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DEVELOPMENT OF A RECOMBINANT PROTEIN VACCINE AGAINST *PLASMODIUM FALCIPARUM* MALARIA

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Abstract

Malaria, caused by *Plasmodium falciparum* is one of the world´s deadliest diseases, killing one to two million children, while another 500 million suffer from clinical attacks every year. Infections caused by *P. falciparum* and culminating in death or in asymptomatic parasitaemia along with the intervening spectrum of severe clinical disease result from the sequestration of infected erythrocytes (iRBCs) in the microvasculature of vital organs. Adhesion to erythrocytes and endothelial receptors is mediated predominantly by *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), while the variation exhibited by this molecule is also responsible for immune evasion. The iRBC's spectrum of interactions with endothelial receptors and erythrocytes is determined by Duffy-binding like (DBL1α) and cysteine-rich interdomain region (CIDR1α) domains, among others, harboured on the PfEMP1 molecule. DBL1α mediated binding to endothelial receptors and erythrocytes, the latter enabling rosetting, is a major virulence factor. Adult residents of endemic regions are semi-immune, wherein much of this protection is afforded by variant and cross-reactive immune responses to PfEMP1. Immunization with recombinant PfEMP1 domains is thus an attractive option for attenuating sequestration and thereby severity of disease.

PfEMP1 domains are notoriously difficult to express as recombinant proteins and thereby constitute a bottleneck for functional and vaccine studies. The parameters that have an impact on the quality and yield of DBL1α domains on recombinant expression in *E. coli* were studied. Induction of recombinant expression at late log phase of *E. coli* growth substantially increases the yield of soluble protein. Additionally, the correlation of sequence specific features to their likelihood for expression as soluble proteins on recombinant expression in *E. coli* was studied. It was experimentally shown that the CIDR and acidic terminal sequence domains are appropriate candidates for recombinant expression in *E. coli*, while the remaining domain types including the DBL domains, constitute a poor choice for obtaining soluble protein on recombinant expression in *E. coli*. These studies provide guidelines for assigning candidates for structural and functional studies to appropriate expression systems and thereby potentially expedite the development of a vaccine against *P. falciparum* malaria.

A prototype recombinant DBL1α vaccine against *P. falciparum* malaria was developed and characterized. It was shown that cross-reactive antibody responses, believed to be an effective counter to antigenic variation, are elicited to heterologous DBL1α variants on immunization with a single variant. Additionally, immunization with phylogenetically distant DBL1α variants, can elicit partial cross-protection in *in vivo* to challenge with parasite strains harbouring a distant variant. It was demonstrated that immunization with DBL1α in the context of its native domain structure, as provided by Semliki forest virus particles elicit antibody responses that are surface reactive, disrupt rosettes and attenuate sequestration in *in vivo*. The stage is thus set for DBL1α to enter clinical trials.

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To my charming and adorable women -

Charlotte Kalpana, Susanne, Poonam and Sudesh

LIST OF PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their roman numerals:

I.
Kirsten Flick, **Sanjay Ahuja**, Arnaud Chene, Maria Teresa Bejarano, Qijun Chen: *Optimized expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli*. Malaria Journal 2004, 3:50-57.

II.
Sanjay Ahuja, Satpal Ahuja, Qijun Chen, Mats Wahlgren: *Prediction of solubility on recombinant expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli*. Submitted manuscript

III.
Sanjay Ahuja, Fredrik Pettersson, Kirsten Moll, Cathrine Jonsson, Mats Wahlgren, Qijun Chen: *Induction of cross-reactive immune responses to NTS-DBL1α/x of PfEMP1 and in vivo protection on challenge with Plasmodium falciparum.* Vaccine 2006, in press.

IV.
Qijun Chen, Fredrik Pettersson, Anna M. Vogt, Berit Schmidt, **Sanjay Ahuja,** Peter Liljeström, Mats Wahlgren: *Immunization with PfEMP1-DBL1α generates antibodies that disrupt rosettes and protect against the sequestration of Plasmodium falciparum-infected erythrocytes.* Vaccine 2004, 22:2701-2712.

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ABBREVIATIONS

ATS	Acidic Terminal Sequence
AMA	Apical Membrane Antigen
BHK-21	Baby Hamster Kidney - 21
BSA	Bovine Serum Albumin
CV	Canonical Variable
CSA	Chondroitin Sulphate A
CSP	Circumsporozoite Protein
CIDR	Cysteine rich Interdomain Region
CR-1	Complement Receptor - 1
DBL	Duffy binding-like
DBP	Duffy-binding protein
EBA-175	Erythrocyte-binding antigen-175
Ebl	Erythrocyte-binding-like
EPI	Expanded Programme of Immunization
GAGs	Glycosaminoglycans
Gb1	Gb1-Domain of Protein G
GLURP	Glutamate Rich Protein
GST	Glutathione S-transferase
Hb	Haemoglobin
HS	Heparan Sulphate
Ht	Haematocrit
His-tag	Histidine Tag
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IB	Inclusion Bodies
ICAM-1	Intercellular Adhesion Molecule -1
IFN	Interferon
IL	Interleukin
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
KAHRP	Knob Associated Histidine Rich Protein
LB plates	Luria-Bertani plates
MACS	Magnet Assisted Cell Sorting
MCM	Malaria Culture Medium
MHC	Major Histocompatibility Complex
MBP	Maltose Binding Protein
MESA	Mature infected Erythrocyte Surface Antigen
MSP	Merozoite Surface Protein
NTS	N-terminal Sequence
NK cell	Natural Killer cell
HEPES	N-Cyclohexyl-2-aminoethanesulphonic Acid
NusA	N-utilizing Substance A
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PECAM-1 (CD31)	Platelet-Endothelial Cell Adhesion Molecule -1 (CD31)
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein-1
RESA	Ring-infected Erythrocyte Surface Antigen
SFV	Semliki Forest Virus

SARS	Severe Acute Respiratory Syndrome
SDS-PAGE	Sodium Dodecyl Sulphate polyacrylamaide gel electrophoresis
TCR	T - Cell Receptor
TSP	Thrombospondin
TRAP	Thrombospondin Related Anonymous Protein
TM region	Transmembrane Region
TBST	Tris Buffered Saline with Tween
TNF	Tumour Necrosis Factor
VSA	Variant Surface Antigen
VCAM-1	Vascular Cell Adhesion Molecule -1
ZZ	Z-Domain of Protein A

INTRODUCTION

Scenario

The infant lay on the hospital bed - unconscious, listless, surrounded by relatives mourning over the loss of another potential bread earner to the bite of the deadly mosquito. Everything from traditional concoctions to the highly proclaimed medications had failed to halt the steady progress of the parasite as it would claim the lives of another two million innocent souls caught in the vicious cycle of poverty, hunger and disease.

From times immemorial to modern days, malaria has continued unabated to trap its unfortunate victims into the vicious cycle of disease and death, a cycle very reminiscent of the natural cycle of life and death, albeit one that is far more short and sinister. For many, competing with an organism equally apt and adamant at survival as its host, the daily struggle for co-existence is a norm with tragic consequences. According to latest estimates, 2.2 billion people worldwide are exposed to *Plasmodium falciparum* malaria, resulting in about 500 million episodes yearly and thereby placing malaria as a major threat to global health and prosperity (Snow et *al*., 2005). The imbalance in the distribution of malaria has forced the affected parts of the world on to a downward spiral of poverty and poor public health, while others are experiencing economic growth and reasonably good health prospects for their inhabitants. In fact, malaria along with human immunodeficiency virus (HIV) infection and tuberculosis are the trio dictating the terms for vast populations inhabiting Africa and Asia. Seventy percent of the burden of malaria is borne by Sub-Saharan Africa although many more areas are under imminent threat as malaria is resurging in areas from which it was eradicated. Global changes in weather accompanied by changes in demographic structures, increasing resistance to prevalent drugs, a vaccine long promised but still due and outright public exhaustion are some factors compounding the scientific challenge of outsmarting an organism fully equipped

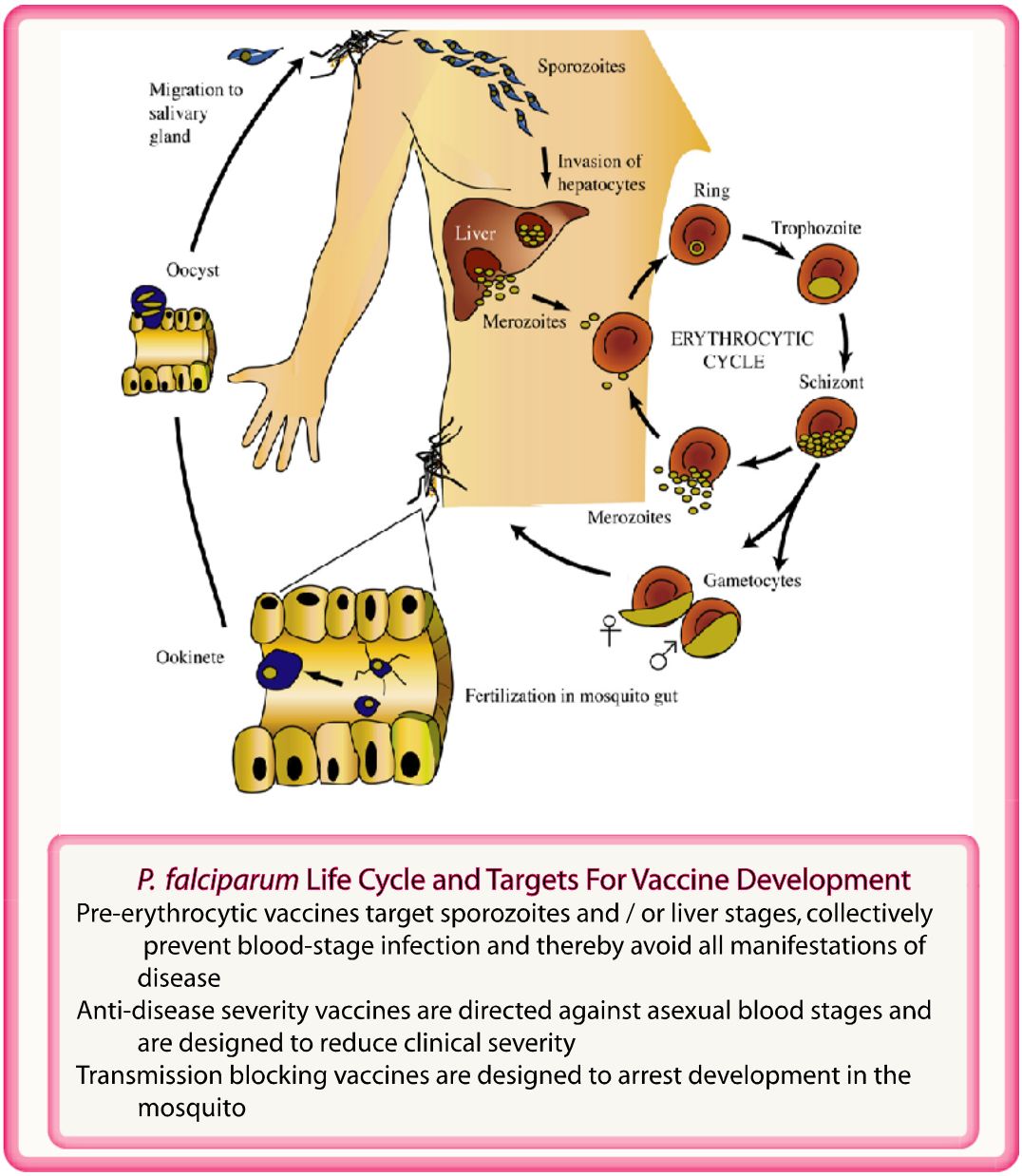
with nature's remarkable set of survival tools. Coupling together public health measures, educational programmes, surveillance, and distribution of bed nets and application of high technology products are for now mankind's best bet to eradicate or at least keep at bay the scourge by the name of malaria.

The Parasite

Members of the coccidian genus *Plasmodium*, totalling 172 species that infect birds, reptiles, and mammals, are protozoan parasites transmitted by the bite of an infected female *Anopheles* mosquito. Four species infect humans - *P. vivax*, *P. malariae*, *P. ovale,* and *P. falciparum.* Recent reports of *P. knowlesi* infecting humans have been made in South East Asia (Singh *et al*., 2004). These parasites can also infect some non-human primates (Garnham, 1966). Both *P. falciparum* and *P. vivax* can infect owl and squirrel monkeys and chimpanzees (Fandeur *et al*., 1995; Contamin *et al*., 2000; Daubersies *et al*., 2000; Sullivan *et al*., 2001). *P. vivax* is a major cause of clinical malaria but is rarely fatal. Absence of the Duffy antigen in African populations precludes the invasion of this particular specie into erythrocytes, thus restricting its occurrence to Asia and South America. *P. malariae* and *P. ovale* are an infrequent cause of clinical malaria, often persisting as low grade parasitaemia with other species and relapse many years after apparent cure. On account of its ability to invade a large proportion of erythrocytes including reticulocytes, utilization of multiple and redundant invasion pathways along with a multitude of virulence factors, *P. falciparum* accounts for the most severe and often potentially lethal forms of malaria. Chronic infections persisting over 2 - 3 years do occur but relapses are uncommon, since no dormant stages, viz. hypnozoites exist in liver.

Life Cycle

The female mosquito, driven by olfactory cues from host derived volatile compounds, homes on to a host and simultaneously injects saliva containing



P. falciparum Life Cycle and Targets For Vaccine Development

Pre-erythrocytic vaccines target sporozoites and / or liver stages, collectively prevent blood-stage infection and thereby avoid all manifestations of disease

Anti-disease severity vaccines are directed against asexual blood stages and are designed to reduce clinical severity

Transmission blocking vaccines are designed to arrest development in the mosquito

sporozoites into the subcutaneous tissue. The sporozoites fairly rapidly leave the site of the bite; penetrate a capillary and travel through the bloodstream to gain access to hepatocytes. Once inside the liver, the sporozoites, transverse a few hepatocytes in their path, fatally wounding them in the process, and finally settle into one of "their choice". They further continue to differentiate and divide into exoerythrocytic schizonts, each containing thousands of infectious merozoites. Six to 16 days after infection (dependent on species), the schizont ruptures releasing merozoites into the bloodstream. These merozoites invade

neighbouring or circulating erythrocytes by a receptor-ligand-mediated mechanism, where they differentiate, grow and multiply in a vacuole to yield more merozoites. This second round of multiplication lasts 48 - 72 hours (contingent on the species) and produces 16 - 32 merozoites per infected erythrocyte. Subsequent rupture, and merozoite release is associated with the characteristic fever and acute symptoms of malaria. The released merozoites further invade new erythrocytes, incessantly propagating the parasite cycle. This cycle of erythrocyte consumption, with its parasite induced sequestration of infected erythrocytes in capillaries within organs, causes the pathological hallmarks of a malaria infection.

A small proportion of merozoites, responding to diverse triggers (Billker *et al*., 1997), follow an alternate developmental pathway that yields a transmissible form, the gametocyte. These long lived, non-dividing parasites circulate in the bloodstream and await uptake by the mosquito vector during a blood meal. Once inside the mosquito's stomach, erythrocytes and asexual blood stage parasites perish, while the gametocytes responding to a drop in temperature and other mosquito associated factors undergo rapid transformation to yield male and female gametes. The ensuing sexual fertilization yields a zygote and then a motile ookinete that invades the mosquito gut wall to establish an oocyst attached to the outside of the gut. During a period of apparent latency lasting 1 - 2 weeks, approximately 10,000 sporozoites are formed in each oocyst. On rupture, these oocytes release the sporozoites into the body cavity of the mosquito, which in turn migrate to invade the salivary glands and are ready to be transmitted to another vertebrate host.

The initial sightings of malaria parasites, what we now know to be gametocytes of the malaria parasite *P. falciparum*, were made in the blood of a French soldier in Algeria in 1880 by Alphonse Laveran (Smith, 1985), and the mode of transmission *via* the mosquito vector was discovered by Ronald Ross while serving in India. The complete cycle was, however, only elucidated in

1948, when Shortt and Garnham (1951) described exoerythrocytic schizonts in liver of infected monkeys.

The Vector

There are approximately 3,500 species of mosquitoes grouped into 41 genera. Human malaria is transmitted only by females of the genus *Anopheles*. Of the approximately 430 *Anopheles* species, only 30 - 40 transmit malaria. These vectors differ in their biology and ecology, resulting in heterologous transmission, varied epidemiological patterns and capability to develop resistance to insecticides. The vectors go through four stages in their life cycle: egg, larva, pupa and the adult. The first 3 stages are aquatic and last 5 - 14 days, depending on the species and ambient temperature. The adult mosquitoes act as the vector and do not live more than 1 - 2 weeks in nature. Both the male and female mosquitoes feed on nectar, the females, however, require a blood meal for development of eggs. *P. falciparum* transmission in Africa is mainly due to *Anopheles gambiae* and *An. funestus*, both of which are anthropophilic and thus two of the most efficient vectors in the world.

As primary vectors, the above mentioned vectors have several common ecological attributes, which include, a wide geographical distribution, a high seasonal abundance, good colonizing ability, efficient adaptation to man made environments, a strong preference for human blood and a high susceptibility to acquire human pathogens (Donnelly *et al*., 2002). Some of the other vectors transmitting malaria are: *An. maculates, An. minimus, An. culicifacies* - in Asia, *An. punctulatus* - in Australia and Papua New Guinea, *An. darlingi, An. albimanus, An. albitarsis, An. pseudopunctipennis* - in Latin America. Several lines of evidence suggest that malaria parasites are under immunological surveillance in mosquitoes (Richman *et al*., 1997), resulting in refractory infection or considerable numerical loss during development inside the mosquito. Additionally, a majority of vectors harbour resistance genes rendering

them unsusceptible to infection (Riehle *et al*., 2006). Centuries of co-evolution have shaped and fine tuned interactions between the parasite and its vector and made them efficient co-partners in crime.

The Human Host

Genetic Resistance

As is the case for all host-pathogen interactions, genetic susceptibility to malaria in humans is complex and multigenic, with a variety of genetic polymorphisms determining the pathogenesis and host immune response to infection. The protective role attributed to polymorphisms that involve erythrocyte-specific structural proteins and enzymes are well documented. Haemoglobin S (Hb S), a structural variant of haemoglobin abundantly observed in the Sub Saharan Africa, protects heterozygous populations against clinical malaria, severe malaria including cerebral malaria and reduces mortality (Allison *et al*., 1961; Aidoo *et al*., 2002). It has been proposed that low oxygen tension as witnessed in heterozygous patients adversely affects parasite invasion, growth and rosetting (Carlson *et al*., 2004). Additionally, erythrocytes carrying the Hb S structural variant of haemoglobin are avidly phagocytosed than their normal counterparts carrying normal haemoglobin (Friedman *et al*., 1978; Ayi *et al., 2004*). Hb C is another structural variant seen frequently in West African populations (Allison *et al*., 1961). While *P. falciparum* grows normally in heterozygous individuals, populations carrying this abnormal variant are still protected against severe malaria to the extent of 30 % (Friedman *et al*., 1978; Modiano *et al*., 2001). Further investigations have confirmed that Hb C affects the display and levels of erythrocyte surface molecules involved in cytoadhesion of parasitized erythrocytes (Fairhurst *et al*., 2005).

Diverse mutations in α and β globin chains of Hb, commonly referred to as thalassaemias, reduce the risk of acquiring severe malaria and offer 50%

protection against hospital admission with malaria (Willcox *et al*., 1983; Allen *et al*., 1999; Mockenhaupt *et al*., 2004; Wambua *et al*., 2006)). Decreased invasion and parasite multiplication, increased expression of parasite derived surface molecules, increased binding to IgG, decreased rosette formation and enhanced phagocytosis have all been proposed as protective mechanisms, though the relative contribution of each varies for the two main types of thalassaemia (Yuthavong *et al*., 1988; Carlson *et al*., 1990a,b; Luzzi *et al*., 1991; Ayi *et al*., 2004).

Earlier ambiguity about the protective effect of glucose-6-phosphate dehydrogenase deficiency has now paved the way for more conclusive evidence that attributes 50% protection against severe malaria (Ruwende *et al*., 1995). The protective effect afforded by deficiencies in pyruvate kinase, an enzyme involved in glycolyis for provision of energy to mature erythrocytes, is presently under investigation, although, studies in mouse models hint that this indeed might be the case (Roth *et al*., 1988; Min-Oo *et al*., 2003). Additionally, mutations in erythrocyte membrane proteins such as erythrocyte band 3 protein, ankyrin, spectrin and glycophorin C have also been shown to confer protection against malaria (Genton *et al*., 1995; Allen *et al*., *1999*; Gallagher *et al*., 2004).

The blood group O has been shown to confer protection against cerebral malaria, in contrast to blood group A, the latter has been reported as a risk factor for severe malaria and cerebral coma (Fischer *et al*., 1998; Lell *et al*., 1999; Barragan *et al*., 2000). Certain human leukocyte antigen (HLA) haplotypes are associated with protection from severe malaria, as illustrated by the high occurrence of HLA-Bw53 and DRB1*1302-DQB1*0501 alleles in West Africa (Hill *et al*., 1991). Polymorphisms in molecules implicated in adhesion and its modulation along with ones involved in determining the innate immune response have all been shown to affect genetic susceptibility to malaria. Three different promoter polymorphisms for tumour necrosis factor α (TNFα) occurring in Gambia and Kenya appear to be independently associated with

severe malaria (Mc Guire *et al*., 1994; Knight *et al*., 1999; Mc Guire *et al*., 1999). Promoter polymorphisms in the gene encoding interleukin (IL) -12p40 has been associated with reduced levels of nitric oxide production and increased mortality from cerebral malaria in Tanzanian but not in Kenyan children (Morahan *et al*., 2002). Polymorphisms in chromosomal regions encoding for cytokines have been shown to affect initial parasitaemia (Garcia *et al*., 1998) and antibody levels (Luoni *et al*., 2001) on infection. Single nucleotide polymorphisms in the nitric oxide synthase promoter region have been associated with protection from severe malaria in Gabon; while contrasting results about increased susceptibility to fatal malaria have been reported from Gambia (Burgner *et al*., 1998; Kun *et al*., 1998a). Complement receptor deficiency, a trait highly prevalent in Southeast Asia and Papua New Guinea populations along with other polymorphisms of this gene encountered frequently in Africa have been associated with resistance to severe malaria (Cockburn *et al*., 2004; Thathy *et al*., 2005).

Immunity to Malaria

Transmission and Naturally Acquired Immunity

Endemic transmission of malaria takes place in most tropical latitudes and seasonally reaches into temporal zones. A clinical indicator of endemicity is the prevalence of splenomegaly in chronically infected 2 - 9 year old children. Hypoendemic, mesoendemic, hyperendemic and holoendemic areas are defined as areas with splenomegaly rates of ≤ 10 %, 11 – 50 %, > 51 % and > 75 %, respectively. Malaria epidemics, as defined by a sharp increase in the malaria transmission that exceeds the inter-seasonal variation normally experienced, tend to occur in areas with low endemicity.

Unlike many acute viral diseases, which produce long lasting immunity to re-infection, immunity to malaria is a function of age, transmission intensity and continuity in exposure to the parasite. Adult residents in malaria endemic areas

are semi-immune, implying that individuals harbour parasites without experiencing an acute illness (Daubersies *et al*., 1996). Their immunity albeit un-sterile is short lived and requires unbroken reinforcement through frequent re-infections. Infants under the age of six months rarely experience clinical episodes of malaria even in areas with intense transmission. As the level of passively acquired antibodies wane at around 6 - 9 months of age, infants in hyperendemic areas experience their first clinical episode (Bruce-Chwatt *et al*., 1952). For reasons presently unknown, often very few infections at any level of transmission suffice to render children immune to the most severe and lethal complications of malaria (Gupta *et al*., 1999). As exposure continues in an unabated manner, immunity is acquired slowly over time with frequent interruptions from parasite strains harbouring antigenic traits different from "in-house" strains previously circulating in blood (Bull *et al*., 1998; Contamin *et al*., 1996; Wahlgren *et al*., 1986; Barragan *et al*., 1998; Giha *et al*., 1999; Ofori *et al*., 2002). These aberrant strains take advantage of the gaps in acquired immunity and expand exponentially causing clinical disease. Immunity to severe malaria develops before protection against uncomplicated malaria, which in turn precedes development of protection against asymptomatic parasitaemia (Gupta *et al*., 1999; Bull *et al*., 2000; Nielsen *et al*., 2002).

In areas with high endemicity, infants and young children are especially prone to severe disease (Snow *et al*., 1997). As intensity of transmission decreases, this vulnerability extends to older children and young adults. Furthermore, with decrease in the intensity of transmission, the cumulative risk of experiencing severe episodes of disease during childhood and likelihood of fatal cerebral complications is increased (Imbert *et al*., 1997; Snow *et al*., 1998). In predictive models, increased antigenic diversity prolongs the time required to achieve immunity irrespective of intensity of transmission, while increased transmission intensity alone decreases the time required to attain immunity (Gatton and Cheng, 2004). Intriguingly, malaria naïve migrant populations

acquire clinical immunity on relatively brief exposure contradicting the concept of natural immunity as a cumulative product of exposure over time (Baird *et al*., 2003). In adults, resurgence in clinical disease occurs at the time of first pregnancy and subsides again with subsequent pregnancies (Brabin, 1983).

Humoral Immunity

Seminal observations on the protective effects of inoculations of purified IgG from adult Africans to African children and Thai adults indicated that humoral mechanisms are important in natural immunity against malaria (Cohen *et al*., 1961; Bouharoun-Tayoun *et al*., 1990). Although immunoglobulins from semi-immune donors can inhibit and reverse cytoadherance, no resolution in cerebral malaria has been observed on infusion with similar preparations (Taylor *et al*., 1992). Cytophilic IgG1 and IgG3 antibodies interacting with monocytes through Fc receptors have been associated with protection in humans (Wahlgren *et al*., 1983; Bouharoun-Tayoun *et al.*, 1990). Elevated anti-malarial IgE levels have been associated with reduced risk for subsequent clinical malaria in asymptomatic individuals (Berecky *et al*., 2004). While antibodies have not been directly implicated in hindering the development of parasites once access to the infected red blood cell is ensured, ample evidence points to the inhibitory role of antibodies on merozoite invasion, blockage of cytoadherance and rosette disruption (Udeinya *et al*., 1983; Wåhlin *et al*., 1984; Carlson *et al*., 1990b; Thomas *et al*., 1990). Although infection *per se* is not prevented, it is however curtailed by altered parasite number dynamics and diminished recruitment of additional erythrocytes for parasite propagation. Malaria infection in residents of endemic areas induces strong humoral responses, reflecting polyclonal B cell activation, wherein diverse fractions of the antibody response are directed against the different developmental stages of the parasite or against parasite encoded antigens translocated on to the surface of the infected erythrocytes. Antibodies opsonize asexual blood stages of the parasite without involvement of

complement and correlate with protection (Celada *et al*., 1983; Groux *et al*., 1990).

While accumulating evidence links antibody dependent immunity with immune responses directed towards particular malaria parasite antigens, only a few are mentioned here. Antibodies to variant surface antigens (VSA) develop after symptomatic malaria and are associated with protection from infection in adults (Wahlgren *et al*, 1986; Marsh *et al*., 1989; Barragan *et al*., 1998; Bull *et al*., 1998; Ofori *et al*., 2002). Antibodies to ring infected erythrocyte surface antigen (RESA), a molecule deposited in the erythrocyte membrane shortly after merozoite invasion, have been shown to inhibit *in vitro* growth of *P. falciparum* and significantly lower parasite densities in adult Liberians (Petersen *et al*., 1990). A high prevalence of stable antibody responses in all age groups against apical membrane antigen-1 (AMA-1) has been observed in holoendemic areas (Udhayakumar *et al*., 2001). Similarly, naturally acquired antibodies have been observed against merozoite surface protein-1 (MSP-1), but their correlation with development of protective immunity is debated (Terrientes *et al*., 1994). Cytophilic immunoglobulin responses against *P. falciparum* glutamate rich protein (GLURP) in cooperation with monocytes have been shown to inhibit *in vitro* growth of the parasite (Theisen *et al*., 1988). Anti-malarial antibodies also seem to play a supportive role in the therapeutic response to anti-malarial drugs during an acute episode of malaria (Mayxay *et al*., 2001; Pinder *et al*., 2006). Interestingly, concurrent HIV infection has been shown to curtail immune responses to AMA-1 and VSA and thereby increase susceptibility to malaria (Mount *et al*., 2004).

Perhaps the strongest evidence for the role of humoral immunity emanates from pregnant women. First time mothers are especially prone to malaria since no acquired immunity exists to certain parasite subsets, which are functionally affinity primed to cellular receptors in placental tissue (Fried and Duffy, 1996). During subsequent pregnancies, women are progressively protected from the

adverse consequences of placental malaria by antibody dependent immunity elucidated against these particular placental strains (Fried *et al*., 1998; Ricke *et al*., 2000; Staalsoe *et al*., 2004).

Cellular Immunity

The role of cell-mediated immunity has been exemplified by extensive studies in rodent models and from epidemiological, immunological and clinical studies of malaria in humans. Antibodies alone are capable of clearing most parasites from circulation, while complete eradication is CD4+ T cell- and B cell dependent (Hirunpetcharat *et al*., 1999). Conversely, CD4+ T cells can limit parasite growth in the absence of B cells (Grun and Weidanz, 1983). Parasite specific CD4+ T cells can adoptively transfer protection (Jayawardena *et al*., 1982; Brake *et al*., 1988). CD4+ T cells from naïve adults, when cultured *in vitro* with *P. falciparum* antigens proliferate and secrete cytokines. CD4+ T cells from malaria-immune humans also proliferate *in vitro* when stimulated with malarial antigens (Troye-Blomberg *et al*., 1994). Analysis of clones responsive to parasite antigens has shown that the CD4+ T cell response consists of two functionally distinct subsets (Taylor-Robinson and Phillips, 1992; Stevenson and Tam, 1993; Troye-Blomberg *et al*., 1994). The initial cellular response is typical of populations secreting cytokines associated with Th1 CD+4 cells ***i.e.*** IL-2, interferon-γ (IFN-γ) and TNF-β. Cell populations isolated during late infection following parasite clearance bear characteristics of Th2 CD+4 T cell subsets, secreting IL-4, IL-5, IL-6 and IL-10. The Natural Killer (NK) cells have also been shown to produce IFN-γ on induction with parasitized erythrocytes (Artavanis - Tsakonas *et al*., 2002). T cell receptor (TCR) β and δ chain knockout mice, lacking TCR αβ+ T cells and γδ T cells, respectively, are unable to eliminate *P. chabaudi* infection independent of each other (Langhorne *et al*., 1995).

Given the central role played by IFN-γ, its use as a correlate of protection is increasingly being investigated (Reece *et al*., 2004). Considering that erythrocytes do not express HLA class I molecules, the role of CD8+ T cells is restricted to protection at the pre-erythrocytic stage. CD8+ T cells have been shown to eliminate parasite-infected hepatocytes in *in vitro* cultures (Hoffman *et al*., 1989) and to adoptively transfer protection from irradiated sporozoite immunized mice to naïve ones (Romero *et al*., 1989).

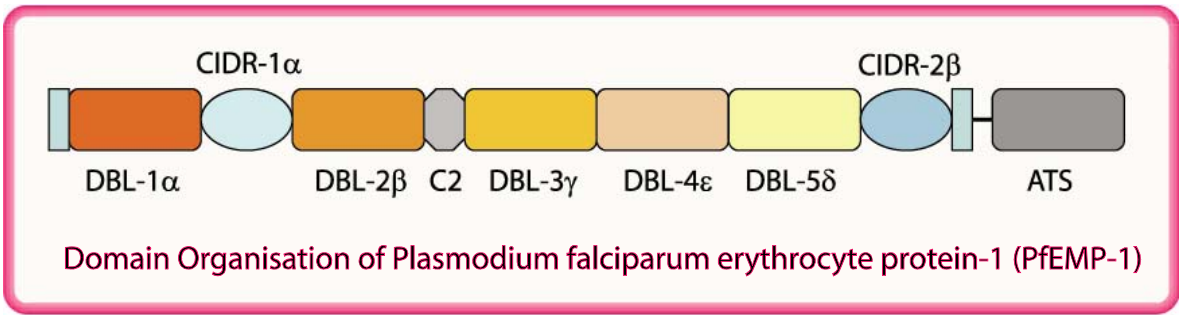
The role of dendritic cells is at the moment unclear. While earlier studies reported that maturation of dendritic cells was suppressed following exposure to *P. falciparum* or *P. yoelli* infected erythrocytes, later studies point towards their efficient role in activating naïve T cells and in particular γδ T cells to produce IFN-γ (Urban *et al.*, 1999; Seixas *et al*., 2001; Ocana-Morgner *et al*., 2003; Perry *et al*., 2004). Unmistakable evidence to the role of cell-mediated immunity in malaria, however, emanates from experiments in which non-immune volunteers were repeatedly challenged with ultra low doses of infected erythrocytes and subsequently developed immunity to further challenge without detectable antibody responses (Pombo *et al*., 2002).

PfEMP1 - a Balancing Act between Sequestration and Immune Evasion

PfEMP1 as an Adhesive Ligand

P. falciparum, as a major human pathogen, owes its success, partly if not predominantly, to the *Plasmodium falciparum* erythrocyte membrane protein-1(PfEMP1). PfEMP1, as a major virulent protein, is knitted intricately into the web of pathogenic events defining the clinical picture of malaria. PfEMP1 variants were originally identified by radiolabelling and immunoprecipitation of mature asexual stages and described as surface associated, variable, trypsin sensitive and Triton X-100 insoluble proteins (Howard *et al*., 1983; Leech *et al*., 1984). Earlier, erythrocytes infected with *P. falciparum* had been shown to develop knob like structures that supposedly facilitated their adherence to endothelium *in vivo* (Luse and Miller, 1971), to human umbilical vein endothelial cells and C32 amelanotic melanoma cells *in vitro* (Udeinya *et al*., 1981; Schmidt *et al*., 1982). PfEMP1 was presumed to be involved in these adhesive events (David *et al*., 1983; Udeinya *et al*., 1983, Magowan *et al*., 1988). Adhesive events eventually culminating in sequestration are brought about by proteins resident on the surface of *P. falciparum* infected erythrocytes, among which PfEMP1 is the most extensively studied protein.

PfEMP1 molecules harbour multiple domains designated as Duffy binding-like (DBL), cysteine rich interdomain region (CIDR) and C2 domains that provide a basis for multiadhesion and immune invasion through ligand and antigenic diversity (Baruch *et al*., 1995; Smith *et al*., 1995; Su *et al*., 1995; Chen *et al*., 2000). These sequence domains are classified by type-specific consensus motifs into seven types of DBL domains (α, α_1, β, γ, δ , ε and x) and four types of CIDR domains (α, α_1 , β and γ) (Kraemer *et al*., 2003).



The DBLα types bind to blood group A, heparan sulphate (HS) and complement receptor - 1(CR1), and all are implicated in rosetting (Rowe *et al*., 1997; Chen *et al*., 1998a; Vogt *et al*., 2003). Binding to CD36, a constitutive feature of field strains (Smith *et al*., 1999; Baruch *et al*., 1997) has been mapped to the CIDR1α domain of PfEMP1 (Baruch *et al*., 1996; Baruch *et al*., 1997), although the latter has been shown to bind additionally to CD31/PECAM-1 and IgM (Chen *et al*., 2000). The DBLβ-C2 tandem accounts for ICAM-1 binding (Smith *et al*., 2000a). DBL2x and DBL6ε are the ligand domains for chondroitin sulphate A (CSA), a receptor for human placental infections by *P. falciparum*, often leading to malaria complications associated with pregnancy (Gamain *et al*., 2005)

The variant DBL domains placed in tandem in PfEMP1, possess sequence features comparable to similarly named domains in the erythrocyte-binding-like (ebl) family of adhesion molecules, which include the *P. falciparum* erythrocyte-binding antigen-175 (EBA-175) and the *P. vivax* Duffy-binding protein (DBP) (Adams *et al*., 1990; Chitnis *et al*., 1994). The DBP has only a single copy of the DBL domain, whereas EBA-175 has two DBL domains that appear to function as a single ligand domain (Sim *et al*., 1994). These domains have evolved within the *Plasmodium* species to have specificity for different types of erythrocyte receptors while still maintaining homologous functions in the invasion process (Michon *et al*., 2002). PfEMP1 DBL domains, although

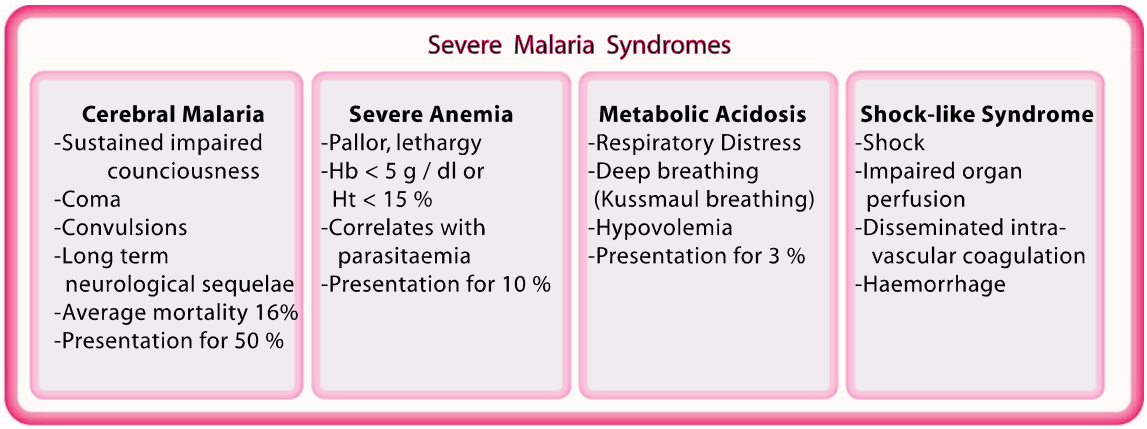
divergent in length and sequence, carry ten invariant cysteine residues distributed unevenly among ten semi-conserved homology blocks, surrounded by an equal number of hypervariable blocks that account for the variance. Transfection experiments with deletion constructs of various domains has revealed that the central region between cysteines 5 and 8 is important for binding of DBL1α, DBLγ and DBLβ–C2 domains and binding is independent of flanking regions (Mayor *et al*., 2005; Russell *et al*., 2005). The CIDR domains are divided into three areas *viz.* MI, M2 and M3 based on the Malayan Camp CIDR1α and together harbour 13 invariant cysteine residues (Smith *et al*., 2000b). A 67 amino acid fragment from FCR3-CSA DBL3γ, located at the C-terminal end of the cysteine 5 to 8 core-binding region has shown the capacity to bind CSA (Gamain *et al*., 2004). Structural studies of EBA-175 and DBP of *P. knowlesi* (Tolia *et al*., 2005; Singh *et al*., 2005) indicate that the invariant cysteines stabilize the scaffold of semi-conserved blocks ensuring binding, while the variable loops enable immune evasion.

PfEMP1 domains are organised in defined patterns *i.e* each DBL1α pairs with CIDR1α to form a semi-conserved head structure followed by DBL2β pairing with C2 or CIDRβ or γ, altogether forming a double tandem that builds the smallest PfEMP1 variants. Additional DBLβ, γ and ε domains are placed in between or after the duplicated tandem. These tandem structures are strikingly conserved during frequent recombination events at telomeric clusters (Freitas-Junior *et al*., 2000; Taylor *et al*., 2000). The above mentioned domain structure has a functional foothold, since a majority of the PfEMP1 variants harboured by the 3D7 *P. falciparum* strain have a type 1 head structure, implying that DBL1α pairs with a CD36 binding CIDR1α domain (Robinson *et al*., 2003).

Sequestration

Approximately 14 - 16 hours post-invasion, the surface of infected erythrocytes undergoes major molecular and structural reorganisation and in the process becomes increasingly rigid and permeable, and exhibits electron dense protrusions, commonly referred to as knobs (Gruenberg *et al*., 1983). Adhesive events, as described below are thought to be initiated at these "out-reaching" points of contact on the infected erythrocyte surface. PfEMP1 in association with other proteins undergoes signal-mediated transport along vesical mediated pathways on to the erythrocyte surface and becomes anchored in these knobs (Baruch *et al*., 1995; Kriek *et al*., 2003; Haeggström *et al*., 2004; Marti *et al*., 2005). Although these knobs are not essential for adhesion, it has been suggested that the biological role of knobs is to augment PfEMP1 adhesion under flow conditions (Udomsangpetch *et al*., 1989a; Crabb *et al*., 1997). Within the confines of the knobs, PfEMP1 interacts with other structural proteins such as knob associated histidine rich protein (KAHRP), PfEMP-3 and mature infected erythrocyte surface antigen (MESA) / PfEMP-2, all of which are involved in ensuring the transport and anchorage of PfEMP1 on the erythrocyte surface (Kilejian *et al*., 1979; Howard *et al*., 1987; Pasloske *et al*., 1993; Horrocks *et al*., 2005).

The spectrum of adhesive events exhibited by *P. falciparum* infected erythrocytes can be categorized into three defined phenomena viz. cytoadherence, rosetting and autoagglutination, all of which however represent an overlap of events that facilitate sequestration of the infected erythrocyte and thereby parasite propagation and survival in the human host. While the ring forms of *P. falciparum* infected erythrocytes are seen abundantly in the bloodstream of infected individuals, relatively few mature stage trophozoites or schizonts circulate, implying that the later stages are actively trapped, "sequestered" in vital organs.



P. falciparum infected erythrocytes are found to be sequestered in various organs including heart, lung, brain, liver, kidney, subcutaneous tissues and placenta (Miller, 1969; Yamada *et al*., 1989). Through successive cycles of sequestration and multiplication, parasites achieve sufficient densities in microvascular beds to cause both organ specific and systemic disease. Sequestration occurs in every *P. falciparum* infection, be it in non-immune individuals with severe malaria or semi-immune individuals with asymptomatic malaria. While flow perturbations that eventually lead to obstruction, hypoxia and haemorrhages in parenchymal tissue, cannot completely account for the wide spectrum of clinical events encountered during *P. falciparum* infection. Other mechanisms such as increased vascular permeability, widespread endothelial cell activation, huge local cytokine surges and metabolic acidosis are all considered to contribute additively to the pathogenesis of severe malaria (Miller *et al*., 1994).

The host spleen seems to play an important role in modulating sequestration. Comparison of adhesive traits of *P. falciparum* infected erythrocytes in spleen-intact and splenectomised monkeys indicated that splenectomy reduced sequestration *in vivo* and that parasitized erythrocytes from splenectomized animals lost their adhesive traits *in vitro* (David *et al*., 1983; Contamin *et al*., 2000).

Cytoadherence

Cytoadherence is defined as the receptor mediated binding of *P. falciparum* infected erythrocytes to post capillary endothelium *in vivo* or to cell line cultures *in vitro*. Cytoadherence, previously considered to be confined only to erythrocytes infected with the mature trophozoite and schizont stages, has lately been shown to involve the earlier ring stage as well (Pouvelle *et al*., 2000, Douki *et al*., 2003). The adhesion process is comparable to adhesion of leukocytes, whereby most infected erythrocytes initially tether followed by rolling before adhering firmly (Cooke *et al*., 1994). Most host receptors are involved with tethering and rolling but are unable to support firm adhesion under conditions of flow on their own. Different parasites can bind to variable numbers and combinations of host receptors, which are believed to affect the tissue distribution and pathogenesis of parasites (Yipp *et al*., 2000; Heddini *et al*., 2001). Definite correlations beween severe malaria and binding to HS and blood group A, as well as between anemia and immunoglobulin binding indicate that cytoadherence is a virulence factor (Scholander *et al*., 1998; Heddini *et al*., 2001). Evidence does also point towards multiadhesion being a feature of strains causing severe malaria (Heddini *et al*., 2001).

The precise involvement of receptors in the phenomenon of cytoadherence exhibited by *P. falciparum* infected erythrocytes has been largely characterised by the availability of cell lines amenable for transfection viz. human umbilical vein endothelial cells (Udeinya *et al*., 1981), C32 melanotic melanoma cells (Schmidt *et al*., 1982), squirrel monkey brain microvascular endothelial cells (Gay *et al*., 1995), transfected CHO (Hasler *et al*., 1993), COS-7 (Rowe *et al*., 1997; Chen *et al*., 2000), BeWo and L cell lines (Treutiger *et al*., 1997; Viebig *et al*., 2006). *In vitro* studies utilizing these cell lines have implicated the following receptors in cytoadherence of *P. falciparum* infected erythrocytes: thrombospondin (TSP), CD36, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, CSA,

platelet endothelial cell adhesion molecule-1 (PECAM-1 /CD31), P-selectin, HS and hyaluronic acid (HA).

Two receptors, CD36 and CSA have been shown to provide stable stationary adherence (Cooke *et al.*, 1994; Rogerson *et al.*, 1997). Binding to CD36 and likewise to TSP seems to be a constitutive feature of most adherent parasites (Hasler *et al*., 1990; Ockenhouse *et al*., 1991; Newbold *et al*., 1997), although binding to these receptors does not correlate with severity of disease (Roberts *et al*., 1985; Turner *et al*., 1994; Newbold *et al*., 1997). ICAM-1 mediated interactions, however feeble, have been shown to play an important role in the rolling phase of attachment of the infected erythrocytes (Craig *et al*., 1997). ICAM-1 expression is up regulated in victims of cerebral malaria (Turner *et al*., 1994), yet isolates from patients with cerebral disease have not significantly higher binding to ICAM-1 than isolates from control groups (Newbold *et al*., 1997). ICAM-1 and CD36 act synergistically in securing adhesion of infected erythrocytes (McCormick *et al*., 1997). Binding to VCAM-1, E-selectin and P-selectin is exhibited by only a minority of patient isolates (Ockenhouse *et al*., 1992; Newbold *et al*., 1997) and their role in sequestration is therefore unclear. Binding to HS is not a predominant feature of field isolates, although recent evidence suggests that a correlation to severe disease might exist (Heddini *et al*., 2001; Vogt *et al*., 2003). Besides binding to CSA and HA (Fried and Duffy, 1996; Beeson et al., 2000), placental isolates bind to placental tissue through non-immune immunoglobulins (Flick *et al*., 2001; Niloofar Rasti, personal communication).

Rosetting

The binding of two or more uninfected erythrocytes to an infected erythrocyte, was first observed and described in *P. falciparum* cultures *in vitro* (Udomsangpetch *et al*., 1989b), and has since been reported in several species of Plasmodia (David *et al*., 1988; Lowe *et al*., 1998). This phenomenon has been

observed in the peripheral blood of patients with acute severe malaria and in blood vessels of autopsy specimens from victims of malaria (Riganti *et al*., 1990; Ho *et al*., 1991; Scholander *et al*., 1996). Serum components such as albumin, fibrinogen, IgG and IgM, are considered to participate in securing and stabilizing the interaction between infected and uninfected erythrocytes and have subsequently been shown to be indispensable for rosette formation (Scholander *et al*., 1996; Clough *et al*., 1998; Treutiger *et al*., 1999; Rogerson *et al*., 2000; Rowe *et al*., 2000, Somner *et al*., 2000). Most importantly, an association between rosette forming capacity and severe malaria has been reported from studies conducted in areas with largely different geographical and epidemiological settings (Carlson *et al*., 1990a; Treutiger *et al*., 1992; Rowe *et al*., 1995; Udomsangpetch *et al*., 1996; Newbold *et al*., 1997; Kun *et al*., 1998b; Heddini *et al*., 2001). Sera from patients with severe malaria harbour a low titre of anti-rosette antibodies, while the latter are abundant in patients suffering from uncomplicated malaria (Carlson *et al*., 1990a; Imbert *et al*., 1997; Treutiger *et al*., 1997; Barragan *et al*., 1998)

Rosetting strains of *P. falciparum* preferentially rosette with erythrocytes bearing blood groups A and B and exhibit larger rosettes and more stable interactions than when seen on invasion of blood group O erythrocytes (Carlson and Wahlgren, 1992; Udomsangpetch *et al*., 1993; Barragan *et al*., 2000). This explains the observation that blood group A is associated with higher risk for development of severe malaria, while blood group O is associated with protection against cerebral malaria (Hill, 1992). Besides the ABO-blood group antigens, rosetting has been shown to be mediated by HS, CR-1 and non-immune immunoglobulins (Udomsangpetch *et al*., 1989b; Carlson *et al*., 1990a; Carlson *et al*., 1992; Rogerson *et al*., 1994; Rowe *et al*., 1994; Scholander *et al*., 1996; Rowe *et al*., 1997; Chen *et al*., 1998a; Barragan *et al*., 1999; Barragan *et al*., 2000; Rowe *et al*., 2000; Vogt *et al*., 2004). A role of CD36 in rosetting has also been suggested (Handunnetti *et al*., 1992).

Giant Rosetting and Autoagglutination

The ability of infected erythrocytes to adhere to neighbouring infected erythrocytes and thereby forming large aggregates without involving any un-infected erythrocytes is termed as autoagglutionation and falls under the terminology of giant rosetting, described initially by Carlson *et al*., (1990). While rosetting seems to occur at 16 - 18 hours post-invasion with a peak at 32 - 36 hours (Treutiger *et al*., 1998), no such observations for autoagglutination have been made so far. Autoagglutination, as an adhesive phenomenon, has only been reported for *P. falciparum* infections in humans and monkeys (Roberts *et al*., 1992; Fandeur *et al*., 1995). This phenomenon is common in natural infections and a correlation with disease severity has been shown (Roberts *et al*., 2000).

Clumping is another adhesive phenomenon exhibited by *P. falciparum* infected erythrocytes, whereby platelets possibly through CD36 receptors interact with adjoining infected erythrocytes to form large clumps (Wahlgren *et al*., 1995; Pain *et al*., 2001). Parasites expressing the clumping phenotype have been shown to occur more frequently in severe malaria than in mild malaria (Pain *et al*., 2001).

Antigenic Variation and PfEMP1 Gene Regulation

Each *P. falciparum* genome carries approximately 60 copies of the *var* gene encoding for PfEMP1 molecules (Gardner *et al*., 2002). Var genes possess two exons intersperced with an approximately 0.8 - 1.2 kb relatively conserved intron. The first polymorphic exon varies between 4.0 and 10.0 kb and encodes for the extracellular binding region and a transmembrane domain, while the second 1.5 kb exon encodes for the cytoplasmic acidic terminal (ATS) segment.

Studies utilizing degenerate DBL1α primers against DBLα homology blocks have shown that the *var* genes exhibit vast inter- and intraclonal diversity

(Kyes *et al*., 1997; Ward *et al*., 1999; Kirschgatter *et al*., 2000; Fowler *et al*., 2002). This is especially true for parasites causing uncomplicated malaria, whereas parasites associated with severe malaria express a restricted subset of PfEMP1 (Bull *et al*., 2005; Nielsen *et al*., 2002; Nielsen *et al*., 2004). Every successive clinical episode in young children induces variant and cross-reactive antibodies against a broad range of PfEMP1 variants (Aguiar *et al*., 1992; Giha *et al*., 1999; Chattopadhyay *et al*., 2003), while simultaneously driving expression against PfEMP1 variants associated with severe malaria. Consequently, PfEMP1 expression is regulated by acquired immunity and is thus subject to modulation by immunization with cross-reactive variants.

Genome analysis of 3D7 has shown that the *var* upstream non-coding sequences are also structured into 3 types of sequences, designated as UpsA, UpsB and UpsC (Gardner *et al*., 2002; Voss *et al*., 2000). UpsA and a majority of UpsB are associated with subtelomeric *var* genes while the central *var* genes are associated with UpsC. This categorization of upstream sequences has been shown to carry functional significance since the *var* genes associated with severe malaria in African children, were transcribed from UpsA (Jensen *et al*., 2004). In Papua New Guinea, UpsB has been shown to be the predominant sequence type in children with clinical malaria as against UpsC in children with asymptomatic malaria (Kaestli *et al*., 2006). No particular upstream sequence was correlated to the occurrence of severe malaria in Kenya (Bull *et al*., 2000), underscoring the fact, that stringent categorization with respect to parasite phenotype and clinical presentation is required, if any meaningful information about certain *var* subgroups corresponding to definite outcomes is to be obtained.

The fact that PfEMP1 has a pivotal role in sequestration and is under constant immunological surveillance, maintenance of the parasite within the organism would be unlikely without the possibility of switching PfEMP1 molecules in tandem with different epidemiological, receptor and

immunological settings. For parasites strains under no selective pressure, such as *in vitro* cultures, switching rates as high as 2 % have been previously described (Roberts *et al*., 1992). Recombination events involving *var* genes localized at subtelomeric regions of heterologous chromosomes have been shown to generate a high level of PfEMP1 variation (Biggs *et al*., 1989; Freitas-Junior *et al*., 2000; Taylor *et al*., 2000). While massive transcription of *var* variants occurs during the early ring stage of development, only one *var* gene is exclusively singled out for expression that ultimately defines the cytoadherent, antigenic and virulent profile of the infected cell (Chen *et al*., 1998b; Scherf *et al*., 1998).

The molecular mechanisms that control this phenomenon of allelic exclusion and mutually exclusive gene expression are under intensive investigation. Changes in *var* gene expression have been shown to be independent of alterations in the sequence of the genes or from the presence or absence of transcription factors and antisense RNA (Scherf *et al*., 1998; Deitsch *et al*., 1999). Studies from reported constructs, indicated that phenomenon of "allelic exclusion" is dependent on the cooperative interaction between regulatory elements in the 5' - flanking region and the intron of *var* genes (Deitsch *et al*., 2001). Additionally, silencing requires strict one to one pairing between *var* promoters and introns, and each intron can silence only a single *var* promoter (Frank *et al*., 2006). Interestingly, a transgene encoding for drug resistance is able to silence all other *var* genes when placed in a *var* locus (Dzikowski *et al*., 2006). This underscores the fact that *var* gene expression is controlled at the transcriptional level and no negative feedback from the expressed PfEMP1 variant exists. All this is in harmony with observations that two adjacent *var* genes that occupy the same chromosomal context can assume different rates of transcriptional activity and that *var* genes without introns *i.e.* *var* $_{common}$ are constitutively transcribed (Winter *et al*., 2003).

The above line of evidence implicating the *var* intron in silencing has been challenged by a study suggesting that an active *var* promoter by itself is sufficient to silence endogenous *var* genes and ensures monoallelic expression by translocation to a unique perinuclear compartment associated with chromosome-end clusters (Voss *et al*., 2006). While the contrasting line of evidence suggested that silencing is independent of chromosomal position, this study and others suggest that changes in gene expression for sub-telomeric *var* genes are associated with epigenetic alterations in the chromatin structure and binding of the telomere associated protein PfSir2, although the processes that initiate these alterations in gene expression are presently unknown (Duraisingh *et al*., 2005; Freitas-Junior *et al*., 2005; Ralph *et al*., 2005). Thus, it remains to be seen, which amongst the two lines of evidence have an overriding role. All this, however, does indicate that PfEMP1 antigenic variation is controlled at multiple tiers and thereby ensures immune evasion and persistent sequestration in the human host.

Vaccine Development

From the Bench to the Bush

Vaccines are the mainstay of the fight against pathogenic organisms. The eradication of smallpox, the success of the polio eradication campaign, as well as the success of the expanded programme of immunization (EPI), and a 39% decrease in the global incidence of deaths caused by measles, all point unambiguously to the benefits of immunization. Effective new vaccines against infections caused by Hepatitis B virus, *Haemophilus influenzae* type B and *Neisseria meningitidis* have been introduced in developed countries, and are now being progressively incorporated into the immunization programme of developing countries.

Vaccines against rotavirus diarrhoea, *Streptococcus pneumoniae*, and human papillomavirus have all shown efficacy in clinical trials and await introduction into prevention programmes. While advances continue to be made in certain sectors, older concerns such as malaria, tuberculosis, influenza along with relatively new ones such as HIV, severe acute respiratory syndrome (SARS), and Hepatitis C continue to defy vaccine efforts. While the cost benefit association for vaccine development against these diseases is well established, huge financial investments usually pivotal in driving research into clinical application have, however, lacked behind when it comes to fighting the diseases of the poor. The low profit margins, high marketing and political risks and absence of a broader unifying vision have created a situation in which less than 10 % of the global expenditure on health related research and development is spent on developing vaccines for health problems affecting 90 % of the world's population.

No other area of research within the malaria field has been so grossly underestimated and wrongly predicted, as the subject of introducing a functional malaria vaccine in the field. While the ultimate goal has been defined decades

ago, researchers are not much nearer their goal as they were at the beginning. This journey has brought about a myriad of supposedly important vaccine candidates, the discovery of each infusing a state of expectancy among an already disenchanted research community eager to see the backside of an increasingly dire situation. These discoveries of yesteryears are finally finding their way beyond the stage of scientific publication and "proof of concept" into clinical trials. While efficacies are thus far marginal, minute improvements translate into lives saved and set a precedent for future success in a field very short on success stories. The uncertainty of outcome associated with a particular vaccine candidate necessitates parallel development of a portfolio of potential candidates. Described below are a few vaccine candidates in various stages of the development pipeline.

Pre-erythrocytic Vaccines

Pre-erythrocytic vaccines, by definition target either the sporozoite stage that is inoculated by the infectious mosquito, or the liver stage that follows subsequently. Both of these are clinically silent stages of infection and parasite clearance or reductions in the parasite burden at these stages can markedly attenuate disease. Such a vaccine would benefit those individuals who have previously not been exposed to the parasite, be it infants or travellers, and would therefore be at a greater risk of severe morbidity and mortality. Such vaccines would need to elicit sustained high antibody responses, since every single sporozoite would need to be neutralised in a very short time window. Once inside the hepatocyte, cellular responses involving both the CD8+ and CD4+ T cells are required to clear the parasite.

Pioneering work in the 1960´s showed that sterile immunity in humans could be attained by immunization with irradiated sporozoites (Nussenzweig *et al*., 1967; Clyde *et al*., 1973). Since the approach itself was considered unfeasible for mass vaccination, considerable efforts were directed towards

identifying sporozoite components targeted by protective immune responses (Herrington et al., 1987; Egan et al., 1993), which implicated the major component of the sporozoite protein coat, namely circumsporozoite protein (CSP) to be the primary target. The central region of CSP contains several specie specific repeats and harbours immunodominant B cell epitopes that are targeted by protective antibodies. The flanking regions are highly conserved and contain CD4+ and CD8+ epitopes (Nussenzweig and Nussenzweig, 1989). As a number of candidate vaccines based on CSP repeat sequences in various adjuvant formulations were progressively tested in humans, it became evident that the immunogenicity of these formulations was low.

The present vaccine candidate, RTS,S/ASO2A is a protein particle vaccine and incorporates improvements from observations made during CSP trials in humans over the last two decades (Herrington *et al*., 1991; Gordon *et al*., 1995; Stoute *et al*., 1997; Alloueche *et al*., 2003; Heppner *et al*., 2005). Of late, two field trials of RTS,S/ASO2A carried out in Gambia and Mozambique marked an important landmark in the history of malaria vaccine development (Bojang *et al*., 2001). The initial Phase IIb trials were conducted in semi-immune Gambian adults, who were immunised with three doses of RTS,S/ASO2A during a period of low transmission and followed up on occurrence of new infections during 16 weeks of active malaria transmission. While estimated efficacy during the first 9 weeks of follow up was 71%, it was zero thereafter. Additionally, protection was not limited to the NF54 parasite genotype from which the vaccine was derived. In another Phase II trial, carried out in children aged 1 - 4 years in Mozambique, RTS,S/ASO2A imparted 30 % reduction in the incidence of clinical malaria, a 45 % delayed time to first infection, and reduced incidence of severe malaria by 58% at a 6 month follow up (Alonso *et al*., 2004). An additional follow up after another 12 months indicated that the efficacy of RTS,S/ASO2A against clinical malaria and severe malaria was sustained at similar levels (Alonso *et al*., 2005)

Another vaccine candidate that emanates from sporozoites (Robson *et al*., 1988) and has progressed to field studies is thrombospondin related anonymous protein (TRAP). Although not necessary for sporozoite formation, TRAP has a pivotal role in ensuring sporozoite motility and thereby successful invasion of mosquito salivary glands and human hepatocytes (Sultan *et al*., 1997). Antibodies against TRAP inhibit *in vitro* hepatocyte invasion by sporozoites and have been shown to correlate with control of parasite densities in *in vivo* (Rogers *et al*., 1992; Scarselli *et al*., 1993).

TRAP attached to a multi-epitope string consisting of CD8+, CD4+ and B cells epitopes derived from six other pre-erythrocytic antigens, and provided in various heterologous prime boost regimes entailing DNA or pox viral particles has been tested in a series of Phase IIa and IIb trials (McConkey *et al*., 2003; Hill, 2006). As a recent trial indicated, the hepatic burden of the parasites could be reduced by 92 % and circulating memory T cells elicited sterile immunity for as long as 20 months in some volunteers (Keating *et al*., 2005; Webster *et al*., 2005). In order to assess protection against febrile malaria in children, another Phase IIb efficacy trial has recently been initiated in Kenya (Hill, 2006).

Asexual Blood Stage Vaccines

Two leading asexual blood stage vaccine candidates are MSP-1 and AMA-1. Both of these have been identified in all *Plasmodium* species examined and the availability of their homologues in rodent and Simian parasites has allowed the vaccine potential of these candidates to be tested in animal models (Waters *et al*., 1990). Merozoites represent one of the developmental stages in which the parasites are extracellular and thus theoretically readily accessible to antibodies during repeated cycles of merozoite release from rupturing infected erythrocytes.

MSP-1 is a 185 - 210 kDa glycoprotein synthesized during schizogony and distributed abundantly at the surface of merozoites. This protein is

proteolytically processed to smaller fragments of varied molecular weights, all held together non-covalently to the merozoite surface. At the time of merozoite release, a 42 kDa fragment undergoes secondary processing to form a 33 kDa product, which is shed, and another 19 kDa fragment, subsequently gains entry into the erythrocyte (Blackman *et al*., 1990). The C - terminal fragments of MSP-1 are of particular interest for vaccine development, since naturally acquired antibodies to MSP-$1_{(42)}$ are associated with resistance to clinical malaria and monoclonal antibodies mapping to the MSP-$1_{(19)}$ fragment, as well as anti MSP-$1_{(19)}$ antibodies affinity purified from human hyper-immune sera, all inhibit merozoite invasion *in vitro* (Thomas *et al*., 1984; Pirson *et al*., 1985, McBride *et al*., 1987; Chang *et al*., 1992; Riley *et al*., 1992; Al-Yuman *et al*., 1994; Blackman *et al*., 1994; Egan *et al*., 1995). Vaccination of mice with the analogous region of *P. yoelli* MSP-1 elicits complete protection against challenge with a lethal strain (Holder and Freeman, 1981).

Immunization with recombinant MSP-$1_{(42)}$ and MSP-$1_{(19)}$ protein antigens has been shown to provide protection from blood stage parasites in malaria monkey models challenged with *P. falciparum* (Egan *et al*., 2000; Kumar *et al*., 2000; Stowers *et al*., 2001a). Protection in challenge models, however, relies upon high antibody titres, which are only obtained on immunization with Freund´s adjuvant, an adjuvant inapplicable for human subjects. On account of the low immunogenicity, limited availability of T cell epitopes (Egan *et al*., 1997) within MSP-$1_{(19)}$, conformational variability (Stowers *et al*., 2001b) and sequence variation previously thought to be non-existent for MSP-1 (Sakihama *et al*, 1999), attention has now shifted to MSP-$1_{(42)}$. Preclinical evaluation of *E. coli* recombinant MSP-$1_{(42)}$ with adjuvants approved for humans, has been shown to be not only safe, but also immunogenic as evidenced by the high antibody titres and high lymphocyte proliferation rates (Pichyangkul *et al*., 2004). Phase IIb clinical trials with these formulations are presently ongoing.

AMA-1 is an antigen expressed at the apical end of the parasite and is postulated to play a central role in erythrocyte invasion by the merozoite (Deans *et al*., 1982; Thomas *et al*., 1990; Triglia *et al*., 2000). It is an 83 kDa integral membrane protein with an ectodomain organised in three domains stabilized by disulfide bonds (Hodder *et al*., 1996). Antibodies against AMA-1 have been detected in populations exposed to malaria and these antibodies have been shown to inhibit merozoite invasion *in vitro* (Thomas *et al*., 1990; Hodder *et al*., 2001). Significant differences in AMA-1 sequences have been documented for asymptomatic and symptomatic *P. falciparum* infections in Papua New Guinea; further implying that AMA-1 is one of the determinants of malaria morbidity (Cortes *et al*., 2003). Immunization with AMA-1 or passive transfer of anti-AMA-1 antibodies protected mice against a lethal challenge with *P. yoelli* blood stage infection (Narum *et al*., 2000). Trials in Rhesus monkeys with native *P. knowlesi* AMA-1 protein conferred protection against homologous infection (Deans et al., 1988). Furthermore, immunization with recombinant *P. fragile* AMA-1, expressed in baculovirus and injected in association with the adjuvant Montanide ISA 720, provided partial protection against homologous as well as *P. falciparum* infection in Saimiri monkeys (Collins *et al*., 1994).

CD4+ T cells have also been implicated in the protection induced by *P. chabaudi* AMA-1, although cellular responses alone without antibodies are unable to provide protection in mice (Xu *et al*., 2000). The efficiency of AMA-1 is highly dependent on the correct folding of the recombinant molecule since immunization with reduced and alkylated AMA-1 does not inhibit invasion or induce protection (Hodder et al., 2001). The epitopes on antibodies inhibiting invasion have been shown to involve more than one sub-domain (Lalitha *et al*., 2004). A large number of point mutations have been observed in AMA-1, suggesting that the polymorphic regions within AMA-1 are targets of protective immune responses (Cortes *et al*., 2003). Antibodies against AMA-1 are considered to be strain specific, although recent evidence suggests that

immunization with a combination of several polymorphic AMA-1 forms can induce protection against a broader spectrum of challenging parasites (Crewther *et al*., 1996; Hodder *et al*., 2001; Kennedy *et al*., 2002; Healer *et al*., 2004; Cortes *et al*., 2005). A recently completed Phase Ia clinical trial on AMA-1 based on sequences from two diverse strains 3D7 and FVO, showed that the formulation is safe and the antibodies induced were functional as judged by *in vitro* studies (Malkin *et al*., 2005)

Erythrocyte Surface Antigen Vaccines

While variable surface antigens on the erythrocyte surface are still undergoing pre-clinical evaluation, accumulating evidence supports their probable utility as vaccines against severe disease and against placental malaria. A prime example in this regard is provided by PfEMP1. Antibodies to the variant infected erythrocyte surface antigens develop after symptomatic malaria infection and are associated with protection against infection in adults and children (Marsh *et al*., 1989; Carlson *et al*., 1990a; Treutiger *et al*., 1992; Giha *et al*., 1999; Dodoo *et al*., 2001). Protective immunity mediated by anti-PfEMP1 responses develops initially to severe malaria followed by uncomplicated malaria (Gupta *et al*., 1999; Bull *et al*., 2000; Nielsen *et al*., 2002; Yone *et al*., 2005). Antibodies present in children residing in endemic areas recognize only a small proportion of circulating variants, but with age and continued exposure, children acquire a much broader range of antibody specificities against PfEMP1 variants. These antibodies disrupt rosettes and inhibit erythrocyte adhesion to various endothelial receptors, thereby suggesting a relationship between anti-PfEMP1 responses and clinical immunity (Carlson *et al*., 1990a; Treutiger *et al*., 1992; Barragan *et al*., 1998; Bull *et al*., 1998; Giha *et al*., 2000). Anti-PfEMP1 IgG responses interspersed with periods of asymptomatic infection have been suggested to reduce new episodes of clinical malaria (Kinyanjui *et al*., 2004).

Variant specific antibodies to DBL1α domains of PfEMP1 confer semi-immune status to populations in hyperendemic regions (Oguariri *et al*., 2001). Additionally antibodies against CIDR1α are associated with marked reduction in parasite densities, fever episodes and overall risk of anaemia (Lusingu *et al*., 2006). Immunization with DBL1α has been shown to disrupt rosettes / autoagglutinates (Chen *et al*., 2004; Pettersson *et al*., 2005) and block *in vivo* adhesion in a rat model (Ahuja *et al*., 2006; Chen *et al*., 2004; - Papers III and IV respectively). Immunization with phylogenetically diverse DBL1α domains elicits cross-reactive responses that provide semi-protection to challenge by the FCR3S1.2 strain, as judged by pulmonary sequestration in an *in vivo* rat model (Ahuja *et al*., 2006 - Paper III). Immunization of monkeys with functional CIDR1α domains has been shown to provide protection against a lethal challenge with a homologous strain and protect against severe malaria on re-infection (Baruch *et al*., 2002; Makobongo *et al*, 2006).

Features of Placental Malaria

-Placental insufficiency
-Low birth weight
-Premature delivery
-Abortion
-Maternal anemia
-Maternal mortality
10 %- 15%

Pregnancy associated malaria arises from the adherence of distinct parasite subpopulations, defined by their ability to adhere to the placental syncytiotrophoblasts through CSA, immunoglobulins and HA; and most importantly, to be recognised by sera in a sex and parity dependent manner (Fried *et al*., 1996; Fried *et al*., 1998; Staalsoe *et al*., 2004; Niloofar Rasti, personal communication). Women in endemic areas uniformly develop resistance over one to two pregnancies as they acquire antibodies against placental parasites, and sera from multiparous women are cross-reactive with placental parasites collected in Africa and Asia (Fried *et al*., 1998). Parasite subpopulations implicated in

placental malaria, show consistently up regulated transcription of a single conserved gene, namely var2CSA (Salanti *et al*., 2003). High anti-var2CSA IgG levels are found in pregnant women in endemic areas and correlate to protection against delivering infants with low birth weight (Salanti *et al*., 2004; Tuikue Ndam *et al*., 2006). Immunization with the var2CSA domains induces antibodies that block placental binding of infected erythrocytes (Viebig *et al*., 2005). Although var2CSA as a vaccine candidate is yet to enter full-scale clinical trials, a vaccine against placental malaria is not far off provided that current understanding holds true.

Sexual Stage Antigen Vaccines

The primary goal of anti-sexual stage vaccines is to prevent parasite transmission, hence the name transmission blocking vaccines. While these vaccines do not protect an individual directly from acquiring malaria, they can provide herd protection by decreasing overall parasite load and hence the transmission. Blocking transmission could potentially reduce the mortality / morbidity associated with malaria, or lead to eradication of parasites in geographically isolated areas or areas with low transmission. The concept is based on the observations that antibodies against sexual stages are frequently encountered in individuals living in endemic areas and antibodies raised against sexual stages correlate with reduction in transmission (Carter, 2001).

The original experiments on malaria transmission blocking immunity were carried out in avian malaria models and indicated that antibodies against gametes act within 5 - 10 minutes after a blood meal, thereby preventing fertilisation whereas developing or mature ookinete were incapacitated by cytophilic antibodies active even 12 - 24 hours following a blood meal (Carter, 2001). While a number of sexual antigens have been discovered and evaluated, only Pfs25 (Kaslow *et al*., 1988), an antigen expressed practically in all developmental forms inside the invertebrate host, has so far made it to Phase Ia

trials. As is evident, a malaria transmission blocking vaccine based on Pfs25 is intended for administration in association with other anti-malaria vaccines.

Whole Organism Vaccine Approaches

It has been known long that sterile immunity lasting for months could be evoked by immunization with irradiated sporozoites (Nussenzweig *et al*., 1967; Clyde *et al*., 1973). Most importantly, protection induced by irradiated sporozoites is strain descending: inoculation with sporozoites of one strain confers protection against heterologous strains (Hoffman *et al*., 2002). In light of the fact that most of the recombinant vaccines that deliver one or a few parasite antigens suffer from inherent problems viz. difficulties in retaining the correct native secondary or tertiary conformation of crucial antibody binding sites, inability to provide the broad range of major histocompatibility complex (MHC) class II binding motifs that are required to induce a T-cell response in human populations with heterogeneous HLA haplotypes, the need for an exogenous adjuvant, their inability to long term antigen persistence and to facilitate long term memory; whole-parasite approaches are gaining increased acceptance.

Lately, it has been shown that immunization with genetically attenuated sporozoites, fully capable of hepatocyte invasion but unable to proceed beyond the schigony stage in hepatocytes, provide complete protection from sporozoite challenge in mice (Mueller *et al*., 2005; van Dijk *et al*., 2005). Although heterologous challenge in these series was not conducted, challenge with infected erythrocytes resulted in a normal blood stage infection. This implies that the immunity induced here is stage specific and sterile immunity can be achieved independent of immune responses against blood stages. While further confirmation in human volunteers is awaited, technical and logistic challenges associated with production, storage, delivery and administration of whole organisms are key questions for both approaches. Another whole organism approach, utilizing ultra-low doses of parasite infected erythrocytes has been

shown to render human volunteers immune (Pombo *et al*., 2002). Protection was characterised by strong CD4+ and CD8+ T cell proliferation responses, IFN-γ production and up regulation of nitric oxide synthase in peripheral blood mononuclear cells, while antibodies, IL-4 and IL-10 were remarkably lacking.

Vaccine Development - an Uphill Battle

The difficulties associated with vaccine development against malaria fall back on the following:

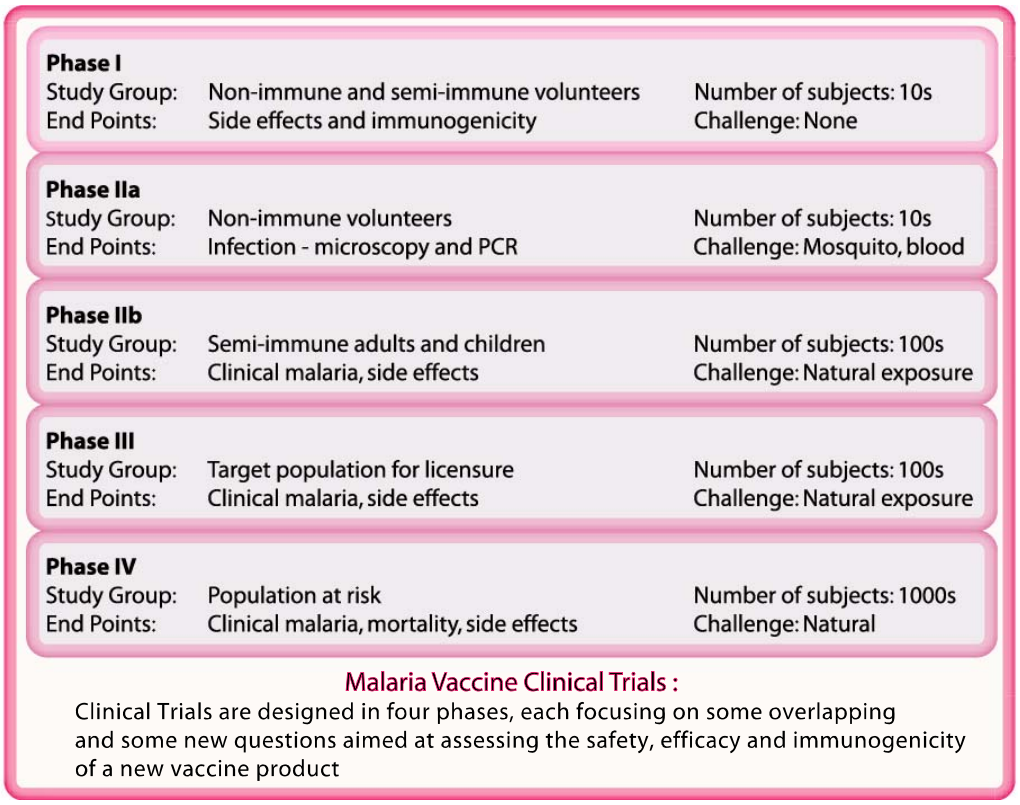
a. While the complexity of the parasite cycle offers a plethora of vaccine candidates, there is considerable uncertainty about whether these invoke native, adaptive immune responses and immunological memory crucial for long-term protection;

b. Antigenic diversity as reflected by allelic polymorphisms and antigenic variation limit the efficacy of malaria vaccines, particularly those targeting the asexual blood stages;

c. Natural immunity to malaria consists of a complex mixture of diverse immune responses, some probably of no protective value and some potentially counter-protective or outright inhibitory (Guevara Patino *et al*., 1997). Subunit vaccines, single component or in combination, need to evoke additive responses that are substantially greater than those generated by years of natural exposure, to afford protection of any relevance;

d. Although functionally key antigens shared by different developmental stages (Silvie *et al*., 2004) present an alternative of combining antigens into a multistage vaccine, there are concerns about the ensuing selective pressure paving way for mutations in target genes and jostling closely related genes with redundant attributes into alternate functional pathways. This would results in parasites insensitive to the antibodies generated to the original antigen(s) and fully capable of completing the developmental life cycle; and

e. No correlates of protection have so far been defined, making comparison of vaccine candidates extremely subjective. Immune correlates of protection allow for optimization of immune responses by adjusting vaccine potency, regimes, dosage and mode of administration. Furthermore, a sound comparison of different antigens can be carried out, ensuring that only relevant candidate antigens proceed along the developmental pipeline.



Recombinant Expression in *E. coli*

All functional studies on malarial proteins, without exception, have been initiated by introducing genes downstream of promoters ported by plasmids and transfection into *E. coli* for recombinant expression. Procedural simplicity, availability of compatible molecular tools, no post-translational modifications, higher yields per unit biomass and low costs have been claimed to be the hallmarks of recombinant expression in *E. coli*.

The enthusiasm for *E. coli* has, however, been marred by the fact, that most of the malarial proteins form insoluble aggregates on recombinant expression. In an extensive *P. falciparum* expression study to date, a 6.3 % overall soluble expression rate was obtained for 1000 open reading frames (ORF) recombinantly expressed in *E. coli* (Mehlin *et al*., 2006). The discrepancy in codon usage and the AT richness of the *P. falc*iparum genes has often been named as an overwhelming problem for expression in *E. coli*

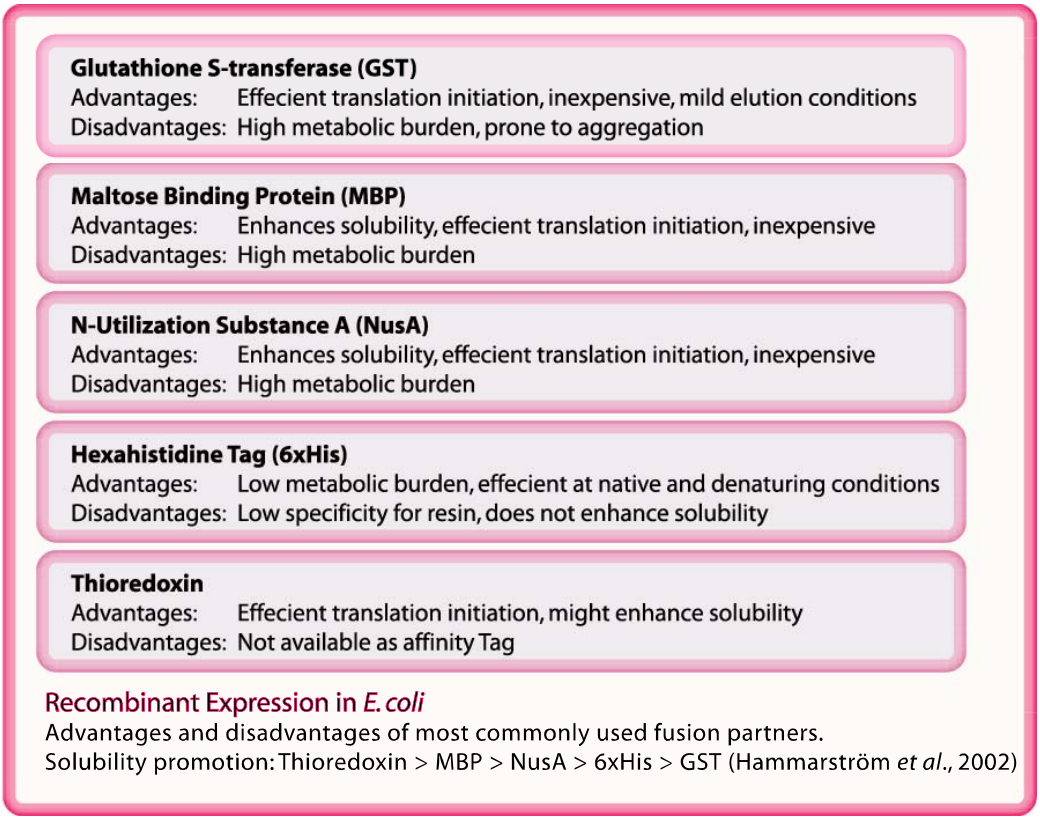
A few approaches meant to sidestep problems associated with recombinant expression of *P. falciparum* proteins have been probed unsystematically and with mixed results. These include use of codon optimization and use of plasmids to expand the tRNA pool. Expression studies on the F2 domain of EBA-175, revealed that the *E. coli* codon optimised gene gave three to four times better total expression yield although no differences in soluble protein obtained were noted for the native and codon optimised gene (Yadava and Ockenhouse, 2003; Zhou *et al*., 2003). An additional approach, recommending induction of recombinant protein expression in late log phase, has been shown to contribute significantly not only to the total amount of protein expressed but also to enhancement of the yield of soluble protein (Flick *et al*., 2004 – Paper I; Galloway *et al*., 2004). The increased yield from late log phase culture induction was not merely due to the greater number of cells, but reflected a metabolic adaptation in these cells, that attenuated the response to foreign and potentially toxic proteins.

Other heterologous expression systems, namely baculovirus and yeast (*Pischia pastoris* and *Saccharomyces cerevisiae*) have also been used for obtaining superior yields of recombinant malarial proteins. These expression systems combine the ability of growth on minimal medium and at very high cell densities, secretion of the heterologous protein and thereby simplifying recovery of recombinant protein. Additionally, post-translational modifications such as protein folding, proteolytic processing, disulfide bond formation and glycosylation are taken care of intrinsically. Codon optimized genes for expression in *P. pastoris* yield approximately 9 times more protein than its counterpart on *E. coli* expression (Yadava and Ockenhouse, 2003). Interestingly, both the yield of soluble protein and functionality were superior for the recombinant protein produced in *P. pastoris* (Yadava and Ockenhouse, 2003). On the contrary, the yield of recombinantly expressed MSP-$1_{(42)}$ was superior in *E. coli* than in any of the other expression systems tested (Singh *et al*., 2003), again underlining the fact that heterologous expression remains a "trial and error" procedure.

E. coli expressed and refolded receptor binding F2 domain of EBA-175 has also been shown to elicit functional antibodies far superior in inhibiting parasite invasion than those obtained from a baculovirus product, indicating that glycosylation in alternate expression systems can compromise immune responses (Pandey *et al*., 2002). Ectodomains of AMA-1 expressed in *E. coli* have been shown on immunization to induce growth-inhibitory antibodies that inhibit invasion, indicating that the disulfide bridging between the domains was retained on recombinant expression (Lalitha *et al*., 2004). These examples show that no particular expression system offers a universal solution.

Another approach, as yet poorly utilised for malarial proteins is the use of solubility enhancing fusion tags. Although traditionally glutathione S-transferase (GST) and Histidine (His) tags have been preferred, yet recent evidence shows that other fusion partners such as maltose binding protein (MBP), N - utilizing

substance A (NusA), Z-domain from Protein A (ZZ), Gb1-domians from Protein G (Gb1) and thioredoxin may be far more effective as affinity tags (Waugh, 2005). Fusion of an aggregation prone polypeptide to MBP has been shown to enhance not only solubility but also promote the proper folding of the polypeptide into its biologically active form (Kapust and Waugh, 1999; Planson *et al*., 2003). In a direct comparison of six different N-terminal fusion proteins and an N-terminal 6* His tag fused to 32 human proteins, thioredoxin, MBP, GB1, and ZZ ranked in the above order as the best fusion proteins with regard to solubility (Hammarström *et al*., 2002)



Inclusion Bodies

A combination of protein engineering and theoretical knowledge has revealed that a folding protein follows multiple pathways to the native-like transition stage, which subsequently collapses to assume the native conformation. These multiple pathways are occupied by intermediate forms of

the native protein that interact with the cellular milieu and continue on the productive pathway for a correctly folded protein, or undergo irregular or incomplete folding processes that usually result in their accumulation as insoluble aggregates, commonly known as inclusion bodies (IB). Amyloid formation, an established cause of many neurodegenerative diseases, is an analogous event in human cells that leads to deposition of insoluble aggregates of biologically relevant proteins into non-functional fibrils (Carrio *et al*., 2005)

Optical microscopy has revealed IB as refractile particles of up to about 2 mm^3 and as rod shaped amorphous particles at higher magnifications (Bowden *et al*., 1991). A major portion of IB are formed by the recombinant protein of interest, while proteolytic fragments of the recombinant protein, heat-shock proteins, membrane proteins, contaminants retained during the purification procedures and phospholipids along with nucleic acids form the remaining constituents (Carrio and Villaverde, 2002). Most proteins, irrespective of their source, occur in both soluble and insoluble fractions on recombinant expression in *E. coli*. The relative proportion of the soluble and insoluble fractions, however, varies between recombinant proteins.

While the nature of the recombinant proteins forming IB is diverse, these proteins are, however, united by common mechanisms that lead to IB formation. These include, the local accumulation in high concentrations of the recombinant protein and its misfolded conformations with decreased solubility, accumulation of unstructured protein fragments following proteolysis, establishment of inappropriate interactions with the bacterial folding machinery, absence of post-translational modifications necessary for solubility of some eukaryotic proteins and the prevention of proper disulfide bridging in the reducing cytoplasmic environment of *E. coli*.

The IB cannot be, however, envisaged as a dead-end, since functional protein can be successfully recovered by *in vitro* preparative refolding from inclusion bodies (Singh *et al*., 2003; Vallejo *et al*., 2004). This involves

disruption and solubilization of the IB followed by refolding of the solubilized protein, procedures many of which are not universal and need to be optimised for every single recombinant protein.

Amino Acid Sequence Determinants of Soluble versus Insoluble Recombinant Proteins

The dramatic influence of point mutations on IB formation suggests that the primary sequence and structure of a polypeptide somehow determines its propensity to aggregate into IB, whereby specific changes have an immense impact on solubility. Several intrinsic sequence related features viz. the size of the polypeptide, its phylogenetic origin, the protein family, the charge average, the proportion of hydrophilic and hydrophobic residues, *in-vivo* half-life, the frequency of occurrence of certain dipeptides and tripeptides within the sequence, high β-sheet propensity, fractions of turn forming residues are the factors which have all been implicated in the propensity of a polypeptide to form IB (Wilkinson and Harrison, 1991; Jenkins *et al*., 1995; Dyson *et al*., 2004; Luan *et al*., 2004; Idicula-Thomas *et al*., 2005; Koschorreck *et al*., 2005; Ahuja *et al*., 2006a - Paper II).

The extent of protein aggregation is also determined, at least partially, by the combination of extrinsic process parameters, which include culture media composition, growth temperature, induction temperature and duration, and production rate as a function of diverse factors such as copy number, promoter strength, mRNA stability and codon usage. Aggregation is thus the net result of several intrinsic and extrinsic factors, and the relative extent to which these are important is determined by the recombinant protein and its expression context.

Earliest attempts, comparing sequence features between experimentally proven soluble and insoluble proteins, revealed that inclusion body formation is correlated, in decreasing order, to charge average, turn forming residues, cysteine fraction, proline fraction, hydrophilicity and molecular weight

(Wilkinson and Harrison, 1991). Analysis of a dataset of 27,000 targets from 120 different organisms revealed that the protein length, composition of negatively charged and polar residues, hydrophobicity, presence of signal sequence and serine percentage composition were important determinants for solubility (Goh *et al*., 2004). In another study, expression experiments of 10,167 ORF from *Caenorhabditis elegans* utilising a single expression vector and *E. coli* strain, determined hydrophobicity to be the key determinant (Luan *et al*., 2004). A study based on literature reports of expression of proteins from viruses, prokaryotes and eukaryotes in *E. coli,* stated that the aliphatic index, frequency of occurrence of Asn, Thr and Tyr, and the dipeptide and tripeptide sequence compositions varied significantly between soluble and insoluble proteins *(*Idicula-Thomas *et al*., 2005). An additional study, testing 30 human proteins in 14 different *E. coli* expression vectors came to a more pragmatic conclusion, whereby proteins smaller than 30 kDa, containing less than one low complexity region and no more than four contiguous hydrophobic amino acids had a fairly good chance of being expressed as soluble protein on recombinant expression in *E. coli* (Dyson *et al*., 2004).

OBJECTIVES OF THE STUDY

Contrary to the commonly accepted view of considering expression of recombinant proteins as a means to an end, this study has aimed at investigating recombinant expression as a legitimate field of scientific inquiry in its own right. The major objective was to develop and characterize a prototype recombinant vaccine against *P. falciparum* malaria. The specific objectives were:

- Contribute to the process optimization task by deriving parameters that have a considerable impact on recombinant product quality in the *E. coli* expression system.

- Derive algorithms for predicting solubility outcomes on recombinant expression in *E. coli.*

- Characterize immune responses on immunization with DBL1α/x provided in context of a recombinant protein and virus particle.

- Characterize cross-reactive immune responses as a means of obviating antigenic variation exhibited by *P. falciparum.*

EXPERIMENTAL PROCEDURES

A full detailed account of experimental procedures is provided in the attached publications and manuscripts (Paper I - Flick *et al*., 2004; Paper II - Ahuja *et al*., 2006a; Paper III -Ahuja *et al*., 2006b; Paper IV - Chen *et al*., 2004.)

Sequence Dataset (Papers I-IV)

The DNA and protein sequences encoding for all PfEMP1 variants from the *P. falciparum* 3D7AH1, FCR3S1.2 and TM284S2 strains were retrieved from the PlasmoDB database maintained by the National Center for Biotechnology Information (Bethesda, MD, USA). In order to determine domain boundaries, PfEMP1 sequences were aligned and conserved stretches corresponding to the N-terminal sequence (NTS), DBL, CIDR, C2 and ATS domains as specified earlier by Smith *et al.* (2000b) were extracted and analyzed. A phylogenetic analysis incorporating NTS-DBL1α domains from the 3D7 and FCR3S1.2 strains was also conducted using McVector default settings.

Solubility Predictions (Paper II)

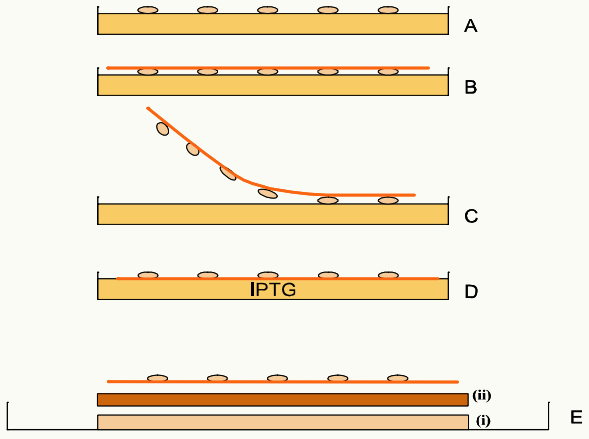
Solubility predictions were based on a statistical model, correlating experimental outcomes of 81 recombinant proteins expressed in *E. coli* and analyzed for their physiochemical features (Wilkinson and Harrison, 1991). A composite parameter (CV-canonical variable) dependent on the contribution of each of the individual amino acid was derived as follows:

$$CV=15.43\ \{(N + G+ P+ S) / n\} - 29.56\ \{[(R+K)-(D+E) / n] – 0.03\}$$

Where N, G, P, S, R, K, D, E are the absolute numbers of asparagine, glycine, proline, serine, arginine, lysine, aspartic acid and glutamic acid residues, respectively, and n is the total number of residues in the whole sequence. A

threshold discriminate CV′= 1.71 (Koschorreck *et al*., 2005) was introduced to distinguish soluble proteins from insoluble ones. A protein is predicted to be soluble, if the difference between CV and CV′ is negative. On the contrary, a CV-CV′ difference larger than zero, predicts the protein to be insoluble. Further a probability of solubility was calculated from the following equation:
$P = 0.4934 + 0.276\ (CV\text{-}CV') - 0.0392(CV\text{-}CV')^2$. The CV-CV′ values, probabilities for soluble expression in percentage, relative number of turn forming residues, charge per residue and length of protein sequence were compared for the PfEMP1 domain types of the 3D7 genome parasite. Additionally mean solubility propensities along with the lower and upper quartiles for each domain group were compared.

Colony Blot Filtration (Paper II)



Colony Filtration Blot Procedure

A. Colonies on LB plate
B. 0.45 µm membrane filter is placed on colonies
C. Membrane filter is peeled off along with attached colonies
D. Membrane filter is turned around and placed on LB plate containing IPTG
E. Following induction, membrane filter is moved on top of a sandwich composed of a nitrocellulose membrane (ii) and Whatmann filter paper pad (i). Cells are lysed on spot and soluble proteins diffuse through the membrane filter and are captured on nitrocellulose membrane

The colony filtration blot method was used for validation of solubility predictions (Cornvik *et al*., 2005). Expression constructs harbouring DBL1α, CIDR1α and ATS domains from the 3D7 genome parasite and FCR3S1.2 were transfected into chemically competent *E. coli* SG10009 and plated onto Luria-Bertani (LB) plates for overnight growth. After overnight growth, a 0.45 µm filter membrane was placed atop the LB plate hosting the colonies. The membrane was subsequently peeled off and placed carefully, with the colonies

facing upwards, on a LB plate containing 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Recombinant protein expression in the colonies "on the membrane" was induced for 6 hours at room temperature. The filter membrane was peeled off and placed on top of a nitrocellulose filter and a Whatman filter paper, both soaked in Tris based lysis buffers containing lysozyme, DNAse I and complete EDTA free proteinase inhibitors. The "filter-sandwich" was incubated at room temperature for 30 min and then freeze-thawed thrice for 10 min each at -80ºC and 37ºC, respectively. The nitrocellulose membrane was subsequently removed from the sandwich and blocked with 1% bovine serum albumin (BSA) in Tris buffered saline with Tween (TBST) for 1 hour. The membrane was washed thrice in TBST buffer and incubated for one hour with a mouse monoclonal anti-His antibody or anti-GST mouse monoclonal antibody in a 1:1000 dilution with TBST buffer. After incubation with the primary antibody, the membrane was washed 3 x 10 min in TBST buffer. Following the washes, the membrane was probed with an alkaline phosphatase-labeled anti-mouse polyclonal secondary antibody and the resultant reactive protein spots were visualized.

Expression in *E. coli* (Papers I-IV)

Chemically competent *E. coli* cells were transformed with expression vectors harbouring various PfEMP1 domains, and grown under antibiotic pressure overnight on an LB plate. Large volume cultures were inoculated and grown under specific conditions and duration, as determined earlier through the optimization of small-scale cultures expressing the respective constructs. Induction with 0.1 mM IPTG was carried out once a cell density corresponding to an $O.D._{600}$ of 1.2-1.5 was achieved. *E. coli* cells were harvested by centrifugation and the cell paste was subsequently frozen overnight at -70ºC.

Downstream Purification of Recombinant Proteins (Papers I-IV)

BL21 Codon Plus (DE3)-RIL *E. coli* cell pellets harbouring GST – recombinants and SG10009 *E. coli* cell pellets harbouring His-recombinants were re-suspended in proprietary BugBuster Protein Extraction Reagent (Novagen, Merck KGaA Darmstadt, Germany) or "in house" Tris based lysis buffer (pH 8.0), respectively. These lysis buffers were spiked with a nuclease, lysozyme, protease inhibitors and a detergent enabling adequate lysis and release of recombinant protein from the cells. When lysis was done with "in-house" lysis buffer, the resuspended cells were snap frozen and thawed twice by swirling the tube contents in a vessel containing liquid nitrogen and an additional sonication step was conducted. The suspensions were incubated at 4ºC for 30 minutes on a roller chamber and subsequently spun at 12,000 rpm for 30 min. The soluble fractions were batch purified by loading the supernatant on Glutathione Sepharose beads (Amersham Biosciences, Sweden) or on a cobalt-based resin (BD Biosciences, Sweden), followed by extensive washing with compatible wash buffers. The bound protein was eluted with 10 mM reduced glutathione (Sigma, USA) or 250 mM imidazole in elution buffers, respectively. Eluted fractions were subsequently analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE) and immunoblotting.

Parasite Culture and DNA Extraction (Papers III- IV)

FCR3S1.2 and 3D7AH1 strains of *P. falciparum* were cultured according to standard procedures (Trager and Jensen, 1976; Ljungström *et al*., 2004). In brief, parasites were grown in blood group O erythrocytes at 5 % haematocrit and maintained in malaria culture medium (MCM) containing RPMI-1640, N-Cyclohexyl-2-aminoethanesulphonic acid (HEPES), 25 mM sodium bicarbonate, 10 mg / ml gentamicin supplemented with 10% heat-inactivated human AB+ sera. Genomic DNA of 3D7AH1 *P. falciparum* strain was extracted by employing QIAamp blood kit (Qiagen, Valencia, CA, USA) and used as a

template to amplify different domain-specific fragments by PCR using standard procedures. The amplified PCR fragments were subsequently gel purified and cloned into *E. coli* expression vectors or into Semliki forest virus (SFV) constructs.

Construction of SFV particles (Paper IV)

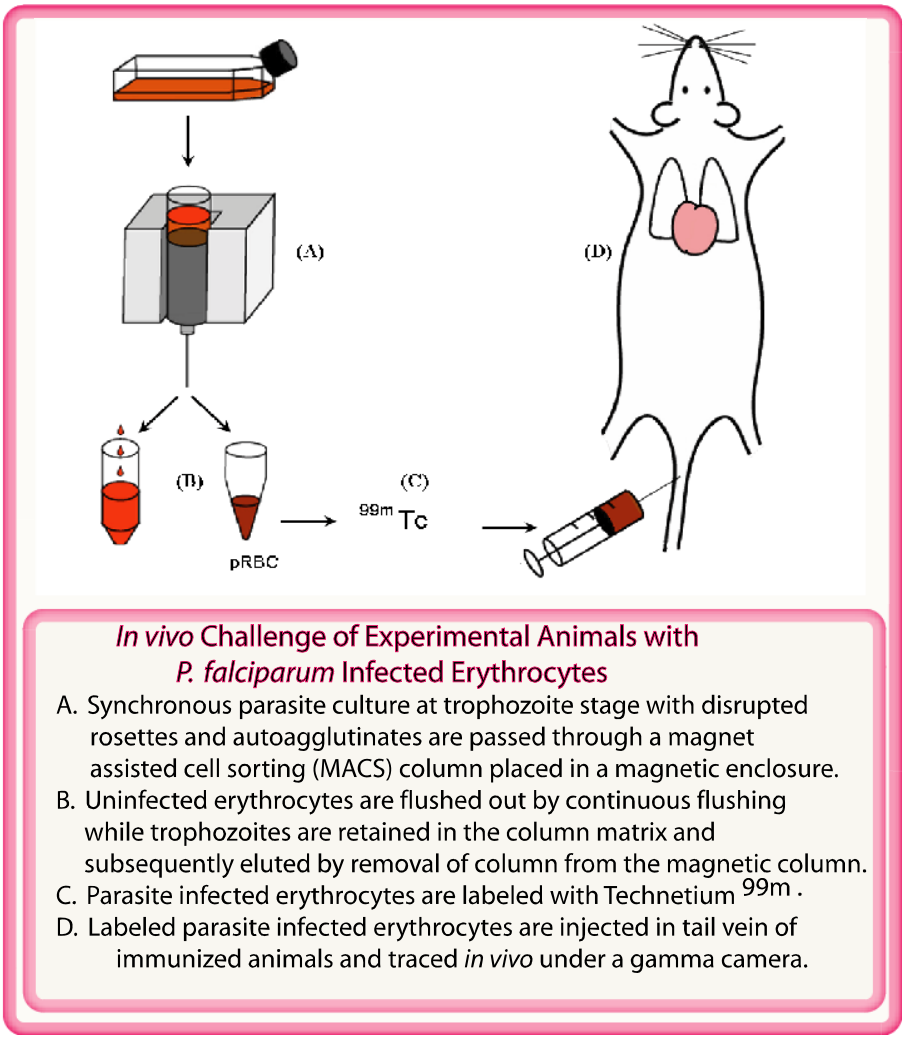
Mini *var* genes were generated by ligation of the transmembrane region (TM) and the ATS sequences to the DBL1α, CIDR1α and DBL2δ domains of *var 1* of FCR3S1.2. The GST sequence was ligated upstream of the mini-*var* genes. These constructs were cloned into SFV derived vector and *in vitro* transcription of RNA from linearised plasmid vectors performed. SFV particles were generated by co-electroporation of baby hamster kidney - 21 cells (BHK-21) with recombinant RNA and two additional helper RNA templates encoding SFV coat and spike proteins. Titres of the harvested viral stocks were determined by infection of BHK-21 cells with serial dilutions of stocks followed by live immunofluorescence.

Immunization of laboratory animals (Papers III-IV)

In order to study the immune responses elicited by the various vaccine formulations, two different primer boost regimens were studied. One regimen investigated intramuscular priming and triple boosting with recombinant proteins only, wherein animals were immunized with different permutations of GST - PfEMP1 domains emulsified in Freund´s complete adjuvant (Paper III) or Montanide ISA 720 (Paper IV). The other regimen investigated subcutaneous priming and double boosting with SFV - PfEMP1 particles plus a final boost with the GST - PfEMP1 recombinant protein (Paper IV). Immunizations aimed at investigating cross reactivity were primarily protein primer and boost regimens (Paper III). All animals were housed in the animal facility maintained

by the Swedish Institute for Infectious Disease Control, Stockholm and adhered to the prevailing ethical and handling regulations.

In vivo Challenge of Immunized Rats with FCR3S1.2 Infected Erythrocytes (Papers III-IV)



In vivo Challenge of Experimental Animals with *P. falciparum* Infected Erythrocytes

A. Synchronous parasite culture at trophozoite stage with disrupted rosettes and autoagglutinates are passed through a magnet assisted cell sorting (MACS) column placed in a magnetic enclosure.
B. Uninfected erythrocytes are flushed out by continuous flushing while trophozoites are retained in the column matrix and subsequently eluted by removal of column from the magnetic column.
C. Parasite infected erythrocytes are labeled with Technetium ^{99m}.
D. Labeled parasite infected erythrocytes are injected in tail vein of immunized animals and traced *in vivo* under a gamma camera.

Synchronous FCR3S1.2 cultures at trophozoite stage were washed thrice in RPMI-1640 and re-suspended in 2 % BSA in phosphate buffered saline (PBS). The resuspended cultures were passed repeatedly through a 23 gauge needle enabling complete disruption of rosettes and autoagglutinates as confirmed under a UV light microscopy. The cell suspension was subsequently

loaded on to a magnet assisted cell-sorting column (MACS, Miltenyi Biotec, Auburn CA, USA) placed in a magnetic enclosure. Continuous washing with 2 % BSA enabled the removal of uninfected and infected erythrocytes at ring stages, leaving mature stages trapped in the column. The column was subsequently removed from the magnetic enclosure and the trapped erythrocytes hosting mature trophozoites were flushed out with 2 % BSA in PBS. The cell suspension was centrifuged and the cell pellet re-suspended in 1 ml of RPMI-1640. Aliquots of 10^7 infected erythrocytes / ml at 85 - 90 % parasitaemia were labeled with ^{99m}Tc, and injected into sedated immunized rats. The fate of the labeled infected erythrocytes was traced over the following 30 minutes by dynamic acquisition of the whole body images with a gamma camera (Pettersson *et al*., 2005). The rats were subsequently sacrificed and the dissected lungs were placed under the gamma camera for the acquisition of minute long images over a total of five minutes. The proportion of parasites bound to the pulmonary vasculature was quantified both from the whole body as well as separate lung acquisitions.

ELISA and Immunoblotting (Papers I-IV)

All ELISA (Papers III-IV) and immunoblotting experiments (Papers I - IV) were done according to standard procedures. For cross reactivity investigations (Paper III), sera were depleted of immunoreactivity against contaminant *E. coli* proteins present in the "vaccine dose" by adsorption twice onto *E. coli* protein agarose (Sigma, Sweden) at a 1:2 dilution, for 30 minutes at room temperature. An additional refinement, designed specifically at apprehending true immunoreactivity, involved the use of different fusion tags for immunization and cross-reactivity readouts *i.e.* while immunizations were done with GST fusions, immunoblotting and ELISA experiments were conduced with homologous or heterologous proteins fused to a His-tag.

Indirect Surface Fluorescence and Rosette Disruption Assay (Paper IV)

Trophozoites of the FCR3S1.2 strain were washed thrice with RPMI-1640. A small aliquot of these infected erythrocytes at 5% haematocrit and 5% parasitaemia was mixed with different dilutions of the sera from immunized animals and incubated at 37ºC for an hour on a rolling chamber. After three subsequent washes, the cells were incubated with Alexa G-488 labeled secondary antibody, washed again and visualized under the UV-lamp for assessment of surface fluorescence (see also Ljungström *et al*., 2004).

For assessment of rosette disruption activity of the sera, different dilutions of the latter were incubated with equal volumes of unwashed FCRS1.2 culture at 5% haematocrit and 80% rosetting. Following an hour-long incubation at 37ºC on a rolling chamber, the rosetting rate was determined under a UV lamp and compared to that ofthe original culture (see also Ljungström *et al*., 2004).

RESULTS AND DISCUSSION

The following series of studies describe the pre-clinical phase in the development and validation of DBL1α of PfEMP1 as a vaccine candidate against severe malaria and for that matter, represent the typical pathway of development for any vaccine candidate prior to clinical validation. Contrary to the commonly accepted view of considering expression of recombinant proteins as a means to an end, the initial studies (Papers I- II) have investigated recombinant expression as a legitimate field of scientific inquiry in its own right. The latter two studies (Papers III-IV) further support the conceptual inclusion of blood stage antigen vaccines as a legitimate component of multi-component vaccines aimed at attenuation of disease severity of malaria.

Optimized Expression of PfEMP1 Domains in *E. coli* (Paper I)

This study describes successful recombinant expression of PfEMP1 domains as a function of the induction strategy i.e. variations in the quality of the product in relation to the time point of induction. IPTG induction at a culture density of A_{600} = 0.6 is a typical recommendation of most *E. coli* expression protocols. An evaluation of the soluble protein yields on recombinant expression of FCR3S1.2 DBL1α, TM284S2 DBL1α and TM284S2 DBL2β domains *E. coli* at various cell densities revealed that the yield of soluble protein increased with induction at higher cell densities (Figure 2 in Paper I). This data suggests that the induction of recombinant protein expression at late log phase of growth of *E. coli* contributes significantly to not only the total amount of protein expressed but also to enhancement of the yield of soluble protein. Similar results have been obtained in a study investigating the influence of IPTG concentrations, growth temperature, duration of induction and specifically time-point of induction on recombinant expression of a 64 kDa RNA binding protein

in *E. coli* (Galloway *et al*., 2003). Interestingly, the yield of soluble protein on induction at post-log cell densities was independent of growth temperature at 30ºC or 37ºC. One might expect the increased number of cells in late log phase cultures to account for larger yields. Equal aliquots of cells from cultures induced at different densities, however, revealed that the increased yield of soluble protein was not merely due to greater cell numbers at higher densities, but that late log phase cells incorporate relatively lesser amounts of the total protein into inclusion bodies.

Not only is the yield of soluble protein higher for *E. coli* cultures induced at higher densities, but also the quality of the soluble protein is appreciably superior for late log cultures (Figure 1 in Paper I). While the above observation is surprising, considering that post-log cultures are often rate-limited by the dwindling sources of energy, it is highly probable that the rapid accumulation of large quantities of heterologous product in the log phase of exponential phase of growth is incompatible with post-translational events. Since folding is a rate-limiting step during protein expression, a higher rate of translation or synthesis of recombinant protein leads to an accumulation of unfolded or incompletely folded intermediates. In a simplistic view, this would result in the newly synthesized protein remaining unfolded for appreciably longer periods, thereby allowing for aberrant interactions between folding intermediates or exposure of residues that can result both in degradation or inclusion body formation. In post-log cultures, *E. coli* growth rates are substantially lower, allowing for a relatively better match between influx of newly synthesised recombinant protein and chaperones or factors that allow the protein to successfully navigate the folding pathway into its final conformation. The formation of inclusion bodies mainly depends on the competition between folding and aggregation rates connected to the rate of recombinant protein biosynthesis. The reduced synthesis rates at higher cell densities lower the concentration of unfolded intermediates in the cell and consequently diminish the likelihood of the protein being

appropriated into inclusion bodies. High production states are indeed accompanied by higher proteolysis and a higher level of inclusion body formation (Rozkov *et al*., 2000; Sanden *et al*., 2003)

If the cellular capacity for total protein production at a given physiological state is constant, the production of recombinant protein in the host cell always stands in direct competition to the production of the *E. coli* cellular protein, thereby exercising a certain level of metabolic burden on the host cell. Acetic acid is a metabolite that has been shown to correlate inversely to product yields (Jensen and Carlsen, 1990). Accumulation of acetic acid results from the influx of carbon into cells that exceeds demand for biosynthesis *i.e* in early log phases.

Most importantly, recombinant proteins obtained for late log induced cultures of *E. coli* are functionally active, as indicated by the binding of recombinantly expressed FCR3S1.2 DBL1α to heparin and blood group A (Figure 3 in Paper I).

Prediction of Solubility on Recombinant Expression of PfEMP1 Domains in *Escherichia coli* (Paper II)

Computational predictions based on correlation of sequence-specific features of proteins with successful soluble expression elucidated considerable heterogeneity as regards to the propensity of individual PfEMP1 domains to be expressed as soluble recombinant proteins in *E. coli*. From amongst all PfEMP1 domain types, the ATS and DBL2-5δ domains occupied extremes of the solubility prediction scale for recombinant expression in *E. coli* (Figures 1 and 2 in Paper II). While 71 % of the ATS sequences were predicted to exhibit high soluble protein expression, almost all of the DBL2-5δ sequences were predicted to exhibit low soluble protein expression in *E. coli* (56.7 % vs. 14.8 % mean solubility for the ATS and DBL2-5δ domains, respectively). A similar polarity

in propensities for soluble expression was evident for the constituent domains involved in PfEMP1 head structure (24.9 % vs. 46.8 % mean solubility for the DBL1α and CIDR1α domains). The CIDR2β domains, which constitute the second largest domains in terms of size, were predicted to give high soluble protein on expression (mean 42.4 %), indicating that expression was largely independent of the sequence length and was in fact dependent on the inherent features of the sequence itself. The DBL2β-C2 domain, a tandem domain implicated in ICAM-1 binding, was predicted to be fairly insoluble (30.6 % mean probability of solubility) on recombinant expression in *E. coli*. The DBL2-4γ and DBL2-7ε domains - domain types encountered infrequently on PfEMP1 molecules, were also predicted to give low soluble protein expression in *E. coli* (mean solubility of 31.6 % and 23.1 % respectively). Taken together, the following decending gradient in probabilities for soluble expression for PfEMP1 domain types could be deduced:

ATS > CIDR1α > CIDR2β > DBL2-4γ > DBL2β+C2 > DBL1α > DBL2-7ε > DBL2-5δ

Two important inferences could be drawn from the colony filtration blots (Figure 4 in Paper II). As for one, the easily visualized gradient in protein spot intensities for *E. coli* colonies harbouring the ATS, CIDRα and DBLα domains provided experimental confirmation for the computed solubility predictions. For the other, easily visualized differences in the protein spot intensities for two equally long but different domain types (DBL1α and CIDR1α) confirmed that the size of the domain, within certain presently unknown limits, was not the primary determinant of solubility on recombinant expression in *E. coli*. A more detailed analysis of the variation in solubility prediction over the domain length indicates that though the variation is confined to approximately 20%, marginal variations in the solubility can be expected, depending on the length of the domain chosen for expression (Figures 3A and 3B in Paper II). No particular

correlations between size and solubility have been similarly acknowledged in another *E. coli* protein expression study (Mehlin *et al*., 2006). The latter study evaluated a dataset of 1000 small, non-membrane proteins from *P. falciparum*. PfEMP1 proteins, well recognized for their poor recombinant expression in *E. coli* and reported in our investigation, were however not included in the study by Mehlin *et al*., (2006).

The present study provides general guidelines for assigning candidates for structural and functional studies to appropriate expression systems. As a generic strategy, ATS and CIDRα/β domain types are suitable for recombinant expression in *E. coli* while all the remaining domain types encompassing the DBL domain constitute a poor choice for obtaining soluble protein on recombinant expression in *E. coli*. It is likely that variations in the induction time-points, as described earlier or alterations in the cloning vector, expression temperature and *E. coli* strain can potentially increase the yield and purity. These strategies, albeit useful for single candidates, are inefficient for large-scale structural studies, considering the disproportionate amount of time and efforts required for defining the optimal set of expression parameters for each recombinant protein. Recent studies by Singh *et al*., (2003) and Mehlin *et al*., (2006) point out that, proteins insoluble on expression in *E. coli* might not necessarily fare better on recombinant expression in alternate expression systems. In fact, one might fairly deduce that the rules governing expression outcomes in *E. coli* might be applicable for other expression systems as well. A number of physiochemical parameters, often with conflicting results, have been correlated to successful expression in soluble form (Dyson *et al*., 2004; Goh *et al*., 2004; Idicula-Thomas *et al*., 2005). These differences might be attributable to the fact that prediction algorithms were applied to highly diverse proteins from equally diverse organisms viz. *C. elegans*, *E. coli* and *Homo sapiens*.

Induction of cross-reactive immune responses to NTS-DBL1α/x of PfEMP1 and in vivo protection on challenge with *P. falciparum* (Paper III)

Cross-reactive responses to PfEMP1 domains are elucidated in natural infections with *P. falciparum* (Marsh and Howard, 1986; Giha *et al*., 1999; Chattopadhyay *et al*., 2003; Nielsen *et al*., 2004) indicating that geographically and temporally separated parasites share antigenic repertoires, probably confined by the functional constraints required for binding. The precise "sequence" targets for cross-reactive responses are however unknown, although recent evidence does suggest that these might lie within small conserved stretches between hypervariable regions of DBL1α (Ward *et al*., 1999, Chattopadhyay *et al*., 2003; Johan Normark, personal communication). A vaccine that elucidates immune responses that collectively target these "molecular determinants" of cross-reactivity has a fair chance of attenuating disease severity.

In the present study, cross-reactivity, as well as, the "molecular range" of cross-reactivity was investigated, by immunizing rats with phylogenetically divergent DBL1 domains (Figure 1 in Paper III). Five widely divergent DBL1α or x domains were used for immunization in various sequential and mixed permutations (Figure 2 in Paper II).

We were aware of the complexity of discerning cross-reactivity with immunization of recombinant fusion proteins expressed in *E. coli* and have used two serial approaches to tackle this issue. Firstly, the rats were immunized with GST-DBL1α/x fusion proteins, while ELISA and immunoblot "read-outs" on immune sera were conducted on DBL1α/x His fusion proteins. This allowed characterization of true cross-reactivity to DBL1α/x domains. Secondly and most importantly, the immune sera were completely depleted of their reactivity to contaminating *E. coli* proteins. This was accomplished by adsorption of sera

onto agarose coated with *E. coli* cellular proteins, prior to ELISA and immunoblot. Thus, these approaches further narrowed down potential interferences on DBL1α/x cross-reactivity measurements by contaminating *E. coli* proteins in the vaccine dose.

Cross-reactivity discerned by ELISA and Western blot (Figures 4 and 5 in Paper III), indicated that cross-reactivity responses were indeed elicited to other variants by immunization by a single variant *viz*. immune responses against DBL1x (Chr 12.3) recognized diverse DBL1α variants (Chr 6.3, Chr 4.5 and FCRS1.2). The relevance of these cross-reactive responses in our study was elucidated in an *in vivo* rat sequestration model wherein cross-reactive responses elicited by a single or multiple variants attenuated lung sequestration of the *P. falciparum* strain (Figure 6A in Paper III). Additionally, the slopes of curves describing lung sequestration were steeper for the DBL1α/x immunized animals indicating that parasite-infected erythrocytes were effectively cleared away from circulation in the lung vasculature (figures 6B and 6C in Paper III). While only one challenge strain was used in the present investigation, an ongoing study suggests that the cross-protective response extends to other challenge strains as well (K. Moll, personal communication). The protective effect of sequestration was, however, statistically similar for the rats immunized sequentially or with composite DBL1α domains, indicating that the cross-boosting effect is mainly directed towards semiconserved regions.

The DBL1α domain of PfEMP1 mediates binding of the parasite-infected erythrocyte to HS present on endothelial cells or on uninfected-erythrocytes, thereby enabling cytoadherence and rosetting respectively (Barragan *et al*., 2000; Chen *et al*., 2000; Vogt *et al*., 2003; Vogt *et al*., 2004). Cross-reactive antibodies against DBL1α domains specifically target this interaction of parasite-infected erythrocytes. The specificity of this effect is also illustrated by the fact that pre-incubation of the parasite-infected erythrocytes with immune

sera prior to passive immunization of naïve rats impedes lung sequestration in a concentration dependent manner (K. Moll, personal communication).

The FCR3S1.2 *P. falciparum* strain is a multiadhesive rosetting strain that exhibits binding to a host of receptors, *viz.* PECAM-1 / CD31, blood group A, CD36, HS (Chen *et al*., 2000; Heddini *et al*., 2001). This parasite strain is also well recognized by sera from endemic areas and especially so by sera from patients with severe malaria (Barragan *et al*., 1998; Johan Normark, personal commmunication), and therefore might be considered to represent a severe malaria prototype parasite. The lung sequestration of this particular strain was reduced to an extent of 37 % by immunization with a DBL1 variant that shares only 29 % similar residues at the sequence level to this challenge strain. Additionally, only one single variable of FCR3S1.2 interaction, namely adhesion mediated by the DBL1α/x domain was targeted in this study. One might be tempted to speculate that a vaccine cocktail, assembling not only variant DBL1α/x domains but also variant CIDR1α and DBL2δ domains, and for that matter any variant domains involved in cytoadherence, can potentially target the whole spectrum of interactions and thereby reduce the sequestration to negligible levels.

The *in vivo* data presented in the study only reflects on the interactions between the immune responses and parasite infected erythrocytes captured over a short interval of time. Limitations imposed by host specificity make it impossible to extend this span to cover the entire parasitic cycle(s) in the rat model. Data available from an alternate model indicates that anti-PfEMP1 antibodies can indeed reduce parasite sequestration in immunized Aotus monkeys (Baruch *et al*., 2002). This implies that anti-DBL1α/x antibodies do not only block parasite sequestration but also can potentially alter parasite dynamics over a certain period of time.

Interestingly, no cross-reactive immunofluorescence was observed on the surface of parasite-infected erythrocytes. A certain degree of conformational

misfolding in the "immunization dose" might account for these observations. It is also likely that cross-reactive epitopes are not abundant and, all the more, are poorly accessible to cross-reactive antibodies that supposedly constitute a relatively small proportion in the immune sera. Additionally, flow conditions and higher levels of cross-reactive antibodies in sera in an *in vivo* situation might also explain the incongruence in findings.

Immunization with PfEMP1-DBL1α Generates Antibodies that Disrupt Rosettes and Protect against the Sequestration of *P. falciparum*-infected Erythrocytes (Paper IV)

The investigations conducted in this "proof of concept" study provide direct evidence about the role of anti-DBL1α antibodies in disrupting rosettes and attenuating sequestration. The study was designed primarily to evaluate immune responses to DBL1α FCR3S1.2, provided as a protein antigen or as a non-infectious SFV particle, in various non-primate species.

In a natural setting, all of the constitutive domains that make up a PfEMP1 molecule are acted upon by a multitude of immune mechanisms and protection is thus a reflection of immune responses directed towards the whole of the PfEMP1 molecule. Although immunization with the whole intact PfEMP1 molecule is at present not achievable, an alternative is to immunize with a composite mix of different domains in one single shot. Additionally, providing the domains in the context of its native domain structure, with the respective domains projecting away from the cell surface while being anchored by the TM and ATS domains to the host cell (mini-*var*), emulates the natural setting to considerable extent. Thus immune responses to various PfEMP1 domains, all implicated in sequestration can be evaluated. This was made possible by genetic modifications in the SFV expression setup thereby allowing mini-*var* antigen

constructs to be displayed on the surface of BHK21 cells (Figures 1 and 2 in Paper IV).

Immunization with *E. coli* recombinant DBL1α, CIDR1α, and DBL2β domains separately or all domains together, generated elicited strong antibody responses, though no surface fluorescence was again observed. On the contrary, immunization with SFV particles, harbouring these domains in the mini-*var* structural context elicited high antibody titres that recognized the homologous recombinant proteins by ELISA and immunoblots. These antibodies not only recognized the native PfEMP1 molecule from parasite-infected erythrocytes immobilized on blots, but also exhibited strong surface fluorescence (Figures 3 and 4 A in Paper IV) on FCR3S1.2 infected erythrocytes. Immunization with a composite mix of SFV-PfEMP1 domains did not elicit consistently higher antibody responses, although a statistically better response was noted in mice (Figure 4C in Paper IV). Importantly, the antibody responses elicited to the NTS-DBL1α, DBL1α and the composite mix of domains disrupted rosettes to an appreciable degree. This translated to a substantially lower sequestration of FCR3S1.2 infected erythrocytes in the lung vasculature of DBL1α immunized rat.

Conclusions

The following conclusions were drawn from the studies presented in this thesis:

- Induction of recombinant protein expression at the late log phase of growth of *E. coli* significantly enhances the yields of soluble proteins.

- Computational predictions based on sequence specific features predict the ATS and CIDR1α/β PfEMP1 domain types to be appropriate candidates for recombinant expression in *E. coli*, while the remaining domain types including the DBL domains, constitute a poor choice for obtaining soluble protein on recombinant expression in *E. coli*.

- Cross-reactive antibody responses to heterologous DBL1α /x are elicited on immunization with a single variant. Immunization with phylogenetically distant DBL1α/x variants, can elicit partial cross-protection to challenge with parasite strains harbouring a distant variant.

- Immunization with DBL1α in a native structural context as provided by SFV particles elicit antibody responses that are surface reactive, disrupt rosettes and attenuate sequestration in *in vivo*.

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Africa, that sublime continent a bare eight miles from Europe´s southern most shores, is so utterly mired in such totality of decay that only a fundamental rethink of all that has gone wrong while trying to put things right will suffice.

Excerpt from Bob Geldof´s article in The Guardian