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# **Comparative Genomics and Proteomics in Drug Discovery**

Edited by

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# Preface

This book arose from a one-day symposium arranged as part of the Annual Meeting of the Society for Experimental Biology (UK) in Barcelona in 2005 and takes up the important question of how emerging genomic and proteomic technologies are making significant contributions to global drug discovery programmes, and in particular the key role that comparative genomic and proteomic strategies play.

Rapid progress in our understanding of cellular and molecular biology has led to the field of biomedical science undergoing a major revolution over the last 30 years or so. Of particular note is the dramatic development of genomic and proteomic technologies and their associated application in biology and medicine.

Genomics is the study of an organism's genome and involves isolation, identification and mapping of genes along with associated functional dynamics and interaction. The Human Genome Project has revealed that the human genome is composed of 20,000 to 25,000 genes. However, since each gene can be translated into a variety of different proteins via a variety of cellular and molecular mechanisms, it is estimated that the human proteome consists of approximately 1,000,000 differently modified proteins. This in turn means that there are significantly more potential biomarkers or drug targets to be discovered using proteomic approaches rather than genomic approaches. Expression of these proteins is known to vary in response to environmental change and is related to genetic history. Unlike variation in gene expression, any change in protein expression usually results in alteration of function. Genomics and proteomics are thus considered as being highly complementary approaches to the molecular study of disease.

Written by widely respected authorities from both academic and pharmaceutical backgrounds, this book is composed of seven concise chapters. In Chapter 1, Sara Melville (Cambridge University, UK), introduces the use of comparative genomics in drug discovery in one of the three main human pathogens associated with kinetoplastids, the trypanosomatids, parasitic protozoa responsible for a wide range of diseases including African sleeping sickness, Chagas disease and leishmaniasis. In Chapter 2, Chris Peacock (Wellcome Trust Sanger Institute, UK) describes how comparative genomics is being used to assist in developing treatments against the kinetoplastids, a remarkable group of organisms that include major pathogens responsible for thousands of deaths each year and serious illness to millions. Maria Mota (Universidade de Lisboa, Portugal) then considers the relevance of host genes in malaria (Chapter 3), a devastating disease that affects extensive areas of Africa, Asia and South/Central America causing up to 2.7 million deaths per year. In Chapter 4, David Sattelle (University of Oxford, UK) describes how comparative genomics has contributed to the study of nicotinic acetylcholine receptors as drug/chemical targets for a number of conditions including genetic and autoimmune disorders. Chapter 5 (Jeff Clare, GlaxoSmithKline, UK) discusses how genomic and proteomic strategies are being used in gene family-based drug discovery projects aimed at isolating novel sodium channel inhibitors. A sub-population of voltage-gated sodium channels are expressed primarily in nerves involved in pain signalling; these are hence of major interest to the pharmaceutical industry as potential targets for improved analgesics. In Chapter 6,

John Bilello (GlaxoSmithKline, USA) discusses how information resulting from genomic and proteomic studies can be translated into disease understanding and effective management of therapy. Finally, in Chapter 7, David Wishart (University of Alberta, Canada) discusses how advances in genomic and information technology have led to the possibility of *in silico* drug target discovery.

The purpose of this book is to provide an introduction to the concepts behind the dynamic and powerful fields of comparative genomics and proteomics and their specific application in drug discovery. To some extent, the book assumes knowledge of basic molecular biology and is targeted at students, researchers and academics in related areas of biomedicine and pharmaceuticals and to a more general readership interested in specific applications of genomic and proteomic technologies.

We would like to thank all of the contributing authors and the Society for Experimental Biology (SEB, UK) for helping us to put this book together. We would also like to extend our thanks to Elizabeth Owen and Kirsty Lyons at Taylor & Francis for their extensive assistance in the final compilation of this book.

Oxford, April 2006.

John Parrington & Kevin Coward

# The relevance of host genes in malaria

Miguel Prudêncio, Cristina D. Rodrigues and Maria M. Mota

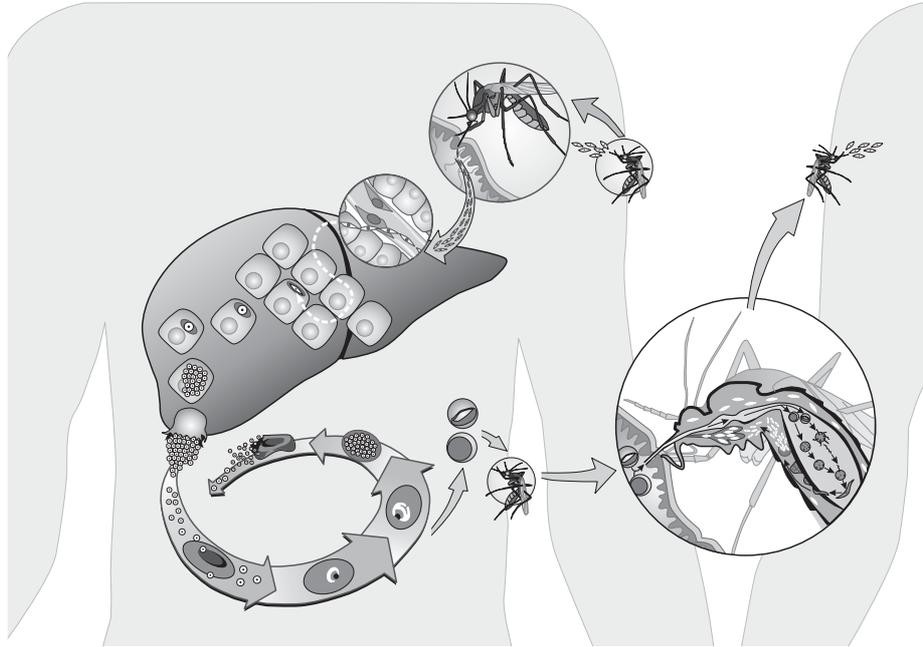
## 1 Introduction

Malaria is a devastating disease that affects extensive areas of Africa, Asia and South and Central America, causing up to 2.7 million deaths per year, mainly children under the age of five (Webster and Hill, 2003). The disease is caused by a protozoan parasite from the genus *Plasmodium* and transmitted through the bite of the female *Anopheles* mosquito. When a mosquito infected with *Plasmodium* bites a mammalian host, it probes for a blood source under the skin and, during this process, deposits saliva containing sporozoites. These sporozoites reach the circulatory system and are transported to the liver. Once there, they migrate through several hepatocytes by breaching their plasma membranes before infecting a final cell with the formation of a parasitophorous vacuole. After several days of development inside a hepatocyte, thousands of merozoites are released into the bloodstream where they invade red blood cells (RBCs), initiating the symptomatic erythrocytic stage of the disease (*Figure 1*).

Malaria infection depends upon the occurrence of interactions between the *Plasmodium* parasite and the host. Every stage of an infection by *Plasmodium* relies, to different extents, on the presence of host molecules that enable or facilitate its invasion, survival and multiplication. Therefore, host genes play a crucial role in determining the resistance or susceptibility to malaria and may constitute potential targets for preventive or therapeutic intervention. Analysis of the genetic basis of susceptibility to major infectious diseases is, arguably, the most complex area in the genetics of complex disease (Hill, 2001). In this chapter, we will examine the progress made towards identifying mammalian host molecules that play a role in the modulation of malaria infections.

## 2 The pre-erythrocytic stage: hepatocyte, liver and beyond

The hepatic stage of a *Plasmodium* infection constitutes an appealing target for the development of an intervention strategy since this would act before the onset of pathology, which only occurs during the blood stage of the parasite's life cycle. In fact, until now, the only demonstrably effective vaccine shown to confer a sterile and lasting protection both in mice (Nussenzweig *et al.*, 1967) and in humans (Clyde *et al.*, 1973;



**Figure 1.** The life cycle of *Plasmodium* in the mammalian and mosquito hosts.

Rieckmann *et al.*, 1974; Herrington *et al.*, 1991) was the inoculation of  $\gamma$ -irradiation-attenuated sporozoites, that are able to invade but not fully mature inside the hepatocyte (see Carvalho *et al.*, 2002; Gruner *et al.*, 2003; Bodescot *et al.*, 2004; Todryk and Walther, 2005; Waters *et al.*, 2005).

Despite being symptomatically silent, the liver stage of a malaria infection is immunologically very complex. Unlike RBCs, liver cells are able to promote cell-mediated immune response mechanisms through expression of class I Major Histocompatibility Complex (MHC) proteins. Class I MHC proteins present antigens to cytotoxic T lymphocytes (CTLs) (Lowell, 1997), which are known to play an important role in the generation of a protective immune response in many microbial infections (Esser *et al.*, 2003). The activation of T cells by antigen-presenting cells (APCs) is required to initiate specific immune responses. Different APCs have been shown to be important in this process, including dendritic cells (DC) (Bruna-Romero and Rodriguez, 2001; Jung *et al.*, 2002; Leiriao *et al.*, 2005) and Kupffer cells (Steers *et al.*, 2005).

Human leucocyte antigens (HLAs) are encoded by genes of the MHC, which are known to be among the most polymorphic of all human genes (reviewed in Williams, 2001). Although most of the human MHC *loci* are relatively stable, the *HLA-B locus* has been shown to undergo rapid changes, especially in isolated populations (McAdam *et al.*, 1994). *HLA-B* encodes an MHC class I heavy chain that is part of the *HLA-B* antigen-presentation complex (Kwiatkowski, 2005). On the other hand, the *HLA-DR* antigen-presenting complex includes an HLA class II  $\beta$  chain, which is encoded by *HLA-DRB1*. *HLA-DR* is found in B lymphocytes, DC and macrophages where it plays an essential role in the production of antibodies (Kwiatkowski, 2005). Both the *HLA-B53* allele and the *DRB1\*1302-DQB1\*0501* haplotype were shown to be

associated with protection against severe malaria (SM) in The Gambia (Hill *et al.*, 1991) and the latter was also found to be associated with protection from malaria anaemia and malarial reinfections in Gabonese children (May *et al.*, 2001).

The analysis of the peptides from a vast range of malaria antigens that bind to HLA-B53-restricted CTLs in malaria-immune Africans led to the identification of a single conserved peptide from liver-stage-specific antigen-1 (LSA-1) (Hill *et al.*, 1992), making LSA-1 an interesting malaria vaccine candidate (Migot-Nabias *et al.*, 2001). Of the four most prevalent allelic variants of the protein recognized by HLA-B53, only two are indeed epitopes, binding this protein *in vitro* (Gilbert *et al.*, 1998). Moreover, these results suggest that cohabiting parasite strains, each of which being an individually effective target for CTLs, may have the ability to use altered peptide ligand (APL) antagonism mechanisms to suppress the CTL response to the other strain, thereby increasing each other's chances of survival. This observation has obvious implications in terms of vaccine development, since it suggests that including all allelic peptide variants in a prospective vaccine might be counterproductive because one given variant may antagonize immunity to other variants.

During *Plasmodium* sporozoite development inside hepatocytes there is an amazing multiplication, with each parasite giving rise to 10,000–30,000 merozoites in 2–7 days (depending on *Plasmodium* spp.). Moreover, there is a high level of specificity of *Plasmodium* sporozoite development, which only occurs in certain types of cells. This strongly suggests an important role for the host cell in supporting the full development of the parasite. However, not much is known about *Plasmodium* requirements, strategies developed to survive and be successful, or how much the host cell contributes to this. An intriguing characteristic of *Plasmodium* sporozoites is their ability to migrate through hepatocytes prior to invading a final one with the formation of a vacuole for further development. Sporozoites breach the plasma membrane of the cell, traverse through its cytosol and leave by wounding the membrane (Mota *et al.*, 2001). This unusual process is frequently observed *in vitro*, where sporozoites traverse mammalian cells at a speed of approximately one cell per minute. Migration through host hepatocytes is also observed *in vivo* in the liver of mice infected with *Plasmodium* sporozoites (Mota *et al.*, 2001; Frevert *et al.*, 2005). Wounding of host hepatocytes induces an alteration in traversed cells which includes the secretion of host cell factor(s), which render(s) neighbouring hepatocytes susceptible to infection. One such factor is hepatocyte growth factor (HGF), which, by activating its receptor MET, seems to be required for the early development of parasites within host cells (Carrolo *et al.*, 2003). These results, however, appear to be contradicted by evidence provided by *spect*-deficient sporozoites, which do not migrate through cells *in vitro* (Ishino *et al.*, 2004). It would be expected that *spect*-deficient sporozoites would not induce host cells to produce HGF and, therefore, infection would be inhibited. However, these sporozoites efficiently infect host cells *in vitro* (Ishino *et al.*, 2004). This apparent discrepancy may be due to particular characteristics of the *in vitro* cell system used for infection (Mota and Rodriguez, 2004), or to alternative ways used by the parasite to fully develop inside host cells.

### 3 The erythrocytic stage and disease

The erythrocytic stage of *Plasmodium*'s life cycle corresponds to the symptomatic phase of a malaria infection. During this phase, *Plasmodium* merozoites invade RBCs and degrade haemoglobin (Hb), releasing heme that is converted into haemozoin

(malarial pigment). The invading merozoites multiply in the RBCs and, upon rupturing the erythrocytic membrane, are eventually released into the blood where they target new RBCs. The interaction between *Plasmodium* and the RBCs occurs in two stages: first, the identification and binding of RBC surface molecules that will enable invasion; subsequently, the intracellular interaction with Hb and the multiplication of the parasite.

### 3.1 Erythrocyte invasion – *P. vivax* versus *P. falciparum*

The human malaria parasite, *P. vivax*, and the related monkey malaria, *P. knowlesi*, use the Duffy blood group antigen as a receptor to invade human RBCs (Miller *et al.*, 1976). The Duffy antigen, encoded by the *FY* gene, is a chemokine receptor on the surface of the RBCs. It belongs to the superfamily of G-protein coupled receptors (GCRs) (Neote *et al.*, 1994) and is also termed DARC (for Duffy antigen receptor for chemokines). The Duffy blood group locus is polymorphic and has three main alleles designated *FY*\*A, *FY*\*B and *FY*\*O. The *FY*\*A allele is very frequent in Asia and the Pacific, whereas in Europe and the Americas the *FY*\*A and *FY*\*B alleles are at intermediate frequencies. The *FY*\*O allele is at or near fixation in most sub-Saharan African populations, but is very rare outside Africa (Hamblin and Di Rienzo, 2000). *FY*\*O arises from a mutation at position -46 in the promoter of the *FY*\*B allele, leading to a Fy(a-b-) phenotype in which RBCs lack both Fy<sup>a</sup> and Fy<sup>b</sup> antigens (Tournamille *et al.*, 1995). Fy(a-b-) RBCs resist invasion *in vitro* by *P. knowlesi* parasites and individuals homozygous for the *FY*\*O allele are completely resistant to *P. vivax* malaria (Miller *et al.*, 1976). The correlation between the Duffy-negative serological phenotype and resistance to *P. vivax* malaria is now clear. *P. vivax* relies on a single pathway to invade RBCs. The lack of redundancy in *P. vivax* invasion pathways may explain the near absence of this parasite from West Africa, where almost 95% of the population have the Duffy-negative phenotype and are resistant to *P. vivax* malaria (Chitnis and Miller, 1994). The extreme degree of between-population differentiation of allele frequency of the Duffy blood group gene shows evidence of human directional selection by *P. vivax* (Hamblin *et al.*, 2002). Furthermore, it has important implications in drug or vaccine design (Yazdani *et al.*, 2004).

Unfortunately, things are a lot more complicated when it comes to the much more deadly *P. falciparum* malaria parasite.

Contrary to *P. vivax*, *P. falciparum* displays the ability to invade RBCs following multiple, alternative pathways, with significant redundancy. Research into the identification of RBC receptors involved in merozoite invasion has made use of RBCs that lack specific surface molecules or enzymes that modify protein and carbohydrate domains on those molecules. The main enzymes used have been neuraminidase (which cleaves sialic acid groups from surface glycoproteins and glycolipids) and trypsin (which cleaves the peptide backbone of a number of surface proteins) (Baum *et al.*, 2003). These studies have revealed several surface receptors that are involved in invasion of the RBC by *P. falciparum* merozoites. Three neuraminidase-sensitive molecules that have received particular attention are glycophorin A (GYPA) (Pasvol *et al.*, 1982), glycophorin B (GYPB) (Dolan *et al.*, 1994), and glycophorins C and D (GYPC/D) (Mayer *et al.*, 2001; Maier *et al.*, 2003). GYPC and GYPD are encoded by the same gene, but use alternative start codons. Deletion of exon 3 in the *GYPC/D* gene

changes the serologic phenotype of the Gerbich (Ge) blood group system, resulting in Ge-negativity. Ge-negative RBCs exhibit a shortened GYPC and lack GYPD (Mayer *et al.*, 2001). This is of particular relevance if we consider that the Ge-negative phenotype is found at high allele frequencies in some regions of Papua New Guinea, which coincide with regions of malaria hyperendemicity. This strongly suggests that selection of Ge-negativity in these populations confers at least partial protection against *P. falciparum* malaria. It should be noted that this is in contrast to a study in which no correlation between the prevalence of *P. falciparum* infection and Ge-negativity in the Wosera region of Papua New Guinea was found (Patel *et al.*, 2001). More recently, the existence of yet another sialic acid-dependent receptor, termed 'Receptor Y', has been demonstrated (Rayner *et al.*, 2001). It would seem apparent that *P. falciparum* shows a near-exclusive preference for sialic acid-dependent (i.e. neuraminidase-sensitive) glycoporphins for RBC invasion. This, however, is now known not to be the case. Several reports have shown that alternative, sialic acid-independent, invasion pathways are commonly used by *P. falciparum* (Mitchell *et al.*, 1986; Hadley *et al.*, 1987; Dolan *et al.*, 1994). Moreover, *P. falciparum* seems capable not only of sialic acid-independent RBC invasion, but also of switching between sialic acid-dependent and sialic acid-independent pathways (Dolan *et al.*, 1990; Reed *et al.*, 2000; Duraisingh *et al.*, 2003). Very recently, the molecular mechanism for this switching process was elucidated (Stubbs *et al.*, 2005).

As we have seen, the surface of the RBC presents various types of sialic acid-dependent and sialic acid-independent receptors that can mediate *P. falciparum* invasion with at least a certain degree of redundancy. Furthermore, it is clear that the relative importance of each receptor is strain-dependent (Rayner *et al.*, 2001). These observations have important implications for vaccine development. While the existence of a single RBC invasion pathway for *P. vivax* yields good reasons to hope for a successful vaccine, this is clearly not the case for *P. falciparum*.

### 3.2 Intraerythrocytic stage – globin and non-globin genes

Following invasion of RBCs, *Plasmodium* parasites develop and multiply, leading to the appearance of the symptoms of malaria infection. Almost 90% of the intraerythrocytic space is taken up by Hb. Therefore, it is to be expected that *Plasmodium* will interact closely with this molecule and be influenced by its overwhelming presence. Thus, it is perfectly conceivable that Hb alterations will affect the development of the parasite, as well as the parasitized RBC itself.

Normal Hbs are tetrameric proteins composed of two pairs of unlike globin chains. After birth, the vast majority of Hb is composed of two  $\alpha$ -globin and two  $\beta$ -globin chains (reviewed in Weatherall and Clegg, 2001; Richer and Chudley, 2005). The molecular pathology of most of the haemoglobinopathies is well defined (Weatherall and Clegg, 2001). The Hb disorders resulting from mutations in the  $\alpha$ - or  $\beta$ -globin gene clusters are the most common single-gene disorders in humans (Weatherall, 2001). Inherited haemoglobinopathies can be divided into two main groups: structural Hb variants, mostly resulting from single amino acid substitutions in the  $\alpha$ - or  $\beta$ -chains, and thalassaemias, arising due to the ineffective synthesis of the  $\alpha$ - and/or  $\beta$ -chains (reviewed in Weatherall and Clegg, 2001). These two classes of Hb disorders constitute one of the most striking illustrations of why malaria is considered

the strongest known selective pressure in the recent history of the human genome (Kwiatkowski, 2005).

*Structural Hb variants.* Of the more than 700 structural Hb variants identified, only those coding for ‘haemoglobin S’ (or ‘sickle haemoglobin’, HbS), ‘haemoglobin C’ (HbC) and ‘haemoglobin E’ (HbE) have reached polymorphic frequencies. Each of the alleles *HbS*, *HbC* and *HbE* results from a single point mutation in the *HBB* gene. Although often lethal in the homozygous state, the unusually high prevalence of these alleles in areas of malaria endemicity has long been attributed to a selective pressure exerted by *Plasmodium* on the human host genome (Min-Oo and Gros, 2005).

In HbS, the glutamate at position 6 of the  $\beta$ -globin chain is replaced by a valine residue. The resulting protein contains ‘sickle’  $\beta$ -globin ( $\beta^S$ -globin) chains and tends to polymerize at low oxygen concentrations, causing the RBC to acquire a sickle-like shape (Brittenham *et al.*, 1985). This results in a condition known as ‘sickle cell’ anaemia, an autosomal recessive genetic disorder characterized by chronic anaemia and periodic vaso-occlusive crises (Shiu *et al.*, 2000). Although the homozygous state (HbSS) is often lethal, the heterozygous state (HbAS) is referred to as sickle cell trait and is usually clinically silent. The HbAS state has been shown to confer significant protection to malaria (reviewed in Kwiatkowski, 2005; see also Aidoo *et al.*, 2002; Williams *et al.*, 2005a). The exact mechanisms through which HbAS protects against malaria are unclear. A few possible explanations have, however, been put forward, including the enhanced sickling of the infected RBCs (iRBCs) (Luzzatto *et al.*, 1970), the suppression of parasite growth in individuals with the sickling disorders (Pasvol *et al.*, 1978) and increased spleen clearance (Shear *et al.*, 1993), enhanced phagocytosis (Ayi *et al.*, 2004) and enhanced acquisition of natural immunity to malaria, possibly due to the accelerated acquisition of antibodies against altered host antigens expressed on the surface of the infected RBCs (iRBCs) and/or against parasite-derived proteins (Williams *et al.*, 2005b).

In HbC, the glutamate at position 6 of the  $\beta$ -globin chain is replaced by a lysine residue. The resulting condition is considerably less serious than sickle cell anaemia. Even in the homozygous state (HbCC), only occasional pathologic developments are observed whereas heterozygotes (HbAC) are asymptomatic (Agarwal *et al.*, 2000; Kwiatkowski, 2005). This Hb variant has been implicated in protection against malaria in both the HbAC and the HbCC states (reviewed in Kwiatkowski, 2005; Min-Oo and Gros, 2005), although contrasting results have been reported concerning the heterozygous state (discussed in Modiano *et al.*, 2001). Three recent reports propose complementary explanations for the protective effect of HbC against malaria, all implying modifications that occur at the host cell surface (Tokumasu *et al.*, 2005; Arie *et al.*, 2005; Fairhurst *et al.*, 2005).

HbE results from a glutamate  $\rightarrow$  lysine mutation at position 27 of the  $\beta$ -globin chain. It is the most common structural variant of Hb and is innocuous both in its heterozygous (HbEA) and homozygous (HbEE) states (Weatherall and Clegg, 2001). There is no unequivocal proof that HbE protects against malaria but it has been suggested that an alteration in the RBC membrane in HbEA cells renders the majority of the RBCs population relatively resistant to invasion by *P. falciparum* (Chotivanich *et al.*, 2002).

*Thalassaemias.* The  $\beta$ - and  $\alpha$ -thalassaemias are disorders of globin chain synthesis that appear as a consequence of deletions or point mutations in the non-coding portion of

the  $\beta$ - and  $\alpha$ - globin genes, respectively (reviewed in Weatherall, 2001; Richer and Chudley, 2005). Because the synthesis of the  $\beta$ -globin chain is determined by two alleles of the *HBB* gene, whereas that of  $\alpha$ -globin is encoded by the four alleles on the equivalent *HBA1* and *HBA2* genes, there is a wide range of possible thalassaemic genetic variants, with different clinical manifestations. In general, homozygous thalassaemia is severe or even fatal, whereas the heterozygous state is clinically benign. However, when either the *HBA1* or the *HBA2* gene, but not both, is defective, a condition termed  $\alpha$ -thalassaemia occurs, for which homozygous individuals show only mild anaemia (Kwiatkowski, 2005). Evidence for protection against malaria by thalassaemia has been shown in different reports (reviewed in Min-Oo and Gros, 2005; Williams *et al.*, 2005a). The nature of protection against malaria by thalassaemias is unclear. Again, several suggestions have been mentioned including impaired parasite growth and increased susceptibility to phagocytosis (Yuthavong *et al.*, 1988, 1990), altered expression of parasite-induced surface neoantigens in  $\beta$ - and  $\alpha$ -thalassaemia allowing greater binding of specific antibody to iRBCs and their subsequent clearance (Luzzi *et al.*, 1991a, 1991b) and enhanced phagocytosis of ring-stage iRBCs of  $\beta$ - (but not  $\alpha$ -) thalassaemia individuals (Ayi *et al.*, 2004).

In addition to Hb, other RBC molecules also seem to play an important role in the parasite's development and in the host's susceptibility to malaria.

*G6PD deficiency.* The haeme liberated in the process of intra-erythrocytic Hb degradation by *Plasmodium* is polymerized to haemozoin or broken down non-enzymatically. Non-enzymatic cleavage of haeme liberates iron and generates hydrogen peroxide, a potential source of oxidative stress (Schwarzer *et al.*, 2003). Glucose-6-phosphate dehydrogenase (G6PD) catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate, while concomitantly reducing nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup> to NADPH). G6PD is the only erythrocytic enzyme that produces NADPH, a compound that is crucial for the defence of the RBCs against oxidative stress (Beutler, 1996). G6PD deficiency is the most common human enzymopathy known, affecting over 400 million people (Beutler, 1990). There are numerous variants of the *G6PD* gene and only those that significantly hamper enzyme activity lead to haemolytic anaemia, a phenotype that is exacerbated under oxidative stress conditions (Kwiatkowski, 2005). Evidence for the geographical correlation between G6PD deficiency and protection against malaria comes from various population-based studies and suggests evolutionary selection by the latter (reviewed in Kwiatkowski, 2005; Min-Oo and Gros, 2005). Although reduced parasite replication in G6PD-deficient RBCs was initially proposed as the mechanism of protection (Luzzatto *et al.*, 1969), the invasion and maturation of the parasite seems not to be significantly different between normal and G6PD-deficient RBCs (Cappadoro *et al.*, 1998). Instead, ring-stage iRBCs present on their surface a higher density of phagocytic removal markers than normal iRBCs and are, therefore, phagocytosed more efficiently. This suggestion has been extended to explain the protective nature of other erythrocytic defects against malaria (Ayi *et al.*, 2004), as previously mentioned.

*Band 3 protein.* Band 3 protein, encoded by *AE1*, is a ~100 kDa membrane protein that acts as an anion permeation channel, exchanging intracellular bicarbonate for chloride through the RBC lipid bilayer (Rothstein *et al.*, 1976; Alper *et al.*, 2002).

A 27-base-pair deletion in the *AE1* gene (known as Band3 $\Delta$ 27), has been shown to cause a condition known as South-East Asian ovalocytosis (SAO), characterized by slightly oval or elliptical shaped RBCs, defective anion transport activity, increased acidosis and haemolytic anaemia (Schofield *et al.*, 1992). Whereas the homozygous state is lethal, population-based studies have shown that the heterozygous state confers protection against cerebral malaria (CM), a condition characterized by progressing coma, unconsciousness, multiple convulsions and, often, death (Rasti *et al.*, 2004; Genton *et al.*, 1995; Allen *et al.*, 1999). Again, the mechanism of protection is unknown but several hypothesis have arisen, such as membrane alterations in SAO RBCs affecting parasite invasion (Kidson *et al.*, 1981) and involvement of Band 3 protein in iRBC cytoadherence, a process described below (Winograd and Sherman, 1989; Winograd *et al.*, 2005; Shimizu *et al.*, 2005).

*PK deficiency.* RBCs do not have mitochondria and are, therefore, dependent on glycolysis for energy. Pyruvate kinase (PK) is a key enzyme in glucose metabolism, catalysing the conversion of phosphoenolpyruvate into pyruvate, with simultaneous production of ATP. Thus, PK-deficient RBCs have impaired glycolysis and difficulty in maintaining normal levels of ATP and NAD. The degree of severity of PK-deficiency depends on the mutation(s) in one or both genes coding for PK in the human genome (Min-Oo and Gros, 2005). No formal evidence exists of a protective effect of PK deficiency against malaria in humans (Min-Oo and Gros, 2005). However, such a correlation is likely to exist, as suggested by recent studies carried out in mouse models (Min-Oo *et al.*, 2003, 2004).

### 3.3 Cytoadherence and sequestration – selective pressures and therapeutic value

*P. falciparum* is the most virulent of all four *Plasmodium* parasites that infect humans. Two features are strongly suggested to contribute decisively to the outstanding pathogenicity of *P. falciparum*: its remarkable potential to multiply to high parasite burdens and its unique ability to cause iRBCs to adhere to the linings of small blood vessels. The latter process is termed cytoadherence and the ensuing sequestration of iRBCs is frequently suggested to be a key feature in the pathogenesis of SM (see Ho and White, 1999; Kyes *et al.*, 2001; Miller *et al.*, 2002 for reviews).

The most widely suggested justification for sequestration in *P. falciparum* malaria is that adhesion of iRBCs to the endothelium allows the parasite to escape peripheral circulation and be cleared by the spleen. Another *Plasmodium* survival advantage is that sequestration in the deep tissue microvasculature provides the parasites with a microaerophilic venous environment that promotes maturation and faster asexual replication (Cranston *et al.*, 1984). Alternative, but not necessarily exclusive, explanations include sheltering of the iRBCs against destruction by the immune system of the host, enhanced survival of the gametocyte and immunomodulation by inhibition of the maturation and activation of DC (reviewed in Sherman *et al.*, 2003).

It has been suggested that parasite accumulation in specific organs is an important factor in malaria pathogenesis. Indeed, post-mortem examinations of people who have died from *P. falciparum* malaria show sequestered iRBCs within the small vessels of several tissues. The best-documented situation regards the sequestration of iRBCs in the endothelium of small vessels of the brain, which can lead to CM

(Kyes *et al.*, 2001; Rasti *et al.*, 2004). The proportion of iRBCs was higher in *P. falciparum* malaria patients dying with CM than in those that showed no CM symptoms (MacPherson *et al.*, 1985; Silamut *et al.*, 1999; Taylor *et al.*, 2004). The most prevalent hypothesis to explain the clinical manifestations of CM seems to be that sequestration obstructs blood flow, and, consequently, decreases the levels of oxygen and nutrients, whilst increasing the accumulation of waste products in the brain (Miller *et al.*, 1994). A complementary hypothesis is that an inflammatory response to the infection activates leukocytes and promotes their adhesion to receptors in the endothelium (Sun *et al.*, 2003). Another organ where sequestration seems to play a particularly important pathogenic role is the placenta. Maternal or placental malaria (PM) in *P. falciparum*-infected pregnant women is associated with disease and death of both mother and child. The characteristic feature of infection during pregnancy is the selective accumulation of iRBCs in the intervillous blood spaces of the placenta, leading to hypoxia, inflammatory reactions and intervillitis (reviewed in Beeson *et al.*, 2001; Andrews and Lanzer, 2002; Duffy and Fried, 2003).

Besides the cytoadherence of iRBCs to endothelial cells (sequestration), other distinctive patterns of iRBCs adherence can be distinguished at the cellular level. These involve different types of interactions between iRBCs and other host cells, such as RBCs (rosetting) (Udomsangpetch *et al.*, 1989; 1992), platelets ('platelet-mediated clumping', formerly known as autoagglutination) (Roberts *et al.*, 1992; Pain *et al.*, 2001a) or DC (Urban *et al.*, 1999, 2001). All these different types of interactions have been suggested to have implications in the course of infection as well as in disease severity (Udomsangpetch *et al.*, 1989; Urban *et al.*, 1999; Pain *et al.*, 2001a).

Over the last few years, significant progress has been made towards the identification of both parasite and host molecules that participate in these interactions. An array of host molecules have been identified that mediate cytoadherence of the iRBCs to various host cells. Among the most important ones identified so far are CD36 (Barnwell *et al.*, 1985), thrombospondin (TSP) (Roberts *et al.*, 1985), intercellular adhesion molecule-1 (ICAM-1) (Berendt *et al.*, 1989), vascular cell adhesion molecule-1 (VCAM-1) (Ockenhouse *et al.*, 1992), E-selectin (endothelial leukocyte adhesion molecule 1, ELAM-1) (Ockenhouse *et al.*, 1992), chondroitin sulphate A (CSA) (Rogerson *et al.*, 1995), platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Treutiger *et al.*, 1997), complement receptor 1 (CR1) (Rowe *et al.*, 1997), hyaluronic acid (HA) (Beeson *et al.*, 2002) and heparan sulphate (HS) (Vogt *et al.*, 2003). Nevertheless, the exact role of each of these molecules in pathogenesis remains largely unclear. Whilst some host ligands appear to be nearly ubiquitous, others seem to be organ- or cell-specific. This specificity has implications in terms of disease severity and adhesion mechanisms. Moreover, sequestration in an organ is likely to involve multiple receptors, and different combinations of specific receptors for adhesion may determine the site at which parasites adhere and accumulate (Beeson and Brown, 2002). Furthermore, receptors can act synergistically in mediating the adhesion of iRBCs (McCormick *et al.*, 1997; Yipp *et al.*, 2000; Heddini *et al.*, 2001b) and *in vivo* sequestration has been shown to involve a multi-step adhesive cascade of events where iRBCs initially roll along the endothelial surface and are subsequently arrested (Ho *et al.*, 2000).

**CD36.** CD36 (encoded by *CD36*), also known as GP88 or platelet glycoprotein IV, is an 88 kDa cell surface class B scavenger receptor and is expressed in endothelial cells,

monocytes, platelets and erythroblasts (Barnwell *et al.*, 1989; Ho and White, 1999). CD36 has been implicated in the interaction with a variety of natural ligands (reviewed in Ho and White, 1999; Serghides *et al.*, 2003) and it was identified *in vitro* as a receptor for iRBCs (Barnwell *et al.*, 1985, 1989). Since then, evidence for the involvement of CD36 in malarial cytoadherence became abundant and unequivocal, although its implications in disease severity are not always clear-cut. Whilst some reports describe a contribution of CD36 to malaria severity (Udomsangpetch *et al.*, 1992; Pain *et al.*, 2001a; Urban *et al.*, 2001; Prudhomme *et al.*, 1996), others suggest that it might be advantageous for host survival (Rogerson and Beeson, 1999; Serghides *et al.*, 2003; McGilvray *et al.*, 2000; Traore *et al.*, 2000). Several *CD36* polymorphisms have been identified. In Africa, the most common of these substitutions is a T1264G stop mutation in exon 10 (Aitman *et al.*, 2000). Studies of the effects of this polymorphism in disease severity have again yielded contradictory results and do not provide definitive answers regarding a possible selective pressure by malaria in endemic areas (Aitman *et al.*, 2000; Pain *et al.*, 2001b; Omi *et al.*, 2003).

The role of CD36 in the pathogenicity of malaria is still ambiguous. This has important implications in terms of the development of therapies or vaccines that target the interaction between PfEMP-1 and the CD36 receptor. The *in vitro*-based assumption that adherence to CD36 contributes to disease severity, and results in negative clinical outcomes, triggered research aimed towards interfering with this interaction that did not always yield agreeing results (Barnwell *et al.*, 1985; Baruch *et al.*, 1997; Cooke *et al.*, 1998; Yipp *et al.*, 2003). One way to try and circumvent this problem is by making use of appropriate animal, ideally rodent, models. The sequences of human and rat CD36 differ in a single amino acid (His242 in humans is Tyr242 in rat) and both are able to bind *P. falciparum*-infected RBCs (Serghides *et al.*, 1998). It has been shown that RBCs infected with the murine parasite *P. chabaudi chabaudi* AS adhere *in vitro* to purified CD36 and are sequestered from circulation in an organ-specific way *in vivo* (Mota *et al.*, 2000). Recently, a novel system that enables real-time *in vivo* imaging luciferase-expressing rodent malaria parasite *P. berghei* was used to monitor sequestration (Franke-Fayard *et al.*, 2005). Using CD36<sup>-/-</sup> mice, it was shown that nearly all detectable iRBC sequestration depends on CD36 and that murine CM pathology still develops in the absence of this receptor, implying that CD36-mediated sequestration in nonerythroid organs does not constitute the molecular basis of rodent CM. Despite the obvious advantages of using animal models to study sequestration, caution should be employed when extrapolating results to the *P. falciparum*/human situation.

*ICAM-1*. Intercellular adhesion molecule-1 (ICAM-1), encoded by *ICAM-1*, is a surface glycoprotein member of the immunoglobulin superfamily. ICAM-1, also known as CD54, plays a central role in immune response generation by functioning as an endothelial and immune-cell ligand for integrin-expressing leukocytes (reviewed in Ho and White, 1999; Kwiatkowski, 2005). ICAM-1 is widely distributed in endothelial cells including, unlike CD36, those of the brain microvascular system (Adams *et al.*, 2000). Also unlike CD36, the expression of *ICAM-1* can be up-regulated by a number of factors, most notably pro-inflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1) and interferon- $\gamma$  (IFN- $\gamma$ ) (reviewed in Dietrich, 2002), which seem to correlate with disease severity.

*P. falciparum* iRBCs have been shown to bind with different affinities to ICAM-1 *in vitro* (Berendt *et al.*, 1989). Unequivocal *in vivo* evidence of the involvement of ICAM-1 in sequestration, mainly in the brain, has been reported in both humans (Turner *et al.*, 1994) and mice models (Willimann *et al.*, 1995; Kaul *et al.*, 1998). The evidence gathered in these studies has led to the notion that ICAM-1 may play an important role in CM by either sequestering iRBCs or adhering to activated leukocytes. Furthermore, this receptor has also been shown to contribute to the cytoadherence of iRBCs within the intervillous spaces of the *P. falciparum*-infected placenta, supporting a possible role of ICAM-1 in PM (Sugiyama *et al.*, 2001), as previously suggested (Sartelet *et al.*, 2000). Moreover, ICAM-1 can synergize with CD36 to promote iRBC adhesion under flow conditions (McCormick *et al.*, 1997; Ho *et al.*, 2000; Yipp *et al.*, 2000). For these reasons, it is generally accepted that ICAM-1 plays a role in iRBC sequestration and in the severity of disease.

A high-frequency coding polymorphism in the *ICAM-1* gene of individuals from Kilifi (Kenya), an area of high malaria endemicity, has been identified (Fernandez-Reyes *et al.*, 1997). The mutant protein, termed ICAM-1<sup>Kilifi</sup>, contains a lysine → methionine replacement at position 29 (Fernandez-Reyes *et al.*, 1997). Intuitively, one would be led to think that the prevalence of this polymorphism in malaria-endemic regions would be associated with protection against severe forms of disease. However, studies attempting to correlate this polymorphism with malaria severity, yielded surprising and contradictory results (Fernandez-Reyes *et al.*, 1997; Kun *et al.*, 1999; Bellamy *et al.*, 1998; Ohashi *et al.*, 2001; Amodu *et al.*, 2005). The obvious difficulties that arise when attempting to reconcile those results constitute an illustration of the extreme complexity of malaria pathogenesis and suggest that different biological selective forces might simultaneously be at play in the studied populations (Craig *et al.*, 2000). In a very recent report, an ICAM-1 exon 6 polymorphism (lysine → glutamate replacement at position 469 in the protein) was seen to positively correlate with an increased risk of SM (Amodu *et al.*, 2005).

*PECAM-1/CD31*. Platelet/endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31), encoded by *PECAM-1*, is a highly glycosylated transmembrane glycoprotein of the immunoglobulin superfamily (Newman *et al.*, 1990). It is expressed on endothelial cells and platelets, as well as on granulocytes, monocytes, neutrophils and naïve T lymphocytes (Newman *et al.*, 1990; Mannel and Grau, 1997).

The role of PECAM-1 as an endothelial receptor for adherence of *P. falciparum*-infected RBCs was demonstrated *in vitro* by Treutiger *et al.* (1997). These authors further demonstrated that binding could be blocked by monoclonal antibodies (mAbs) specific for the N-terminus of PECAM-1 whereas it could be increased by IFN- $\gamma$ , a proinflammatory cytokine associated with the development of CM. In addition to serving as an endothelial receptor, the fact that PECAM-1 is expressed on platelets raises the possibility that it may also be involved in platelet adhesion of iRBCs, although no direct evidence exists for this. Platelets have been proposed as important effectors of neurovascular injury in CM, both in mice and in humans (reviewed in Mannel and Grau, 1997). Furthermore, the PECAM-1 receptor is quite commonly recognized by wild *P. falciparum* isolates (Heddini *et al.*, 2001a). In this particular study, over 50% of the fresh patient-isolates tested recognized PECAM-1, a number that is not matched by any other endothelial receptor, with the exception of CD36.

A functional mutation in codon 125 of the *PECAM-1* gene (leucine → valine replacement in the protein) was analysed in terms of its possible influence on malaria resistance (Casals-Pascual *et al.*, 2001). This study, carried out in Madang (Papua New Guinea) and Kilifi (Kenya) revealed no association between codon 125 polymorphism and disease severity in either of the populations. In another study, carried out in Thailand, a *PECAM-1* haplotype that is significantly associated with CM was reported (Kikuchi *et al.*, 2001).

It seems clear that our knowledge of the role of PECAM-1 in the pathogenicity of malaria is, at present, quite limited. This may be partly explained by the fact that, despite being an apparently high affinity receptor for iRBCs, PECAM-1 has been identified as such more recently than, for example, CD36 and ICAM-1. The use of appropriate animal models and larger-scale assessment of human *PECAM-1* polymorphisms would be a welcome source of potentially valuable information concerning this molecule.

*CR1*. Complement receptor 1 (CR1; C3b/C4b receptor; CD35), encoded by *CR1*, is a glycoprotein expressed on the surface of human RBCs and leukocytes (see Moulds *et al.*, 1991). CR1 was first implicated in malaria when its involvement in rosette formation, a phenotype associated with disease severity, was shown (Rowe *et al.*, 1997). CR1-dependent rosette formation is common in *P. falciparum* field isolates and the region of CR1 involved in rosetting was mapped (Rowe *et al.*, 2000).

The common blood group antigens, Kn(a)/Kn(b) (Knops); McC(a)/McC(b) (McCoy); Sl(a)/Vil (Swain-Langley/Villien) (now known as Sl:1/Sl:2); and Yk(a) (York) have been shown to be expressed on CR1 and, thus, correspond to alleles that encode polymorphisms in this receptor (Moulds *et al.*, 1991, 2000, 2001). The null phenotypes for the CR1-related blood group (also known as the Knops blood group) antigens are associated with the expression of a low number of CR1 molecules on the erythrocytic surface (Moulds *et al.*, 1991). The Sl(a<sup>-</sup>) and the McC(b<sup>+</sup>) phenotypes had a significantly higher prevalence in a Malian population when compared to other African and European-American populations (Moulds *et al.*, 2000). However, no correlation was found between either the Sl(a<sup>-</sup>) or the McC(b<sup>+</sup>) phenotypes (or, in fact, the Sl:2/McC(b<sup>+</sup>) allele) and protection against malaria in The Gambia (Zimmerman *et al.*, 2003).

CR1 is also polymorphic with respect to molecular weight (Wilson and Pearson, 1986; Wong *et al.*, 1989). A *HindIII*-RFLP (restriction fragment length polymorphism), was identified and shown to correlate with differential quantitative expression of CR1 on RBCs in Caucasian populations (Wong *et al.*, 1989). Homozygotes for a 7.4 kilobase (kb) *HindIII* genomic fragment (the H allele) have high RBC CR1 density, whereas homozygotes for a 6.9 kb *HindIII* genomic fragment (the L allele) have low CR1 expression, with HL heterozygotes having intermediate CR1 levels (Wong *et al.*, 1989). Surprisingly, in a Thai population, malaria severity was most prevalent in individuals homozygous for the L allele, compared with heterozygous individuals and individuals homozygous for the H allele (Nagayasu *et al.*, 2001). However, a recent study has found no correlation between the level of erythrocytic CR1 and the H and L alleles in an African population (Rowe *et al.*, 2002), raising doubts about the *HindIII*-RFLP importance in determining CR1 density on RBCs. To add to the confusion, a recent report in Papua New Guinea showed that HH individuals were at most risk of

developing SM whereas HL individuals were significantly protected and LL individuals showed statistically insignificant, albeit reduced, odds (Cockburn *et al.*, 2004).

It is clear that further studies are needed in order to fully understand the actual role of CR1-mediated rosetting in determining malaria severity and in establishing a clear correlation between CR1 polymorphisms and protection against severe disease. Studies involving murine models are unlikely to provide any further insights into this issue. In fact, although mouse leukocytes have been shown to contain CR1, it has been shown that mouse RBCs are CR1-negative, in contrast to human RBCs (Rabellino *et al.*, 1978; Kinoshita *et al.*, 1988). Moreover, recent work carried out with the rodent malaria laboratory model *P. chabaudi* showed that rosetting does occur in mice but suggested that the molecules involved may differ from those in human-infecting parasite species (Mackinnon *et al.*, 2002).

The results obtained when studying adhesion molecule polymorphisms in relation to malaria severity were, in all cases described, ambiguous, if not contradictory. This raises intriguing questions regarding *Plasmodium*-driven selection as well as important issues of therapeutic relevance.

### 3.4 Host's immune response

Any infectious disease is characterized, at the host level, by a complex reaction from the host immune system. The counteraction of host invasion by replicating pathogens demands a rapid response, generally provided by components of the innate immune system, which develops promptly and precedes the time-consuming clonal expansion of antigen-specific lymphocytes (Ismail *et al.*, 2002). Recent research has collected evidence of the importance of innate immunity in shaping the subsequent adaptive immune response to malaria blood-stage infection. It is now known that during blood-stage infection there is a 'cross-talk' between the parasite and cells of the innate immune system, such as DC, monocytes/macrophages, natural killer (NK) cells, NKT cells, and  $\gamma\delta$  T cells. The activity of NK cells was found to be high in uncomplicated cases of malaria while in patients suffering CM there was a profound depression of NK activity (Stach *et al.*, 1986). Murine malaria studies also presented evidence that NK cells play a role in providing protection against the early stages of *P. berghei* or *P. chabaudi* infections (Solomon *et al.*, 1985; Mohan *et al.*, 1997). Recently, using the murine model *P. berghei* ANKA, it was shown that the natural killer complex (NKC) regulates CM, pulmonary edema and severe anaemia, and influences acquired immune responses to infection (Hansen *et al.*, 2005).

Although several recent studies have partially elucidated the role of NK cells during malaria infection (Orago and Facer, 1991; Artavanis-Tsakonas *et al.*, 2003; Artavanis-Tsakonas and Riley, 2002; Baratin *et al.*, 2005), the ligands and receptors responsible for NK-cell activation are still unknown. However, there are some data suggesting an association between NK cell reactivity to *P. falciparum*-infected RBCs and killer Ig-like receptors (KIR) genotype (Artavanis-Tsakonas *et al.*, 2003). This observation raises the fascinating possibility that genetic variation at the *KIR* locus might explain heterogeneity of human NK cell responses to parasitized iRBCs and that the parasite might express ligands to inhibit or activate KIR (Stevenson and Riley, 2004). Nonetheless, these findings highlight the need for large-scale population-based

studies in order to address associations between KIR genotype, NK responses and susceptibility to malaria.

The host's immune response to a malaria infection involves not only molecules from the rapid innate immune response but also molecules from a more specific immune response.

*IL4*. Interleukin-4 (IL4) is a cytokine that regulates the differentiation of precursor T helper cells into the T<sub>H</sub>2 subset, which enhance the antigen-presenting capacity of B cells and specific antibody production (Romagnani, 1995). IL4 serves as an important regulator in isotype switching from IgM/IgG to IgE. Several studies have pointed towards IL-4 as an important factor in malaria resistance. A causal relationship between the activation of IL4-producing T-cell subsets and production of the anti-Pf155/RESA-specific antibodies in individuals with immunity induced by a natural malaria infection has been established (Troye-Blomberg *et al.*, 1990). More recently, it was shown that in the Fulani (Burkina Faso), known for having a lower susceptibility to *P. falciparum* infection than their neighbours, the IL4-524T allele (SNP C → T transition at position -524T from the transcription initiation site) was associated with high IgG levels against malaria antigens (Luoni *et al.*, 2001). However, others did not find this association (Verra *et al.*, 2004).

The possible association between three polymorphisms in the IL4 gene with SM in Ghanaian children has been addressed (Gyan *et al.*, 2004). One of these polymorphisms is located in the repeat region (intron3) of the *IL4* gene while the other two are in the promoter region (IL4+33T, SNP at position +33 relative to the transcription initiation site and the previously mentioned IL4-589T). A significantly higher frequency for +33 and -589 loci (IL4+33T/-589T allele) was observed in patients with CM and this was associated with elevated levels of total IgE.

Studies using the murine *P. chabaudi* and *P. berghei* models and IL4-deficient mice failed to show any role for this molecule in parasitaemia control or resistance to infection (von der Weid *et al.*, 1994; Saefel *et al.*, 2004).

*CD40L*. CD40 ligand (CD40L) is a glycoprotein expressed in activated T cells. When it binds to CD40 in B cells it regulates their proliferation, antigen-presenting activity and IgG class switching (Durie *et al.*, 1994). CD40L is encoded by the gene *TNFSF5*. The study of CD40L-726 (C/T mutation in the promoter region) and CD40L+220 (C/T mutation in exon 1) polymorphisms association with resistance to SM in *P. falciparum* infections led to the observation that Gambian males with the CD40L-726C allele were protected from CM and severe anaemia (Sabeti *et al.*, 2002). This observation provides evidence to implicate CD40L as a factor in immunity or pathogenesis of malaria infections. The role of CD40L in the course of malaria infections was also explored in *P. berghei* infection, a mouse model of SM (Piguet *et al.*, 2001). CD40L-deficient mice are protected from CM, which seems to occur because the CD40/CD40L system is involved in the breakdown of the blood-brain barrier, macrophage sequestration and platelet consumption.

*Fc receptors*. The Fc receptors (FcRs) are a family of cell-surface molecules that bind the Fc portion of immunoglobulins. FcRs are widely distributed on cells of the immune system and establish a crucial connection between the humoral and the cellular immune

responses (Ravetch and Kinet, 1991). There are three families of Fc $\gamma$ R (Fc $\gamma$ RI, Fc $\gamma$ RII and Fc $\gamma$ RIII) that contain multiple distinct genes and alternative splicing forms. Few studies have focused on polymorphisms in different Fc $\gamma$ Rs. The Fc $\gamma$ RIIA presents a polymorphism at position 131 that consists of a single histidine  $\rightarrow$  arginine amino acid substitution (Fc $\gamma$ RIIA-131H/R). It was shown that infants with the Fc $\gamma$ RIIA-R/R genotype were significantly less likely to be at risk from high-density *P. falciparum* infection, compared with infants with the Fc $\gamma$ RIIA-H/R. A later study focused on this same polymorphism and on another receptor polymorphism, Fc $\gamma$ RIIB-NA1/NA2, known to influence the phagocytic capacity of neutrophils (Omi *et al.*, 2002). This work, performed in northwest Thailand, has shown that malaria severity in this area is not associated with the Fc $\gamma$ RIIA-131H/R or the Fc $\gamma$ RIIB-NA1/NA2 polymorphisms individually. However, the Fc $\gamma$ RIIB-NA2 allele together with the genotype Fc $\gamma$ RIIA-131H/H was shown to be associated with susceptibility to CM. It has been also shown that in West Africa the polymorphism Fc $\gamma$ RIIA-131H/R is related to disease severity since the Fc $\gamma$ RIIA-131H/H genotype is significantly associated with increased susceptibility to SM (Cooke *et al.*, 2003).

The importance of Fc receptors in a malaria infection was addressed using transgenic mice in two different studies using *P. yoelii yoelii* and *P. berghei* XAT rodent models that showed that host resistance is mediated by antibodies (Rotman *et al.*, 1998; Yoneto *et al.*, 2001). Overall, the different studies indicate that Fc receptors have an important role in malaria infections.

**TNF- $\alpha$ .** Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a pro-inflammatory cytokine, produced by macrophages, NK cells and T cells, involved in local inflammation and endothelial activation (Janeway, 2001). This cytokine plays a pivotal role in the modulation of immune functions to infection and it was shown to limit the spread of pathogens (reviewed in Beutler and Grau, 1993). In a malaria infection TNF- $\alpha$  production by monocytes/macrophages mainly occurs during the blood stage when RBCs rupture and the schizonts, together with parasite-soluble antigens, are released into the bloodstream (Bate *et al.*, 1988; Kwiatkowski *et al.*, 1989; Taverne *et al.*, 1990a, 1990b; Pichyangkul *et al.*, 1994; Kwiatkowski, 1995).

TNF- $\alpha$ 's role in malaria has been extensively studied and both a protective and a pathogenic role for this cytokine have been observed during infection. TNF- $\alpha$ 's dual actions are related to its ability to activate endothelial cells, leading to the release of pro-inflammatory cytokines (Poher and Cotran, 1990), to the impairment of the blood-brain barrier (Sharief *et al.*, 1992; Sharief and Thompson, 1992) and to the up-regulation of adhesion molecules (reviewed in Meager, 1999). Of all up-regulated adhesion molecules, TNF- $\alpha$ 's interplay with ICAM-1 seems to be extremely important to malaria pathogenesis (Dietrich, 2002; reviewed in Gimenez *et al.*, 2003). TNF- $\alpha$  also induces nitric oxide (NO) release by endothelial cells (Lamas *et al.*, 1991; Rockett *et al.*, 1992) which seems to play an important role during malaria infections (see NO section below). TNF- $\alpha$  was shown to play a beneficial role by inhibiting *P. falciparum* growth *in vitro* (Haidaris *et al.*, 1983; Rockett *et al.*, 1988). However, this effect was not observed using recombinant TNF (Taverne *et al.*, 1987). TNF was also reported to inhibit infection in murine malaria *in vivo* (Clark *et al.*, 1987; Taverne *et al.*, 1987). These and other later studies suggest that TNF is important in the control of malarial parasites (Ferrante *et al.*, 1990; Kumaratilake *et al.*, 1997). Nevertheless,

TNF- $\alpha$  has also an important role in SM pathogenesis (reviewed in Richards, 1997; Mazier *et al.*, 2000; Odeh, 2001; Gimenez *et al.*, 2003; Clark *et al.*, 2004). High TNF- $\alpha$  levels and malaria severity have been associated in several clinical studies (Grau *et al.*, 1989b; Kwiatkowski *et al.*, 1990; el-Nashar *et al.*, 2002; Esamai *et al.*, 2003) and the ratio of the antagonistic cytokines TNF- $\alpha$  and Interleukin-10 (IL-10) has been shown to be important to the outcome of malaria infection (May *et al.*, 2000) (see IL-10 section). It was shown that the balance between the protective and pathological actions of TNF- $\alpha$  depends on several factors, namely the amount, timing, and duration of TNF- $\alpha$  production, as well as the organ-specific site of synthesis (Beutler and Cerami, 1988; Jacobs *et al.*, 1996b).

TNF- $\alpha$ 's unquestionable role in malaria pathogenesis has led to research focused on *TNF* polymorphisms and their association with malaria severity. Several polymorphisms in the *TNF* gene, with special emphasis for 2 promoter polymorphism, the TNF-308 and TNF-238, located -308 and -238 nucleotides relative to the gene transcriptional start, have been shown to be important for infection severity (McGuire *et al.*, 1994; Wattavidanage *et al.*, 1999; Aidoo *et al.*, 2001; Wilson *et al.*, 1997; Abraham and Kroeger, 1999; McGuire *et al.*, 1999; Knight *et al.*, 1999; Meyer *et al.*, 2002; Mombo *et al.*, 2003; Flori *et al.*, 2003). While many studies suggest a positive association between the mentioned TNF- $\alpha$  polymorphisms and malaria severity, other studies have failed to find these associations (Ubalee *et al.*, 2001; Stirnadel *et al.*, 1999; Bayley *et al.*, 2004). However, most evidence supports the idea that TNF polymorphisms may be part of the genetic determinants for human malaria resistance/susceptibility. More studies and functional analysis need to be carried out in order to understand the mechanisms involved. Nevertheless the TNF polymorphisms studies performed to date have provided more biological evidence for the role of TNF- $\alpha$  in human malaria.

A different set of studies, following a more therapeutic approach, has assessed the clinical outcome of SM using antibodies to neutralize TNF- $\alpha$  levels (Kwiatkowski *et al.*, 1993; van Hensbroek *et al.*, 1996; Looareesuwan *et al.*, 1999; Jacobs *et al.*, 1996b; Hermsen *et al.*, 1997; Grau *et al.*, 1987) and pentoxifylline to inhibit TNF- $\alpha$  (Sullivan *et al.*, 1988; Strieter *et al.*, 1988; Kremsner *et al.*, 1991; Stoltenburg-Didinger *et al.*, 1993; Di Perri *et al.*, 1995; Hemmer *et al.*, 1997; Looareesuwan *et al.*, 1998; Das *et al.*, 2003) both in human infections and rodent models of infection. A recent report explores a new way to treat CM by inhibiting TNF- $\alpha$  through the use of LMP-420, an anti-inflammatory drug (Wassmer *et al.*, 2005). Data from this report suggest that LMP-420, through the inhibition of endothelial activation, should be considered as a potential way to treat CM. However, the experimental results were based only on *in vitro* assays and further *in vivo* experiments are required to assess LMP-420 as a new therapeutic treatment.

While some observations suggest that TNF- $\alpha$  inhibition therapies would be of great value for malaria pathology control, others show that they may not hold any benefit. This lack of consensus may reflect the dual action that TNF- $\alpha$  seems to have during malaria infection.

*IFN- $\gamma$* . Interferon- $\gamma$  (IFN- $\gamma$ ), mainly produced by NK cells and helper T cells is a pro-inflammatory cytokine involved in macrophage activation, Ig class switching, increased expression of MHC molecules and antigen processing components (Mohan *et al.*, 1997; Janeway *et al.*, 2001; Seixas *et al.*, 2002). IFN- $\gamma$  plays a central role in the

immune response to several infectious diseases (Pfefferkorn and Guyre, 1984; Suzuki *et al.*, 1988; Rossi-Bergmann *et al.*, 1993; van den Broek *et al.*, 1995).

IFN- $\gamma$  has been shown to be produced during malaria infections (Brake *et al.*, 1988; Meding *et al.*, 1990; Waki *et al.*, 1992; Mohan *et al.*, 1997; Artavanis-Tsakonas *et al.*, 2003; Artavanis-Tsakonas and Riley, 2002; Hailu *et al.*, 2004), and both a protective and a pathological function have been reported. Concerning its protective effect, it was demonstrated that an early IFN- $\gamma$ -driven Th1-type response is required for an effective control of parasite multiplication in a primary blood-stage infection (Clark, 1987; Shear *et al.*, 1989; Stevenson *et al.*, 1990b; Taylor-Robinson, 1995; Favre *et al.*, 1997; Choudhury *et al.*, 2000; Kobayashi *et al.*, 2000). During infection, IFN- $\gamma$  activates monocytes/macrophages (Bate *et al.*, 1988) and neutrophils (Kumaratilake *et al.*, 1991), which were shown to be involved in the recognition and removal of either merozoites or iRBCs (Khusmith *et al.*, 1982; Kharazmi *et al.*, 1984; Kharazmi and Jepsen, 1984; Ockenhouse *et al.*, 1984; Bouharoun-Tayoun *et al.*, 1995). This protective effect is strongly supported by several studies with rodent models using exogenous IFN- $\gamma$  (Clark, 1987; Shear *et al.*, 1989) or an anti-IFN- $\gamma$  mouse antibody (Kobayashi *et al.*, 2000). Furthermore, others have shown a correlation between IFN- $\gamma$  production and a more positive outcome of malaria infections in both *P. chabaudi* and *P. yoelii* (Shear *et al.*, 1989; Stevenson *et al.*, 1990a). Endogenous IFN- $\gamma$  has also been shown to play a role in the development of protective immunity using IFN- $\gamma$  and IFN- $\gamma$  receptor-deficient mice (van der Heyde *et al.*, 1997; Favre *et al.*, 1997). Altogether, these studies with different mouse strains combined with different *Plasmodium* spp. support a beneficial role for IFN- $\gamma$ . Several human studies also point toward a protective role for IFN- $\gamma$  in a malaria infection (Luty *et al.*, 1999; Torre *et al.*, 2001, 2002).

Despite this, several studies have clearly linked IFN- $\gamma$  to the onset of pathology in mice as well as in humans. The detrimental effects of IFN- $\gamma$  are thought to be due to its ability to activate macrophages, which, in turn, produce TNF- $\alpha$ , IL-1 and IL-6, leading to activation of an inflammatory cascade (Kern *et al.*, 1989; Day *et al.*, 1999). Grau *et al.* (1989a) showed that treatment of *P. berghei* ANKA CBA infected mice with a neutralizing anti-IFN- $\gamma$  mouse antibody prevented development of CM and was associated with a significant decrease of TNF- $\alpha$  serum levels. This study supports the idea that there may be a fine balance between the levels of IFN- $\gamma$  and TNF- $\alpha$  and protective immunity or pathological consequences. Furthermore, a synergy between IFN- $\gamma$  and TNF- $\alpha$ , particularly with respect to the effects on endothelial cells, has also been observed (Poher *et al.*, 1986). Additional data correlating IFN- $\gamma$  production with the susceptibility to CM are provided by the observation that CM-susceptible mice exhibit a preferential expansion of Th1-like clones characterized by a marked production of IFN- $\gamma$  (de Kossodo and Grau, 1993). In addition, IFN- $\gamma$  receptor deficient mice are completely protected from CM (Rudin *et al.*, 1997; Amani *et al.*, 2000). IFN- $\gamma$  association with malaria pathology in humans is based on the fact that patients with acute *P. falciparum* malaria present high IFN- $\gamma$  serum levels (Ringwald *et al.*, 1991; Ho *et al.*, 1995; Wenisch *et al.*, 1995; Nagamine *et al.*, 2003) and individuals at risk for SM produce more IFN- $\gamma$  in an antigen-specific manner (Chizzolini *et al.*, 1990; Riley *et al.*, 1991).

IFN- $\gamma$  and its receptor are encoded by the *IFNG* and *IFNGR1* genes, respectively. It was shown that in the Mandika, the predominant Gambian ethnic group, those that are heterozygous for IFNGR1-56 polymorphism (SNP at position -56 (T $\rightarrow$ C) in the promoter region), presented a two-fold protection against CM and a four-fold

protection against death resulting from CM (Koch *et al.*, 2002). Later, it was shown that this polymorphic allele reduces *IFNGR1* gene expression (Juliger *et al.*, 2003).

Only recently has research focused on *IFNG* polymorphisms. SNPs in the region of *IFNG* and the neighbouring *IL22* and *IL26* genes were analysed but no evidence of a strong association between SM and *IFNG* markers was found (Koch *et al.*, 2005). Recently it was shown that in children from Mali the -183T allele is associated with a lower risk of CM (Cabantous *et al.*, 2005). This allele is the polymorphic form of the IFNG-183G/T (G replaced by a T) polymorphism located in the gene promoter region and has been shown to create an AP1-binding site for a nuclear transcription factor, leading to the increase of gene transcription (Bream *et al.*, 2002; Chevillard *et al.*, 2002). IFN- $\gamma$  clearly plays an important role during malaria infection, which can lead towards protection or pathology. The knowledge of IFN- $\gamma$  exact involvement in malaria is important for the understanding of infection progression and, consequently, for the assessment of possible therapies against malaria.

**NO.** Nitric oxide (NO), a labile and highly reactive gas, results from the oxidative deamination of L-arginine to produce L-citrulline through a reaction catalysed by the enzyme inducible nitric oxide synthase (iNOS) (Leone *et al.*, 1991). NO has been shown to inhibit the growth and function of diverse infectious agents, such as bacteria, fungi and protozoan parasites, by inactivating some of their critical metabolic pathways (reviewed in James, 1995). Increased NO production has been reported in murine (Taylor-Robinson *et al.*, 1993; Jacobs *et al.*, 1995) and human infections (Kremsner *et al.*, 1996). NO is produced by macrophages (Ahvazi *et al.*, 1995; Tachado *et al.*, 1996) but also by hepatocytes (Mellouk *et al.*, 1991; Nussler *et al.*, 1991) and endothelial cells (Oswald *et al.*, 1994; Tachado *et al.*, 1996). Cytokines such as IFN- $\gamma$  and TNF- $\alpha$  are critical for the regulation of NO production (Jacobs *et al.*, 1996a). Tachado *et al.* (1996) demonstrated that glycosylphosphatidylinositols (GPI) induce NO release in a time- and dose-dependent manner in macrophages and vascular endothelial cells, and regulate iNOS expression in macrophages. In addition, it was shown that another parasite metabolite, haemozoin, is able to increase IFN- $\gamma$ -dependent NO production by macrophages (Jaramillo *et al.*, 2003).

Both beneficial and pathological roles have been assigned to NO during malaria infection (Sobolewski *et al.*, 2005a). It is generally accepted that NO kills intraerythrocytic malarial parasites (Brunet, 2001; Clark and Cowden, 2003; Stevenson and Riley, 2004). However, it has been recently proposed that the blood stages are virtually immune to the cytotoxic effects of NO and other reactive oxygen species as a consequence of Hb NO scavenging and reactive oxygen species (ROS) suppression within RBCs (Sobolewski *et al.*, 2005b). Excess of NO can also contribute to malarial immunosuppression (Rockett *et al.*, 1994; Ahvazi *et al.*, 1995) as well as to CM development in *P. falciparum* infections (Clark *et al.*, 1991; Al Yaman *et al.*, 1996). It was proposed that overproduction of NO in the brain might affect local neuronal function by mimicking and exaggerating the physiological effects of endogenous NO (Clark and Cowden, 2003; Clark *et al.*, 1992). However, this hypothesis is not consensual as there is evidence suggesting that NO production may be limited in malaria due to the presence of hypoargininaemia in patients (Lopansri *et al.*, 2003). Furthermore, both plasma and urine NO<sub>x</sub> levels, as well as the iNOS protein and mRNA levels, correlate inversely with disease severity (Anstey *et al.*, 1996; Boutlis *et al.*, 2003; Perkins *et al.*, 1999; Chiwakata *et al.*, 2000).

A genomic approach, based on the association of *iNOS* gene polymorphisms with malaria infection has been applied to address NO role in malaria. Two SNPs, -954G → C and -1173 C → T, and a pentanucleotide (CCTTT) polymorphism have been studied in terms of malaria clinical outcome. However, reports show controversial data regarding the association of these *iNOS* gene promoter polymorphisms with severe *P. falciparum* malaria. Several reports show that NOS2A-954C allele is associated with protection from SM and resistance to reinfection both in Gabon and Uganda (Kun *et al.*, 1998, 2001; Parikh *et al.*, 2004). However, data from The Gambia, Tanzania and Ghana did not detect this association (Levesque *et al.*, 1999; Burgner *et al.*, 2003; Cramer *et al.*, 2004). A protective role for the *iNOS* -1173 C → T polymorphism has been shown in Tanzania (Hobbs *et al.*, 2002) but not in The Gambia and Ghana (Burgner *et al.*, 2003; Cramer *et al.*, 2004). Both these SNPs were shown to increase NO synthesis (Kun *et al.*, 2001; Hobbs *et al.*, 2002).

A pentanucleotide (CCTTT) microsatellite -2,5 kb of the transcription start site has also been studied. Short microsatellites (<11) have been shown to occur more frequently in fatal CM in The Gambia (Burgner *et al.*, 1998) while longer microsatellite alleles (≥13) were shown to be associated with SM in Thailand and Ghana (Ohashi *et al.*, 2002; Cramer *et al.*, 2004).

These contradictory results may be explained by the fact that the studies reported were carried out in different regions and again by the dual effect that host some molecules seem to have during malaria infection.

*IL-10*. Interleukin-10 (IL-10) is an anti-inflammatory cytokine produced by T cells and macrophages (Janeway *et al.*, 2001). Expression of IL-10 was shown to increase in response to high TNF- $\alpha$  levels and to down-regulate the production of the latter *in vivo* (Gerard *et al.*, 1993; Howard *et al.*, 1993), possibly representing an attempt of the host to counteract excessive activity of pro-inflammatory cytokines (van der Poll *et al.*, 1994).

Evidence for a beneficial role of IL-10 in malaria stems from an array of different population-based and animal model-based studies. IL-10-deficient mice infected with the rodent model parasite *P. chabaudi chabaudi* have been shown to have higher disease severity and mortality than their wild-type counterparts (Li *et al.*, 1999, 2003; Sanni *et al.*, 2004). The levels of both IL-10 and TNF- $\alpha$  are increased in patients with SM (Day *et al.*, 1999). Several studies have attempted to correlate the IL-10:TNF- $\alpha$  ratio with disease severity (Kurtzhals *et al.*, 1998; Day *et al.*, 1999; Kurtzhals *et al.*, 1999; May *et al.*, 2000; Nussenblatt *et al.*, 2001). The general consensus seems to be that ratios <1 constitute a risk factor for severe anaemia, suggesting that an imbalance between the anti- and the pro-inflammatory responses may constitute a determinant of mortality. Furthermore, a study involving the simian model parasites *P. cynomolgi* and *P. knowlesi*, a role for IL-10 in controlling anaemia during primary infection has been suggested (Praba-Egge *et al.*, 2002).

The available evidence of a protective role for IL-10 against malaria would suggest that strategies to increase the production of this cytokine might hold therapeutic value. However, this may be less simple than it appears. Studies with rodent models have shown that an early pro-inflammatory response may be required to enhance the mechanisms that are essential for elimination of the parasites (Shear *et al.*, 1989; Stevenson *et al.*, 1995; De Souza *et al.*, 1997). In fact, a positive correlation between the IL-10 levels and parasitaemia in *P. falciparum*-infected individuals before the start

of anti-malarial treatment has been found (Luty *et al.*, 2000). These results were extended in a recent study that showed a clear association between IL-10 levels and reduced parasite clearance ability in a Tanzanian population undergoing four different treatment regimens with distinct parasite clearance rates (Hugosson *et al.*, 2004). In another study using the rodent parasite model *P. yoelii yoelii*, it was found that mice injected with anti-IL-10 antibody had significantly prolonged survival, suggesting that IL-10 is associated with disease exacerbation rather than protection. Taken together, these observations indicate that enhancing the level of IL-10 might actually be beneficial for the parasite since it could interfere with production of pro-inflammatory cytokines and enable *Plasmodium* to escape effective killing (Hugosson *et al.*, 2004).

Genetic factors substantially influence production of cytokines and may account for as much as 75% of inter-individual differences in IL-10 production (Westendorp *et al.*, 1997). Furthermore, a clear association exists between a variety of IL-10 polymorphisms and susceptibility to several inflammatory diseases, such as rheumatoid arthritis (Eskdale *et al.*, 1998) and systemic lupus erythematosus (Gibson *et al.*, 2001). Thus, the possible correlation between five IL-10 polymorphisms (defining six haplotypes) and malaria severity in a population in The Gambia was investigated (Wilson *et al.*, 2005). One of these haplotypes, termed HAP3, defined by three SNPs at positions +4949, -1117 and -3585, showed a significant association with protection against CM and severe anaemia. However, this case-control association between malaria and HAP3 was not confirmed by a transmission disequilibrium test analysis of the same population. No evidence was found of a correlation between disease severity and any of the individual polymorphisms under study, suggesting that resistance either depends on other SNPs not addressed in this study or on specific combinations of *IL10* polymorphisms (Wilson *et al.*, 2005).

It seems clear that a certain level of IL-10 is required to balance the effect of pro-inflammatory cytokines. Still, an early pro-inflammatory response may be required to enhance the mechanisms that are essential for elimination of the parasites. The balance between pro- and anti-inflammatory cytokines during a malaria infection is clearly difficult to ascertain and has important consequences in terms of disease progression. If it is true that fine tuning the inflammatory responses during disease appears as an attractive way to improve its outcome, it is nonetheless obvious that our current knowledge of the complex immunological response that takes place during infection needs to be improved before this can be done in a manner that ensures a promising outcome.

#### **4 Final remarks: exploring the host potential in the ‘post-omics era’**

The contact between *Plasmodium* and its mammalian host involves a number of interactions that result in a series of different scenarios that range from complete or partial protection to infection and/or disease to SM leading to death. Both sides of this relationship are quite variable and many different possibilities of interaction may occur in the same population. Still, the survival of the *Plasmodium* parasite depends not only on its interaction with host molecules but also on efficiently coping with the host’s immune responses. Thus, not only host-driven genetic selection acts on malaria parasite populations, but also *Plasmodium* is likely to exert evolutionary pressure on

human gene frequencies. In malaria endemic regions, we might guess that infection contributes positively to the allele frequency of variants associated with protection. The gene conferring the Duffy blood group is one of the most striking examples of this selection pressure; people of this blood type are completely resistant to *P. vivax* blood-stage infection, as they lack a RBC receptor required for *P. vivax* invasion (Hamblin and Di Rienzo, 2000; Chitnis and Miller, 1994). Another striking example is the sickle cell anaemia allele. While severely deleterious in the homozygous state, this allele is associated with malaria protection in the heterozygous state, although the protection mechanisms involved are not fully understood (Aidoo *et al.*, 2002). Importantly, as one might expect, host components involved in the symptomatic phase of infection (blood stage) do not seem to be the only ones implicated in determining the severity of malaria infection. In fact, some alleles on the MHC Class I B53 loci are associated with protective clinical responses in African populations, due to interactions occurring during the silent liver stage of infection (Hill *et al.*, 1991). Presumably this association has contributed to the high frequency of MHC-B53 observed in populations living in areas of high malaria endemicity. Moreover, one should keep in mind that the blood and liver stages of infection can coexist in populations living in endemic areas. Therefore, the final result observed in human populations should not be extrapolated without taking into account possible interactions or common pathways that may occur between the two infection stages.

The sequence of both mice (rodent models of malaria infection) and human genomes can prove to be very useful. Moreover, the use of high-throughput technologies to determine the host molecules altered during the course of an infection, already used for some systems with interesting results (Delahaye *et al.*, 2006), together with systems that allow functional genomics studies, such as RNA interference, will be powerful tools to determine the role of these host molecules during the course of malaria infections.

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