*. Recombinant PfMIF expression in *E. coli* was induced by IPTG and visualised on SDS-PAGE by coomassie a) before and b) after purification and concentration.

Non-reducing acrylamide gel electrophoresis of recombinant PfMIF indicated that the protein may form dimers and trimers (figure 4.2). This is consistent with cross-linking experiments of huMIF, and occurred despite the possible influence on subunit association of the presence of a 5kD N-terminal tag (Mischke *et al.*, 1998). Evidence that PfMIF may form trimers, combined with the prediction that PfMIF shares a similar structure to huMIF (see section 3.2.3), leads to suggestions that PfMIF could form hetero-trimers with huMIF resulting in the potential modulation of huMIF activity.

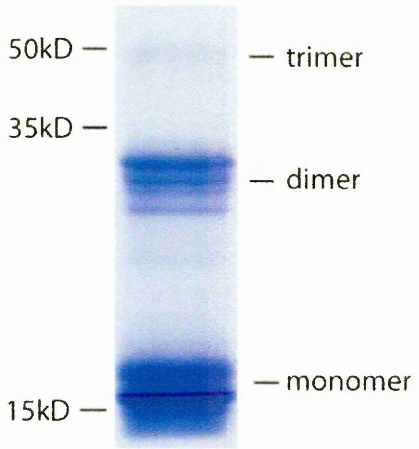


Figure 4.2: Recombinant PfMIF forms dimers and trimers. PfMIF run on a non-reducing SDS-PAGE gel appears to form both dimers and trimers on non-reducing gels, although monomers and dimers predominate. This is consistent with cross-linking experiments with huMIF but will be complicated in the present case by the presence of a 5kD N-terminal tag.

4.2.2 Generation of anti-PfMIF antibodies

In order to provide further tools to examine the role of PfMIF, two different anti-PfMIF antisera were generated in rabbits during the course of this project (see section 2.3). The first anti-serum was generated using purified recombinant PfMIF protein as the antigen. The second was generated against a peptide sequence specific to PfMIF (NRSNNSALADQITKC). This sequence was chosen due to low sequence homology with huMIF in this region and because the 3-dimensional structure prediction indicated that it would be exposed on the outer surface of the protein, thereby providing good antibody access. Western blotting membranes that only carried recombinant PfMIF and huMIF were used to determine the specificity of these two anti-sera (figure 4.3). The anti-peptide anti-serum specifically recognised PfMIF at all dilutions. Anti-PfMIF anti-serum recognised both PfMIF and huMIF but with a higher affinity for PfMIF, with

huMIF not detected at 1:500 dilutions or less.

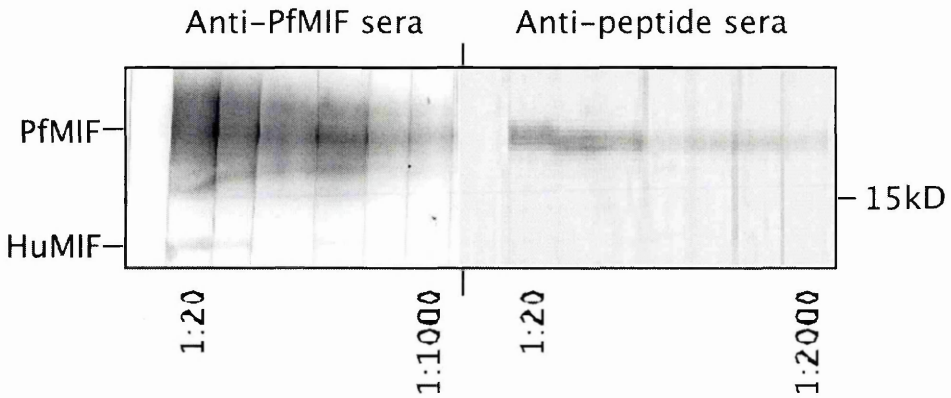


Figure 4.3: Specificity of anti-PfMIF and anti-peptide antisera. Equal quantities (130 ng) of PfMIF and huMIF were separated by electrophoresis on a 12% gel and then transferred to nitrocellulose membranes. After blocking, membranes were incubated with different dilutions of the two antisera raised against PfMIF. Anti-PfMIF antiserum shows some crossreactivity with huMIF at the higher concentrations, whereas the anti-peptide antiserum recognises PfMIF exclusively.

4.2.3 Modulation of monocyte activation by PfMIF

4.2.3.1 PfMIF affects migration of monocytes in vitro

The initial step in the assessment of the activity of recombinant PfMIF was to determine its influence on the random migration and chemotaxis of monocytes *in vitro*. Random migration is the cellular movement occurring in the absence of a known stimulus. Alternatively, chemotaxis is directed cellular movement under the influence of a chemoattractant.

Unsuccessful attempts were made to generate recombinant huMIF protein as a control for PfMIF activity, especially in migration assays (see section 2.2.6). Therefore, LPS was used as a control in migration assays due to the ability of LPS to consistently inhibit monocyte migration.

The effect of PfMIF on cellular movement across a membrane was measured by flow cytometry (see section 2.6.3). It was observed that random monocyte migration was significantly inhibited by treatment with 100ng/ml PfMIF (figure 4.4). This was consistent with previous data showing that mammalian MIF and parasite MIF proteins inhibit random migration (David, 1966; Pastrana *et al.*, 1998). In this instance, LPS was included as a positive control for each assay due to its known ability to inhibit random monocyte migration.

To assess whether chemotaxis was also inhibited by PfMIF, the chemotactic agent MCP-1 was used. MCP-1 is a chemoattractant that, in this experimental system, promotes the movement of cells across a membrane. MCP-1 induced around a four-fold increase of migration by monocytes compared to random migration. The addition of PfMIF inhibited MCP-1 chemotaxis in a dose-dependent manner. However, MCP-1 induced migration was still above baseline level even in the presence of 500 ng/ml PfMIF (figure 4.4).

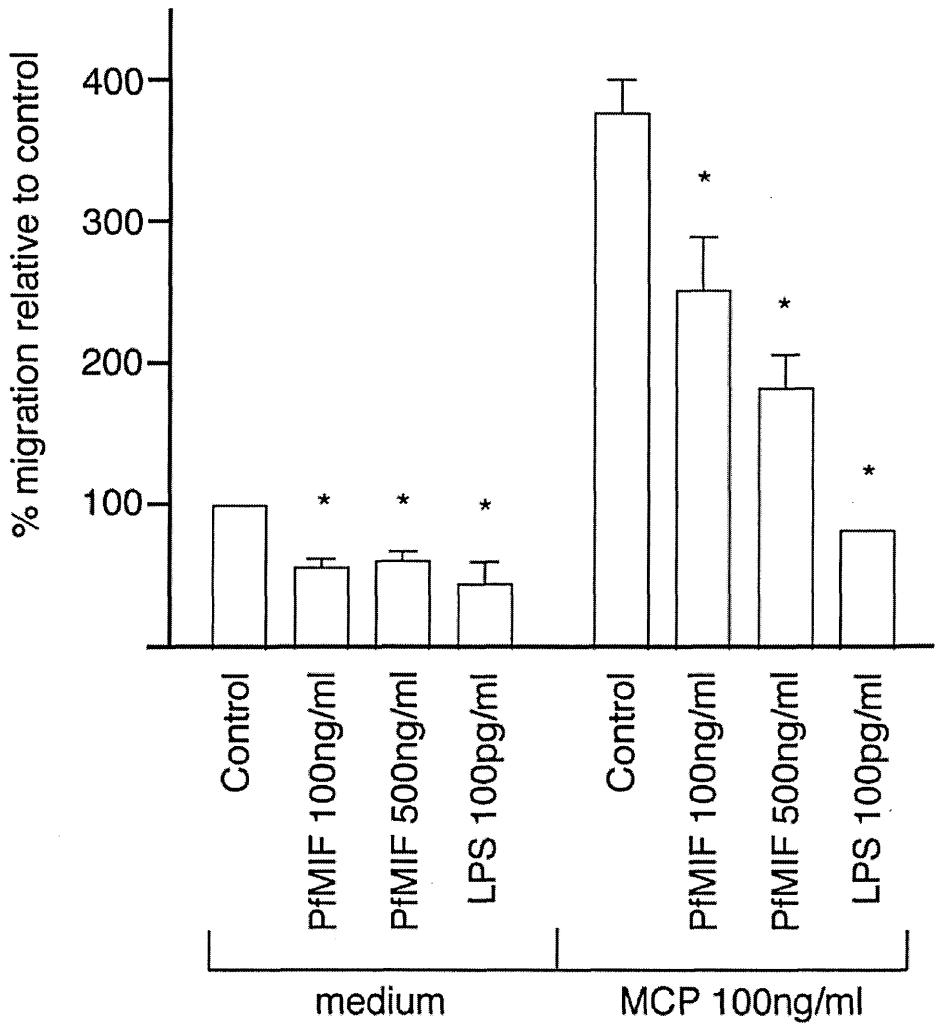


Figure 4.4: *In vitro* migration of monocytes in the presence of PfMIF. The random migration of monocytes across a membrane was assessed in the presence of recombinant PfMIF or LPS and compared to untreated monocytes (medium). Monocyte chemotaxis in response to MCP-1 was also assessed in the presence or absence of PfMIF or LPS ($N \geq 3$ for each condition, performed in duplicate for each experiment; *$p<0.01$, paired t-test).

These results confirm that PfMIF does indeed act in a similar fashion to other previously described MIF proteins, inhibiting both random migration and chemotaxis.

This is despite the presence of an N-terminal tag on recombinant PfMIF that is likely to inactivate tautomerase activity (see section 1.6.4.1). Therefore the inhibition of monocyte migration by PfMIF is unlikely to be mediated by tautomerase activity and must be mediated by another mode of action of the protein.

The effect of PfMIF on random migration of monocytes was found to be unstable and lost approximately 10 days after purification. It was therefore confirmed that PfMIF maintained its effect on random monocyte migration before being used in each subsequent experiment.

4.2.3.2 Cytokine secretion in monocytes exposed to PfMIF

In order to assess the effect of PfMIF on cytokine secretion, monocytes were cultured in the presence or absence of 100ng/ml or 500ng/ml PfMIF (section 2.6.5). After 24 hours exposure to PfMIF, culture supernatants were harvested and the levels of IL-8, IL-10, IL-12 and TNF-α were measured by ELISA.

Figure 4.5 shows that exposure to PfMIF had no effect on the release of IL-8, TNF-α or IL-12 from monocytes within 24 hours. There was a tendency for PfMIF treatment to increase IL-8 secretion but this did not reach significance and was low in comparison to IL-8 secretion induced by 100pg/ml LPS (figure 4.5). Preincubation of monocytes with PfMIF had no significant influence on subsequent cytokine release in response to LPS (data not shown). No changes in IL-10 release were detected under any condition and therefore the IL-10 data is not shown.

4.2.3.3 Surface molecule expression in monocytes treated with PfMIF

As a further indicator of monocyte modulation, I went on to determine whether the expression of the surface molecules HLA-DR, ICAM-1, CD40, CD86, TLR2 and TLR4

was altered upon incubation with PfMIF. Expression of these surface molecules on monocytes cultured in the presence or absence of PfMIF (100ng/ml or 500ng/ml) for 24 hours was assessed by flow cytometry (see section 2.6.4).

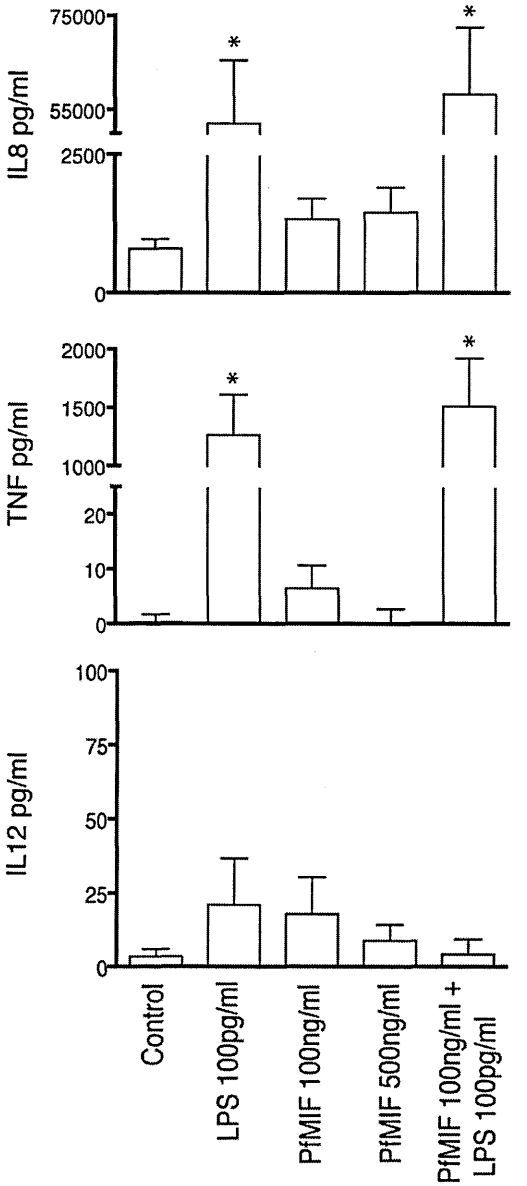


Figure 4.5: Effects of *in vitro* PfMIF treatment on cytokine release from monocytes. Monocytes were cultured in the presence or absence of recombinant PfMIF (concentrations as indicated) and/or LPS (100 pg/ml). After 24 hours of treatment, culture supernatant was collected and assessed for IL-8, TNF-α and IL-12 release by

ELISA (N ≥ 5 for each condition, performed in duplicate for each experiment; *p<0.05, paired t-test).

TLR2, TLR4 and CD86 surface expression was significantly reduced in response to PfMIF, whereas all other surface markers remained unchanged (figure 4.6a). By contrast, LPS treatment of monocytes resulted in upregulation of CD40, CD86 and ICAM-1 and a significant decrease in expression of TLR4 ($p<0.05$ paired t-test; figure 4.6b). Together these data would indicate that PfMIF alters monocyte function but has no effect on LPS-mediated activation of monocytes.

Due to the reduction of TLR expression induced by PfMIF, subsequent responses to TLR ligands were examined. Monocytes were preincubated with PfMIF for 12 hours followed by stimulation with either the TLR4-ligand LPS or the TLR2-ligand peptidoglycan (PGN). As mentioned above, LPS alone upregulated CD40, CD86 and ICAM-1, whilst significantly downregulating TLR4. Pre-incubation of monocytes with PfMIF before a 24 h treatment with LPS did not change expression levels for any of the surface markers compared with LPS alone, although TLR2 showed a trend towards downregulation ($p=0.09$ paired t-test for PfMIF 500 ng/ml; figure 4.6b). Treatment of monocytes with the TLR2-ligand PGN alone increased expression of CD86 only ($p<0.05$ paired t-test; Figure 4.6c). Pre-incubation with PfMIF before a 24h incubation with PGN had no effect on the surface expression of any of the markers analysed compared with PGN alone. Together these data would indicate that PfMIF alters monocyte function but has no significant effect on LPS- or PGN-mediated activation of monocytes.

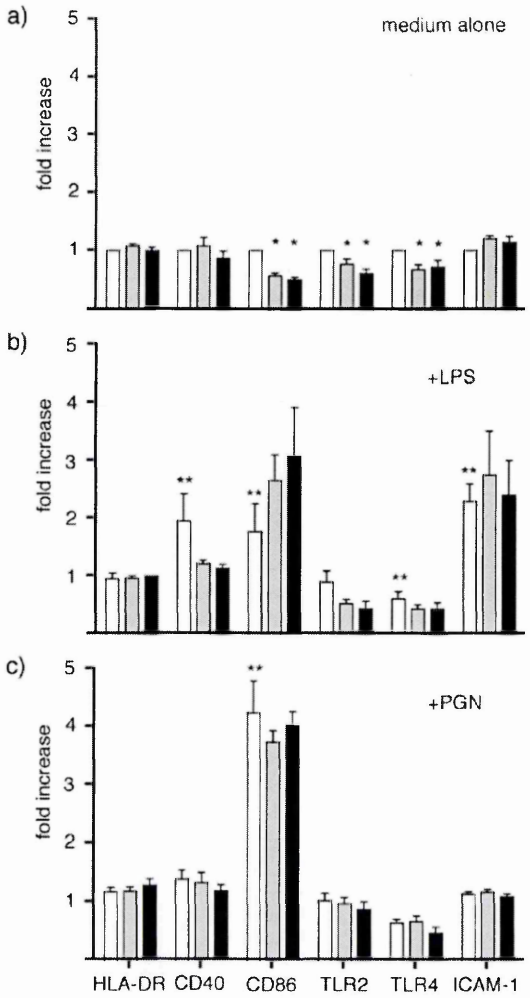


Figure 4.6: Monocyte surface marker expression after PfMIF treatment. Monocytes were untreated (white bars), or exposed to 100 ng/ml PfMIF (grey bars) or 500 ng/ml PfMIF (black bars) for 12 hours before incubation in medium alone (a), LPS (b), or PGN (c) for another 24 hours, followed by staining for the expression of surface molecules. The mean fluorescence intensity (MFI) for a given marker was divided by the MFI of the control (monocytes alone panel a, white bars) to normalise for variations in expression levels of a given marker between individuals (N≥3 for each condition, performed in duplicate for each experiment; *$p<0.05$ paired t-test, PfMIF treated monocytes versus control; **$p<0.05$ paired t-test, LPS or PGN treated monocytes versus control).

4.2.3.4 NO production in monocytes induced by PfMIF

Nitric oxide has been suggested to be important for the pathogenesis of malaria. I tested whether PfMIF was capable of inducing NO production *in vitro*, by the measurement of nitrite levels in monocyte culture supernatant (see section 2.6.6). In monocytes there was no concentration of PfMIF that induced a significant change in NO production (figure 4.7). If anything, PfMIF appeared to show a tendency to inhibit basal and LPS stimulated NO release. The levels of nitrite detected in monocyte culture supernatant were however not high enough to suggest a physiologically significant change in NO production under any condition.

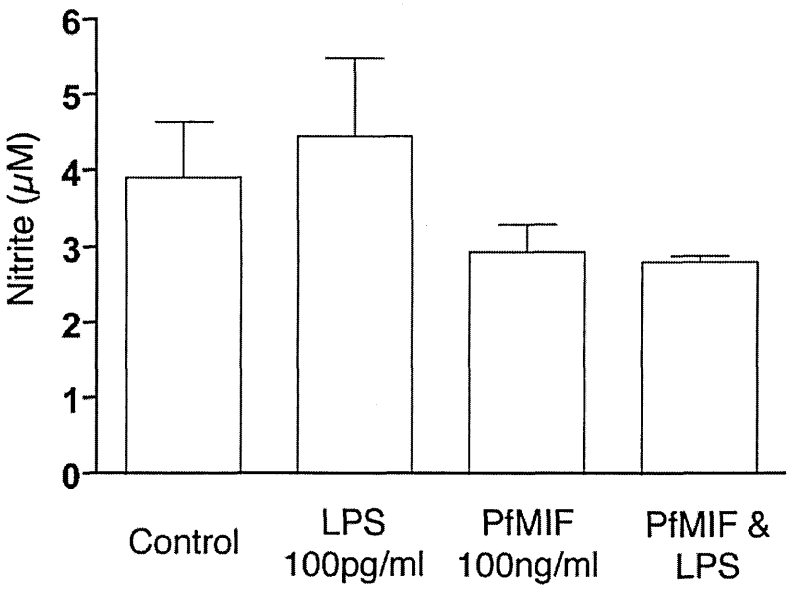


Figure 4.7: NO production by monocytes in response to PfMIF and LPS treatment (n=3 for each condition, performed in duplicate for each experiment).

4.2.4 Dendritic cell activity and PfMIF

Following the assessment of the effect of PfMIF on monocytes, similar experiments were then carried out on monocyte-derived dendritic cells (DC) to see if PfMIF might have equivalent effects in these cells. The potential modulation of DC activity by PfMIF would be of interest in relation to the finding that DC migration is disrupted in the spleen of patients that have died during the course of *P. falciparum* infection (see section 1.5.7).

4.2.4.1 Dendritic cell migration in response to PfMIF

The random migration of dendritic cells in response to PfMIF was tested using the same protocol as used for monocytes (see section 2.6.3). Exposure to PfMIF resulted in no significant difference on random DC migration compared to controls (data not shown).

4.2.4.2 Cytokine secretion in dendritic cells exposed to PfMIF

The release of the cytokines IL-8, IL-12 and TNF-α from dendritic cells after treatment with PfMIF was tested as described in section 2.6.5. Figure 4.8 shows that there was no significant difference in cytokine release from DC induced by incubation with PfMIF, at any concentration.

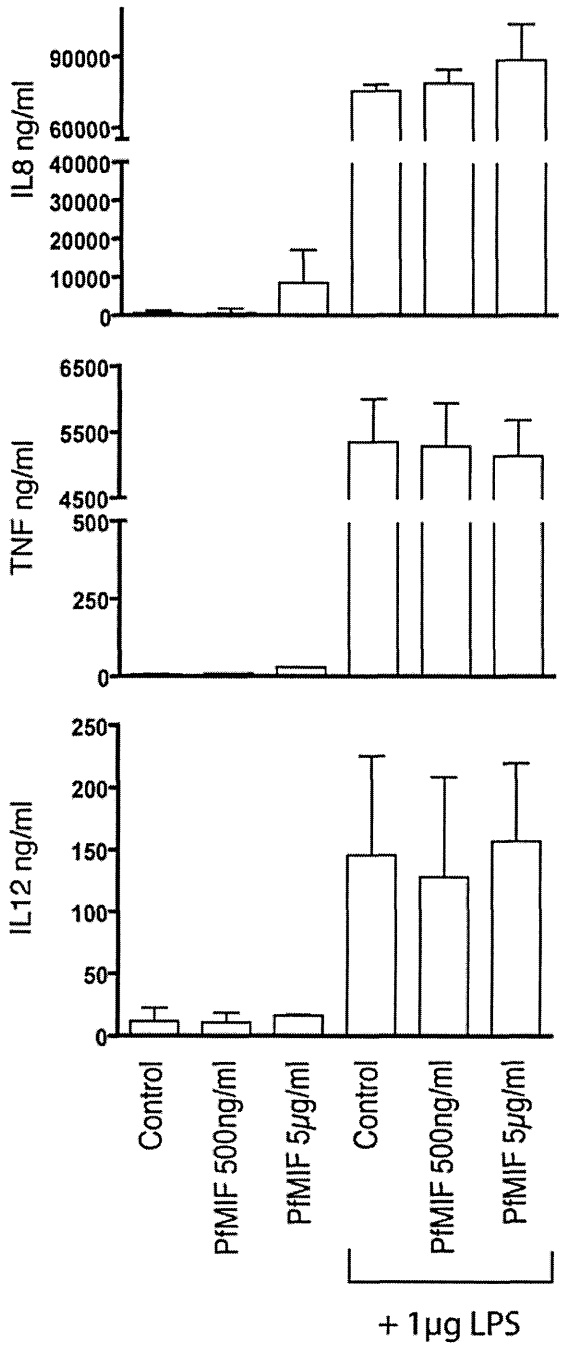


Figure 4.8: Effects of *in vitro* PfMIF treatment on cytokine release from DC. DC were cultured in the presence or absence of recombinant PfMIF (concentrations as indicated) and/or LPS (1 μg). After 24 hours of treatment, culture supernatant was collected and assessed for IL-8, TNF-α and IL-12 release by ELISA (N = 3 for each condition, performed in duplicate for each experiment).

4.2.4.3 Surface molecule expression in dendritic cells treated with PfMIF

The effect of PfMIF on the expression of surface molecules that was examined in monocytes was also measured in DC. There was no significant difference in the expression of CD40, CD86 or ICAM-1 upon exposure to PfMIF. As was seen in monocytes, PfMIF exposure did cause a reduction in TLR2 expression (figure 4.9). However, this effect was only evident at the highest concentration of PfMIF tested (5μg/ml), which is ten-fold higher than any concentration tested on monocytes. There was no indication of a reduction in TLR4 expression as was seen in monocytes; however, only two assays gave reliable results for TLR4 expression (figure 4.10), the third assay gave results that were below baseline and therefore were not used. The two available assays were quite variable and did not suggest a consistent pattern to TLR4 expression in response to PfMIF on DC.

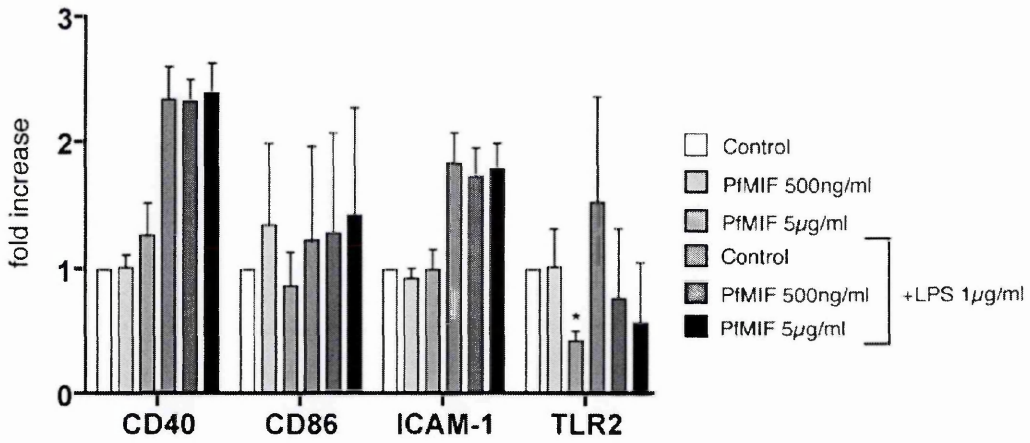


Figure 4.9: Dendritic cell surface marker expression after *in vitro* treatment with PfMIF. Of the surface markers tested only TLR2 was significantly reducing in response to PfMIF exposure (* $p<0.05$, paired t-test, PfMIF treated DC versus control; n=3 for each condition, performed in duplicate for each experiment).

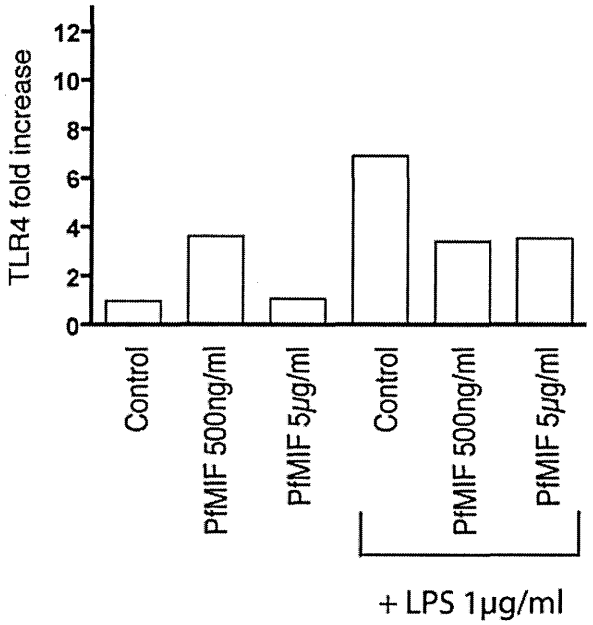


Figure 4.10: TLR4 surface expression on dendritic cells in response to PfMIF and LPS (n=2 for each condition, performed in duplicate for each experiment).

4.2.4.4 NO production in dendritic cells induced by PfMIF

A single nitrite assay was carried out to determine NO production with no suggestion of a difference in NO production between DC treated with LPS, PfMIF or control cells (figure 4.11). This is similar to the results seen with monocytes, with the very low levels of nitrite detected in the culture supernatant not suggesting a physiologically significant change in NO production under any of the study conditions. For this reason PfMIF-induced NO production in DC was not pursued any further.

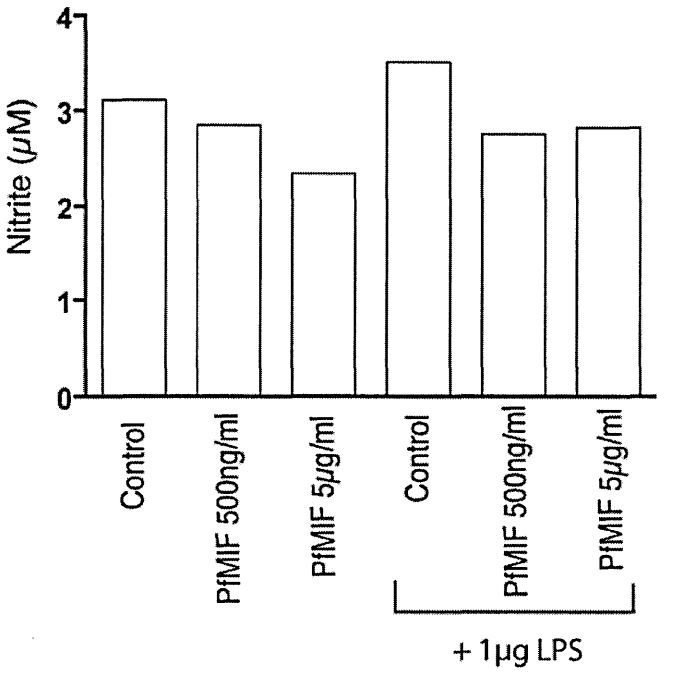


Figure 4.11: NO production by DC in response to PfMIF (n=1, done in duplicate).

4.3 DISCUSSION

The previous chapter provided evidence that PfMIF is capable of coming into direct contact with myeloid cells upon schizont rupture and raised the possibility that it may affect myeloid cell function. In this chapter, the ability of PfMIF to affect myeloid cell activities was investigated *in vitro*. These experiments demonstrated that exposure of monocytes to PfMIF resulted in modulation of some of the activities tested.

The initial challenge faced in these experiments involved the generation of pure and active recombinant PfMIF. A bacterial expression system and sequential purification steps were employed to produce recombinant PfMIF which was shown in cellular migration assays to inhibit random migration and chemotaxis of monocytes in a similar manner to other MIF proteins, thus confirming its activity.

With inhibition of migration confirmed, recombinant PfMIF could then be used to assess other *in vitro* activities of monocytes and DC. Both malaria and MIF activity have been shown to be associated with changes in cytokine production, surface molecule expression and NO generation, which can be used as indicators of myeloid cell activities. The current studies showed no effect of PfMIF on the secretion of any cytokines tested nor NO generation by monocytes or DC. On the other hand, PfMIF was found to affect the expression of a number of surface molecules in myeloid cells. PfMIF resulted in a significant reduction of CD86, TLR2 and TLR4 surface expression on monocytes, indicating that PfMIF can influence myeloid cells and may therefore have an effect on the host immune system during the course of *P. falciparum* infection.

TLR2 surface expression was also downregulated in response to PfMIF in DC. Although consistent with the effect seen in monocytes, a significant reduction in TLR2

expression did not appear to be dose-dependent in DC and was only seen at a PfMIF concentration much higher than those used on monocytes. This may indicate that this effect was due to an increase in contaminating LPS on a cell type that is more endotoxin-sensitive. Generally, DC were much less responsive to PfMIF treatment than monocytes across the range of activities tested.

The expression of ICAM-1 was examined because of its importance as an anchor for cytoadherence when the parasite expresses one of a subset of ICAM-1 specific PfEMP-1 molecules (section 1.5.4). It has also been shown that ICAM-1 cell surface expression is increased upon treatment with MIF in monocytes (Amin *et al.*, 2006). ICAM-1 was therefore considered an ideal candidate for modulation of expression by PfMIF but surprisingly, showed no change in expression levels on monocytes in response to recombinant PfMIF.

The moderate but significant downregulation of TLR2 and TLR4 expression on monocytes in response to PfMIF is an interesting and novel finding. Endogenous MIF has previously been shown to be required for the expression of TLR4 in mouse macrophages (Roger *et al.*, 2003), but no prior study has looked at the effect of exogenous MIF on TLR4 expression in human macrophages, or any other cell type. It has long been recognised that TLR tolerance can be induced in that pre-treatment of cells with LPS renders them unresponsive to further stimulation through TLR4 (the TLR specifically activated by LPS) or other TLRs (Dobrovolskaia *et al.*, 2003). It was therefore hypothesised that a twelve-hour pre-incubation of cells with PfMIF would alter monocyte activation by the TLR2-ligand PGN or the TLR4-ligand LPS. However this was not found to be the case. Pre-incubation with PfMIF at two different concentrations had no effect on the TLR2– or TLR4-mediated activation of monocytes,

suggesting that PfMIF does not play an active part in the TLR tolerance that has been previously described during *Plasmodium yoelii* infection in mice (Perry *et al.*, 2005).

A reduction in TLR expression on myeloid cells has also been demonstrated in patients with filarial infections (Babu *et al.*, 2005, 2006). T and B cells and monocytes from filarial-infected individuals were shown to exhibit significantly less TLR1, TLR2 and TLR4 expression compared to uninfected controls. The mechanism for reduced expression has not been elucidated but, given the results shown in this chapter, it is feasible that the MIF homologue produced and secreted by filarial nematodes may contribute to this phenomenon.

The reduction in CD86 expression on monocytes treated with PfMIF also demonstrates that PfMIF can influence monocyte activity. CD86 is an important co-stimulatory molecule and a reduction in expression could affect the strength of subsequent T cell activation.

In conclusion, this chapter demonstrates that PfMIF can influence the activity of myeloid cells *in vitro* based on the limited number of factors tested. This would suggest that PfMIF has the potential to modulate the immune response in malaria patients.

CHAPTER 5

PfMIF in patients

5.1 INTRODUCTION

The results from previous chapters have shown that PfMIF is expressed during parasite blood stages and that it is released upon schizont rupture. It has also been shown that PfMIF can alter the function of myeloid cells *in vitro*. The next major objective in this project was therefore to determine the possible relevance of PfMIF in patients with malaria.

Through a collaboration with the Kenya Medical Research Institute/Wellcome Trust Centre based at the Kilifi District Hospital in coastal Kenya, I had access to a unique, large and well-established cohort of children (see section 2.7). Access to this population gave me the opportunity to look at PfMIF *in vivo*. The aim was to collect blood from children before, during and after a malarial episode and determine if there is any relationship between circulating PfMIF levels and the immune responses to malaria or the severity of infection. In order to carry out these objectives it was necessary to develop assays to measure PfMIF-specific antibodies and PfMIF protein levels in patient samples.

5.2 RESULTS

During the malarial season, symptomatic patients from the cohort were identified by active surveillance and blood samples taken. Convalescent samples were also collected two weeks later where possible. Additionally, cross-sectional surveys were carried out in the low transmission seasons when patients from the cohort were asked to provide blood samples in order to generate data on this group when not infected with *P. falciparum*.

5.2.1 PfMIF antibody responses in patients

In order to detect anti-PfMIF IgG in patient circulation, an enzyme-linking immunosorbent assay (ELISA) was developed using plates coated with recombinant PfMIF as a capture antigen (section 2.7.1).

Antibody responses to PfMIF were examined in blood samples taken from Kenyan children with acute malaria, the same patients during convalescence, and from healthy Kenyan children during the low transmission season (see table 5.1).

Table 5.1: Malaria patient characteristics and PfMIF IgG levels.

	Healthy	Acute malaria	Convalescent
Number	117	80	35
Age months	57	29	26
(range)	(12-107)	(4-138)	(6-70)
Number positive for anti-PfMIF IgG	62 (53%)	65 (81%)	35 (100%)
Anti-PfMIF IgG OD	0.161	0.7215	1.0649
(range)	(0.01-0.86)	(0.1-2.1)	(0.4-2.2)

Patients with acute malaria were significantly younger than healthy subjects ($P< 0.05$, Table 5.1). Within each group however, there was no correlation between age and PfMIF IgG levels (Spearman's correlation coefficient, acute malaria: $r=0.076$, $p=0.547$; convalescent: $r=0.173$, $p=0.328$; healthy children: $r=0.16$, $p=0.221$).

In the healthy control group 62 children (53%) presented antibody responses to PfMIF. Within this responder group, PfMIF antibodies were not associated with age and therefore with recent exposure. By contrast, in the acute malaria and convalescent groups, 65 (81%) and 35 (100%) children respectively, were found to have circulating antibodies against PfMIF. Therefore, compared to healthy controls, there was a larger than expected proportion of positive antibody responses in acute and convalescent samples (Pearson's Chi-square, $p<0.001$). When only responding children from each group were taken into account, healthy children had significantly lower levels of circulating PfMIF antibodies than seen during and immediately subsequent to *P. falciparum* infection (figure 5.1; acute: OD=0.7215, range 0.1-2.10; convalescent: OD=1.0649, range 0.4-2.20; healthy: OD=0.1610, range 0.01-0.86; $p<0.001$ Mann Whitney test). This result suggests a rapid decrease in PfMIF antibody concentration following acute infection, and is consistent with the antibody profiles seen in response to other malaria antigens (Cavanagh *et al.*, 1998).

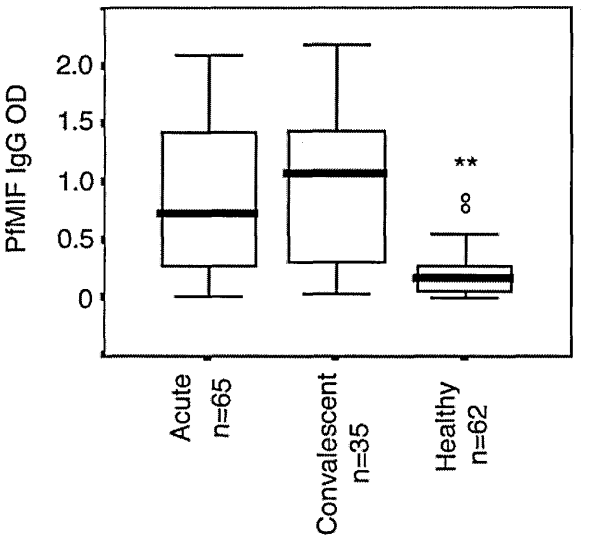


Figure 5.1 Antibody responses to PfMIF in Kenyan children. Samples were taken from acute malaria patients, convalescent patients and healthy controls and assessed for PfMIF IgG levels. Acute and convalescent malaria patients had significantly higher anti-PfMIF IgG levels than healthy children (**p<0.0005, Mann-Whitney U test).

Cross-sectional surveys were carried out on the cohort in two consecutive low malaria transmission seasons. A total of 96 patients had samples available from both cross sectional surveys to assess changes in antibody levels over the course of a year. Average antibody levels were found to be significantly lower in blood samples collected from these subjects in October 2004 compared to samples collected in October 2003 ($P < 0.05$, Figure 5.2). This drop may be explained by a reduced exposure to *P. falciparum* parasite. As described above, antibody responses to malarial antigens tend to decrease over time. Usually it has been estimated that people living in this region of coastal Kenya can expect 10 infectious bites/year. However, due to low rainfall in the study area between the cross-sectional surveys there were greatly reduced numbers of malaria cases than expected during the normal malaria season. This lack of transmission

between the surveys would explain the drop in antibody levels, as most children would not have been exposed to parasites in order to boost their responses over that period.

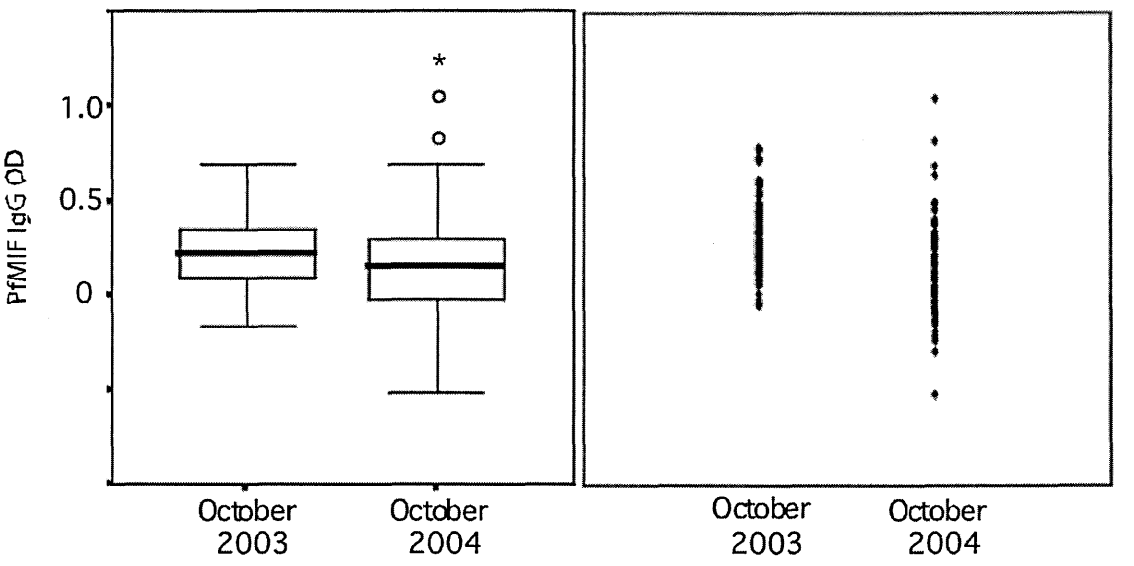


Figure 5.2: Antibody levels in Kenyan children during the low malaria transmission season in consecutive years. The box plot on the left shows a significant drop in PfMIF specific IgG in paired samples between October 2003 and October 2004 (n=96, *p<0.05, paired t-test). The plot on the right shows the raw data, more clearly demonstrating a drop in PfMIF IgG levels over a year.

5.2.2 Detection of PfMIF in malaria patient samples

The ideal way to study the role of PfMIF in human malaria patients would be to measure the circulating level of parasite protein itself and investigate the relationship between parasitaemia, disease severity and host immune responses. To allow detection of PfMIF protein, the two antisera generated against PfMIF (section 2.3) were used in an effort to develop a sandwich ELISA. Purified IgG from the antisera was used in order to assess their suitability for use as capture or detection antibodies. Unfortunately,

neither combination of these antibodies was successful in detecting PfMIF protein by sandwich ELISA. Additionally, when these antibodies were used to detect PfMIF protein bound to the ELISA plate as a capture antigen they still did not recognise the protein. One factor contributing to the difficulties experienced in trying to establish this method may be that both antibodies were generated in the same species, which is known to be problematic.

5.3 DISCUSSION

The studies described in this chapter aimed to determine if PfMIF plays a role in human malaria. PfMIF IgG was detected in the circulation of subjects in a cohort of Kenyan children. A rapid loss of anti-PfMIF antibody levels was evident during the low transmission season compared to acute or convalescent malaria patients. This pattern of antibody response has been shown for other malaria antigens (Cavanagh *et al.*, 1998). There was no indication that PfMIF antibody responses were involved in protection from subsequent disease or linked to disease severity.

The significant drop in low transmission season PfMIF antibody levels from October 2003 to October 2004 can be attributed to the failure of the rains in 2003 that resulted in very few presentations of malaria from the cohort area in the subsequent malaria season. The reduction in antibody levels in this instance is therefore likely to be due to a lack of exposure rather than any other factors.

The inability to generate a method to detect PfMIF in patient samples severely limited the ability to analyse the possible role of PfMIF in malaria pathogenesis. There is a large amount of data available for the cohort of Kenyan children that has been collected

over many years. These data range from demographic data, such as age and sex, the number and severity of malaria episodes, through to immune responses, such as cytokine levels during malaria episodes and subsequent convalescence. It was therefore frustrating not to be able to compare these data with PfMIF protein levels in the subjects and determine if there is an association between PfMIF concentration in patients with the immune response to a malaria episode or disease severity.

In conclusion, in this chapter it is shown that PfMIF antibodies could be detected in the circulation of a cohort of children living in a malarial region and that these antibodies follow a similar pattern of response to those seen against other malaria antigens previously tested. The levels of PfMIF protein present in the circulation of these children could not be determined due to technical difficulties. In order to be able to develop a successful ELISA assay, new antibodies specific for PfMIF need to be generated in other species. This would then allow comparison of PfMIF protein levels with the broad range of immunological, epidemiological and demographic data that have been collected over several years from the cohort of children in coastal Kenya. Such analyses would greatly boost the strength of this study and would confirm whether PfMIF plays an important role in the development of immunity to falciparum malaria, or the progression and severity of the disease.

CHAPTER 6

General discussion

Malaria is responsible for 1-3 million deaths per year worldwide, with half the global population at risk of infection. *Plasmodium falciparum* is the parasite responsible for severe malaria and accounts for almost all fatal cases. In order to develop strategies for the treatment or prevention of malaria, a better understanding of the interaction between parasite and host is required. This project aimed to characterise the structure and function of the putative MIF homologue identified during sequencing of the *P. falciparum* genome and to investigate the possibility that PfMIF plays a role in influencing the host immune system during the course of *P. falciparum* infection.

Sequence analysis and modelling techniques were used to suggest that PfMIF shares important structural similarities to MIF proteins from other species. Such similarities suggest that enzymatic activities attributed to other MIF proteins may be conserved in the *P. falciparum* homologue. Additionally, there were indications that recombinant PfMIF formed homotrimers, as has been reported for other MIF proteins (Mischke *et al.*, 1998). Studies of parasites in culture demonstrated that PfMIF mRNA and protein are expressed during ring and trophozoite stages of the parasite life cycle. Furthermore, PfMIF was found to be exported into the cytosol of the infected erythrocyte and released upon schizont rupture, thus providing an opportunity for PfMIF to interact directly with the host immune system. During the preparation of this thesis, another study characterising PfMIF was published and confirmed the above findings (Augustijn *et al.*, 2006). Augustijn and colleagues characterised aspects of both *P. berghei* MIF (PbMIF) and PfMIF activity. In addition to the results described above, these authors report that *Plasmodium* MIF protein is expressed during all stages of the parasite lifecycle. This study also reported that both species of *Plasmodium* MIF have tautomerase and oxidoreductase activities, albeit significantly less activity than huMIF.

The lack of a complete CXXC motif, that was also identified in the present study, was shown to reduce but not completely ablate oxidoreductase activity of the *Plasmodium* MIF proteins.

Augustijn *et al.* also carried out *in vivo* experiments in which mice were infected with *PbMIF* gene knockout *P. berghei* parasites (Augustijn *et al.*, 2006). These mice demonstrated higher levels of circulating reticulocytes. Although this was the only significant difference identified in mice infected with PbMIF knockout parasites it does suggest that *Plasmodium* MIF homologues may play a role in malarial anemia. The mechanism of this effect was not reported but may involve indirect inhibition of erythropoiesis, for instance by influencing cytokine responses, or a direct effect that *Plasmodium* MIF may have on erythropoietic cells. The effects of PbMIF deficiency in these parasites may become more apparent when the *P. berghei* knockout parasites are used to infect the thicket rat (*Grammomys saurdaster*), the natural host of *P. berghei*.

The localisation of PfMIF to the Maurer's cleft in the cytosol of iRBC (section 3.2.4) is of particular interest as it raises the possibility that, in addition to its effect on monocyte function, PfMIF may play a role in protein trafficking within in the iRBC. This is based on the recent finding that mammalian MIF may have chaperone-like properties (Cherepkova *et al.*, 2006) and that the Maurer's cleft has been implicated in the trafficking of parasite proteins to the surface of the erythrocytes (Cooke *et al.*, 2006). Studies on protein trafficking in iRBC using PfMIF knockout parasites could address this question and would be an interesting direction for future studies. Alternatively, considering that PfMIF does not have an export Pexel/VTS motif like some other exported *P. falciparum* proteins (see section 3.2.1), the association of PfMIF with the Maurer's cleft may represent a mechanism of protein export from the parasite, which is

independent of the Pexel/VTS motif.

To address the potential ability of PfMIF to modulate immune responses, recombinant PfMIF protein was generated and used to treat monocytes *in vitro*. These experiments demonstrate that PfMIF inhibits the random migration and chemotaxis of monocytes and influences surface molecule expression, as evidenced by the downregulation of TLR2, TLR4 and CD86. This supports the hypothesis that PfMIF can influence immune responses.

The effect of exogenous MIF on TLR expression had not been previously examined. In contrast to the effect of endogenous MIF, which has been shown to be required for the expression of TLR4 in mouse macrophages (Roger *et al.*, 2003), during the present study it was observed that TLR2 and TLR4 expression on monocytes was moderately but significantly reduced in response to PfMIF. PfMIF was not found to alter monocyte activation by the TLR2-ligand PGN or the TLR4-ligand LPS and therefore may not play an active part in the TLR tolerance that has been previously described during *Plasmodium yoelii* infection in mice (Perry *et al.*, 2005). Interestingly, TLR expression on myeloid cells has also been found to be decreased in patients with filarial infections through an unknown mechanism (Babu *et al.*, 2005, 2006). Considering the results from the present study, it is tempting to speculate that the MIF homologue produced and secreted by filarial nematodes may contribute to this phenomenon.

The downregulation of CD86 on the surface of monocytes after exposure to PfMIF raises another potential mechanism by which PfMIF may influence the immune response to malaria. CD86 is an important co-stimulatory molecule necessary for T cell activation. A previous study looked at CD86 expression on DC in response to *Plasmodium* infection. Using monocyte-derived DC, it was shown that binding to iRBC

lowered DC surface molecule expression, including CD86, and led to a suppression of subsequent T cell responses (Urban *et al.*, 1999). Although there was no reduction in CD86 expression on DC in response to PfMIF in this study, the reduction in CD86 levels on monocytes may also result in a similar reduction in the strength of subsequent T cell activation. This possibility should be addressed in future studies.

Based on MIF activities described in other systems, there are a number of potential mechanisms for modulation of immune responses that have not been investigated in the present study and which may be of interest for future studies into the actions of PfMIF. These include modulation of expression of matrix metalloproteinases and effects on eosinophil recruitment and activity.

The expression of MMP-9 has been shown to be induced by MIF in rat osteoblasts and has also been implicated in the pathogenesis of malaria (see sections 1.6.4.8 and 1.7.2) (Onodera *et al.*, 2002; Prato *et al.*, 2005). Disruption of basal lamina and endothelial alterations, especially at sites of iRBC sequestration, have been described in cerebral malaria (Brown *et al.*, 2000). It is possible that this disruption may be due to the activity of MMP-9, the expression of which was increased in monocytes by the phagocytosis of trophozoites and haemozoin (Prato *et al.*, 2005). Although trophozoites and haemozoin induce MMP-9 in monocytes this does not preclude a role for PfMIF in MMP induction. PfMIF is expressed in trophozoites, and haemozoin isolated from parasites binds many lipids and proteins, which may include PfMIF. PfMIF may therefore be responsible, at least in part, for the induction of MMP-9 in monocytes and may thereby play an important role in cerebral malaria pathogenesis.

Another potential mechanism of action of PfMIF during the course of malarial disease is its influence on eosinophils. Although the role of eosinophils has not been extensively

studied during the course of *Plasmodium* infection, one study suggests that eosinophils in circulation decrease during acute malarial disease and are increased during asymptomatic infection (Kurtzhals *et al.*, 1998b). Markers of eosinophil activity however are increased during acute malaria. This may suggest that eosinophils are recruited to a specific tissue during infection. Interestingly, a study in mice has shown that administration of recombinant parasite MIF (specifically *Brugia malayi*-MIF-1) into the peritoneal cavity led to eosinophil recruitment in a similar fashion to *B. malayi* infection itself (Falcone *et al.*, 2001). This suggests a potential role for PfMIF in the course of malaria in respect to eosinophil recruitment.

Analysis of the pathophysiological role of host-derived MIF during *Plasmodium* infection has so far been limited to its role in the development of malarial anaemia (McDevitt *et al.*, 2006). MIF knockout mice infected with *P. chabaudi* developed less severe anaemia, had better erythroid development and improved survival compared to controls. Following from this observation it was shown that human MIF (huMIF) levels in plasma were significantly increased during acute malarial disease in patients from Zambia (McDevitt *et al.*, 2006). This finding is however in direct contrast to a study by Awandare and colleagues that showed a significant decrease in circulating huMIF in acute malaria patients from Gabon (Awandare *et al.*, 2006a). Neither of these studies in patients addressed the likely involvement of PfMIF during acute malaria. The present study highlights the important issue that future studies should take into account the presence of parasite MIF in the circulation, and hence any potential cross-reactivity between reagents detecting PfMIF and huMIF.

The measurement of PfMIF in patient samples is an important step in determining the potential role of the protein in the influence of the host immune system. Access to a

cohort of children in Kenya allowed examination of patient antibody responses to PfMIF in this study. Antibody responses to PfMIF were found to follow a pattern consistent with antibody responses against other malaria antigens that have been previously described (Cavanagh *et al.*, 1998). Attempts to develop a sandwich ELISA to detect PfMIF in patient circulation during the course the project were unsuccessful. An alternative method for PfMIF detection may be possible by developing a radioimmunoassay using the antibodies available and radio-labelled recombinant PfMIF protein, however, this was not feasible with the facilities available during this project. The inability to develop a method to detect PfMIF in patient samples with the resources available reduced the ability to fully utilise the data from the patient cohort. The development of a method to detect PfMIF in patient samples would be the main priority in any future study as comparisons with available cohort data would be invaluable in determining whether PfMIF plays a role in immune regulation during *P. falciparum* infection.

The influence of concomitant *Plasmodium* and nematode infections should be taken into consideration in studies investigating the influence of *Plasmodium* MIF in malaria patients. Regions of the world that have high rates of malaria transmission also generally have high rates of nematode infection. For instance, in a study carried out in Malindi district of coastal Kenya, just north of the study area used in this study, 34.4% of males over 15 years of age showed signs of *Wuchereria bancrofti* infection (Njenga *et al.*, 2006). This increased to 55.4% of males over 40. Future studies associating PfMIF and patient responses or malarial disease severity should therefore consider the potential influence of concomitant nematode infection. Hypothetically, if PfMIF released during the course of infection was responsible for pushing a host pro-

inflammatory MIF response into an anti-inflammatory response (like the bell-shaped dose response seen previously, see section 1.6.2) then nematode MIF may cause the same response regardless of levels of *P. falciparum*. The present study only looked at antibody responses and did not compare these to other host immune responses, so the influence of nematode MIF molecules is not an important consideration here.

During the course of this project several unique tools have been developed to examine the role of PfMIF during the course of *P. falciparum* infection. Initially, active recombinant PfMIF protein was produced to examine the effect of this protein on myeloid cells *in vitro*. Antibodies were generated against this protein and used in Western blots and immunofluorescence to examine the expression and localisation of PfMIF within the parasite. Additionally, an ELISA was developed to measure anti-PfMIF IgG levels in patient samples. These resources will be of use in future studies examining other aspects of PfMIF expression and actions.

In summary, this project has investigated the potential role of PfMIF in the pathogenesis of malaria. PfMIF is shown to be expressed by blood stage parasites and released from the infected erythrocyte upon schizont rupture, allowing direct contact with the host immune system. Recombinant PfMIF was found to be capable of modulating the function of myeloid cells *in vitro*, specifically by inhibiting the migration of monocytes and decreasing their surface marker expression. These results taken together suggest that PfMIF could be an important molecule involved in the interaction between the parasite and the host immune system. Although detection of PfMIF in patient circulation was not possible in the course of this study, major advances have been made towards determining the role that this *Plasmodium* MIF homologue plays in modulating the host immune system during the course of malarial disease.

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