Recombinant expression of *Plasmodium falciparum* Erythrocyte Membrane Protein 1 fragments and serological studies

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Summary

The intraerythrocytic stages of the *Plasmodium falciparum* life cycle are exclusively responsible for all clinical symptoms of malaria. Both children and adults that are infected with *P. falciparum* can either have symptoms of variable severity or be asymptomatic. However, it is mostly young children who suffer from severe symptoms ranging from severe anaemia to cerebral malaria, and it is mostly adults from endemic areas who experience comparatively mild episodes with headache and sometimes fever.

The observed morbidity is largely associated with sequestration of parasitized erythrocytes (iRBCs) on endothelial cells of host blood capillaries. This cytoadherence prevents late stage iRBCs from being cleared by the spleen. Instead, iRBCs bind to various host cell receptors such as CD36, ICAM, or CSA leading to obstruction of blood vessels, impaired oxygen delivery in affected host organs and immunological reactions of the affected tissues.

The key mediator of sequestration found is the *P. falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1). This large parasite derived protein is exported from the parasite and trafficked through various membranes and through the host cell cytosol until becoming inserted into the erythrocyte cell membrane. It is located at the interface between parasite and host immune system, and undergoes antigenic variation. *Pf*EMP1 is encoded by approximately 60 *var* genes per haploid genome, and is expressed in a mutually exclusive manner, i.e. only one gene is expressed at any one time. As one of its sophisticated immune evasion strategies, the parasite can switch to another *Pf*EMP1 variant and thus becomes no more recognizable by the host immune system.

It is believed that protection against severe malaria is the result of the development of immune responses against various variants of *Pf*EMP1. However, immunity to malaria is never sterile but instead only reduces parasite density and morbidity. We have based our work on the hypothesis that not all variants of *Pf*EMP1 are equally pathogenic i.e. have the same affinity to host cell receptors. We believe that only a certain subset of *Pf*EMP1 variants is able to confer solid cytoadherence, and consequently is responsible for severe malaria. Possessing an antibody repertoire against these specific variants therefore will protect from severe episodes.

In this work we have chosen a multiple approach to generate molecular tools and to test this hypothesis. Firstly, we elaborated on the generation of pan-specific non-cross reactive *Pf*EMP1 antibodies using both recombinantly expressed domains both from the molecule's head structure (NTS domain) and synthetic peptides corresponding to the semi-conserved intracellular part of *Pf*EMP1 (ATS peptides). By means of various molecular methods, however, we found that none of the generated sera recognized full length endogenous *Pf*EMP1 exclusively.

Secondly, we attempted expression of large fragments of *Pf*EMP1 in *E.coli* to test the recognition of sera from different malaria cases. At the same time we wanted to exploit the possibility to express random fragments of *Pf*EMP1 in a bacterial library to similarly test these sera on. Insuperable obstacles with large recombinant protein expression forced us to divert our approach towards smaller domains.

For this we isolated *var* mRNA from samples from several individuals presenting either with asymptomatic infections or experiencing severe malaria episodes. 14 *var* DBL domains were recombinantly expressed in *E. coli* and used to measure antibody titers in sera from 100 semi-immune Papua New Guinean adults. The frequency of recognition (FoR) for these antigens of was assessed and compared between FoR of DBL domains deriving from severe cases and from asymptomatic samples. We found that DBL domains deriving from severe cases were significantly more often recognized by sera from semi immune Papua New Guinean adults than DBL domains derived from asymptomatic samples. This is indicative for semi-immune adults not suffering from clinical malaria because being better protected against parasites expressing "severe" DBL domains of *Pf*EMP1.

We also tested 34 sera from children with asymptomatic infections collected during a longitudinal study in Tanzania. We selected sera that were collected at two time points 6 months apart to assess the development and dynamics of antibodies against those DBL domains. FoR increased significantly over time in these children but only for DBL domains deriving from severe cases. As these children did not suffer from clinical episodes between the two sampling dates, these results also indicate that acquisition of antibodies against "severe" DBL domains is faster and can confer protection.

In summary, our findings support the notion that development of antibodies against *Pf*EMP1 variants (in this case against DBL domains) is associated with protection

against severe disease and thus contributes as an important factor to the acquired clinical immunity to severe malaria. These findings raise hope in the feasibility of a putative protective vaccine against the major virulence factor *Pf*EMP1.

Zusammenfassung

Alleine der intra-erythrozytäre Lebenszyklus von *Plasmodium falciparum* ist für die klinischen Symptome von Malaria verantwortlich. Die Infektion kann sowohl bei Kindern, als auch bei Erwachsen symptomlos oder mit verschieden starken Symptomen ablaufen. Jedoch sind es meist Kinder, die an den schlimmen Symptomen wie schwere Anämie oder cerebraler Malaria leiden. Erwachsene aus endemischen Gebieten haben vergleichsweise milde Symptome wie Kopfschmerzen und manchmal Fieber.

Die schweren Krankheitsfolgen sind grösstenteils mit dem Anheften von infizierten Erythrozyten an Endothelzellen der Wirtskapillaren verbunden. Diese Zellanheftung verhindert, dass die Milz die späten Blutzellstadien herausfiltriert. Infizierte rote Blutzellen binden Wirtsrezeptoren wie zum Beispiel CD36, ICAM oder CSA was zu einer Verstopfung der Blutgefässe, mangelhafter Sauerstoffzuführung und immunologischen Reaktionen im betroffenen Gewebe führt.

Eine Schlüsselrolle im vermitteln von Zellkontakten hat Plasmodium falciparum Erythrozyten Membran Protein 1 (*Pf*EMP1). Dieses grosse Protein, hergestellt durch den Parasiten, wird durch mehrere Membranen und das Zytosol des Erythrozyten geschleust, bis es in die Erythrozytenmembran eingebaut wird. Es ist so genau an der Grenze zwischen dem Parasit und dem Wirtsimmunsystem lokalisiert und macht Antigenvariation. *Pf*EMP1 wird von ungefähr 60 *var* Genen pro haploiden Parasiten codiert und wird auf einer sich gegenseitig ausschliessenden Art und Weise exprimiert, dass heisst es wird immer nur ein Gen abgelesen. Damit der Parasit das Immunsystem umgehen kann, ist er in der Lage auf ein anders var Gen umzuschalten. Diese kann dann vom Immunsystem nicht mehr erkannt werden.

Man glaubt, dass der Schutz vor schwerer Malaria ein Ergebnis der Entwicklung von Antikörpern gegen mehrere Varianten von *Pf*EMP1 ist. Aber die Immunität gegen Malaria ist nie steril, sondern reduziert nur die schwere der Erkrankung und die Parasitendichte. Die Grundlage unserer Arbeit ist, dass nicht alle Varianten von *Pf*EMP1 gleich pathogen sind, dass heisst, nicht alle haben dieselbe Affinität zu den Wirtsrezeptoren. Wir glauben, dass nur eine kleine Gruppe von *Pf*EMP1 Varianten die Möglichkeit hat eine stabile Zellanheftung auszulösen und dadurch schwere

Malaria verursacht. Der Besitz eines Antikörperrepertoires gegen diese Varianten würde deshalb gegen schwere Malaria schützen.

In dieser Arbeit haben wir einen vielfältigen Ansatz verfolgt um molekulare Werkzeuge herzustellen und unsere Hypothese zu testen. Als Erstes haben wir versucht spezifische, nicht kreuzreaktive Antikörper gegen *Pf*EMP1 zu generieren. Wir haben die Kopfstruktur (NTS Domäne) als rekombinantes Protein und als synthetische Peptide den teilweise konservierten intrazellulären Teil (ATS Domäne) von *Pf*EMP1, als Antigen verwendet. Trotz der Anwendung mehrerer molekularbiologischer Methoden konnten wir jedoch kein Serum finden, dass das ganze endogene *Pf*EMP1 erkennt.

Als Zweites, versuchten wir grosse Fragmente von *Pf*EMP1 in *E.coli* zu exprimieren um dann Seren von verschieden Malariafällen darauf zu testen. Zur selben Zeit wollten wir zufällige Fragmente von *Pf*EMP1 in einer *E.coli* Bibliothek exprimieren, um die selben Seren darauf zu testen. Unüberwindbare Probleme mit der Expression von grossen, rekombinanten Proteinen zwangen uns dazu unseren Ansatz in die Richtung kleinerer Domänen zu konzentrieren.

Hierfür isolierten wir var mRNS von Proben die aus asymptomatischen und schweren Malariafällen stammten. Aus dieser RNS wurden 14 DBL Domänen rekombinant exprimiert und die Antikörpertiter von 100 Seren von Erwachsenen aus Papua Neu Guinea gemessen. Die Frequenz der Antigenerkennung wurde gemessen und zwischen DBL Domänen die aus asymptomatischen und schweren Malariafällen isoliert wurden, verglichen. Wir haben herausgefunden, dass DBL Domänen die aus schweren Malariafällen stammen signifikant öfter erkannt werden, als DBL Domänen, die aus asymptomatischen isoliert wurden. Das ist bezeichnend für teilweise immune Erwachsene, die keine klinischen Malariasymptome mehr zeigen, da sie gegen die DBL Domänen, die in schweren Fällen exprimiert sind, geschützt sind.

Des Weiteren wurden 34 Kinderseren aus einer logitudinalen Studie aus Tansania getestet. Wir haben Seren getestet die an zwei Zeitpunkten gesammelt wurden, diese lagen sechs Monate auseinander. Damit konnten wir die Dynamik der Antikörperentwicklung beobachten. Die Frequenz der Antigenerkennung ist nur für die Gruppe der DBL Domänen die aus Schwerkranken isoliert wurden, gestiegen. Da diese Kinder nicht an klinischen Symptomen litten, zeigen diese Resultate, dass

Antikörper gegen DBL Domänen die in schweren Fällen exprimiert werden, schneller aufgebaut werden und möglicherweise vor schwerer Malaria schützen.

Zusammenfassend, unsere Ergebnisse unterstützen die Empfindung, dass die Entwicklung von Antikörpern gegen *Pf*EMP1 Varianten (in diesem Fall die DBL Domäne) die in schweren Malariafällen exprimiert sind, schützend wirken und so zu einem wichtigen Faktor der klinischen Immunität beitragen. Diese Ergebnisse geben Hoffnung zur Annahme, dass es möglich ist ein Impfstoff gegen den wichtigsten Virulenzfaktor *Pf*EMP1 herzustellen.

Chapter 1: Introduction

Introduction

The disease called malaria

In the year 2002 2,2 billion people were at risk of malaria infection, resulting in over 500 million clinical cases and more than one million deaths. Sub-Saharan Africa has the largest burden of malaria and it accounts for 70% of all malaria cases worldwide. Malaria is both a disease of the poor and causes poverty. Poor people cannot afford measures to prevent or treat infection and have often no instant access to health facilities. In addition, absence from workplace or school because of malaria episodes diminishes income and education, which turns the spiral of poverty [1, 2].

Protozoan parasites from the genus *Plasmodium* cause malaria. *Plasmodium* parasites are transmitted by a bite of an infectious female *Anopheles* mosquito. Malaria is a threat for almost all vertebrates including humans, monkeys, birds, reptiles and rodents. Among the numerous different *Plasmodium* species only four can establish a clinically relevant infection in humans: *Plasmodium falciparum*, *P.vivax*, *P. malariae* and *P. ovale*. P. *falciparum* (malaria tropica, later abbreviated malaria) causes the most severe clinical symptoms in humans. The reason for the higher virulence compared to the other species is that P. *falciparum* has the ability to adhere to the endothelium of blood vessels thus blocking microcirculation. Additionally, it has the highest reproduction rate, releasing up to 24 merozoites per cycle and it is able to invade all-age erythrocytes resulting in very high parasitaemia. There is no periodicity of fever as it is usually observed in infections in other *Plasmodium* species. *P.vivax* and *P.ovale* cause the comparably mild malaria tertiana were two fever days are followed by one fever-free day normally without major complications. However, *P.vivax* morbidity and mortality are underestimated with up to 80 million cases per year and an increasing trend in mortality in Venezuela [3, 4]. Hypnozoites in the liver can cause relapses years after the first manifestation. Malaria quartana is induced by *P.malariae* and causes a four-day fever interval were 2 days with fever are followed by 2 day without fever. It is also a mild variant of malaria but can cause renal complications especially in children.

Human infections with *P.knowlesi* and *P.semiovale* are possible but very rare (personal communication).

Plasmodium falciparum life cycle

Plasmodium falciparum is a protozoan parasite from the phylum apicomplexa. These protists contain a unique set of organelles assembled in the apical complex. The apical complex contains the rhoptries, micronemes and dense granules, which are vesicular structures that contain enzymes and lipids secreted upon invasion. Another distinct and essential organelle is the apicoplast or plastid. The apicoplast is surrounded by 4 membranes and is thought to origin from a second endosymbiosis of a cyanobacterial chloroplast [as reviewed in 5]. However, in some publications an algae is mentioned as ancestor (for example [6]). The apicoplast has its own genome and is part of the lipid synthesis system. Most of the apicoplast proteins are encoded in the nucleus. It has no photosynthetic activity.

The lifecycle of *P. falciparum* is very complex and includes sexual and asexual reproduction. It involves two different hosts: a vertebrate e.g. a human and a mosquito from the genus *Anopheles*. The female anopheles injects saliva during a blood meal to prevent blood coagulation. Sporozoites are transmitted from the mosquito`s saliva into the blood stream of the human. The blood and probably the lymphatic system [7] transports the sporozoites into the liver where they invade hepatocytes. There they differentiate into hepatic schizonts and multiply (Figure 1 A). After 5-14 days the schizont ruptures and releases thousands of merozoites into the blood stream, which then invade red blood cells. In the erythrocytes the asexual reproduction begins. After invasion the merozoite grows and develops into a small ring. The cell is in the G-phase and increases in size. This is the trophozoite stage. The transition into the S-phase with DNA duplication and membrane separation leads to the schizont, which is the last stage in the erythrocyte. The infected red blood cell (iRBC) ruptures and releases around 24 merozoites into the bloodstream. The cycle in the red blood cells lasts approximately 48 hours (*P.falciparum*). All clinical symptoms and severe effects are caused by the asexual cycle in the red blood cells. Not all merozoites will develop into schizonts. A small number will differentiate into male and female gametocytes (Figure 1 B). A female anopheline mosquito takes up these gametocytes during another blood meal. In the mosquito's

midgut the female gametocytes develop into macrogametes. The male gametocytes exflagellate and form microgametes which fertilize the macrogametes by fusion, forming motile zygotes called ookinetes. The diploid ookinetes cross the midgut membrane, undergo meiosis and adhere onto the exterior site of the gut wall. Here they undergo several rounds of mitosis to form oocysts (sporogony). Each oocyst releases thousands of motile haploid sporozoites into the mosquito's body cavity. From there sporozoites migrate into the mosquito's salivary glands. During a next blood meal they are injected together with the saliva into a new host and the cycle is completed.

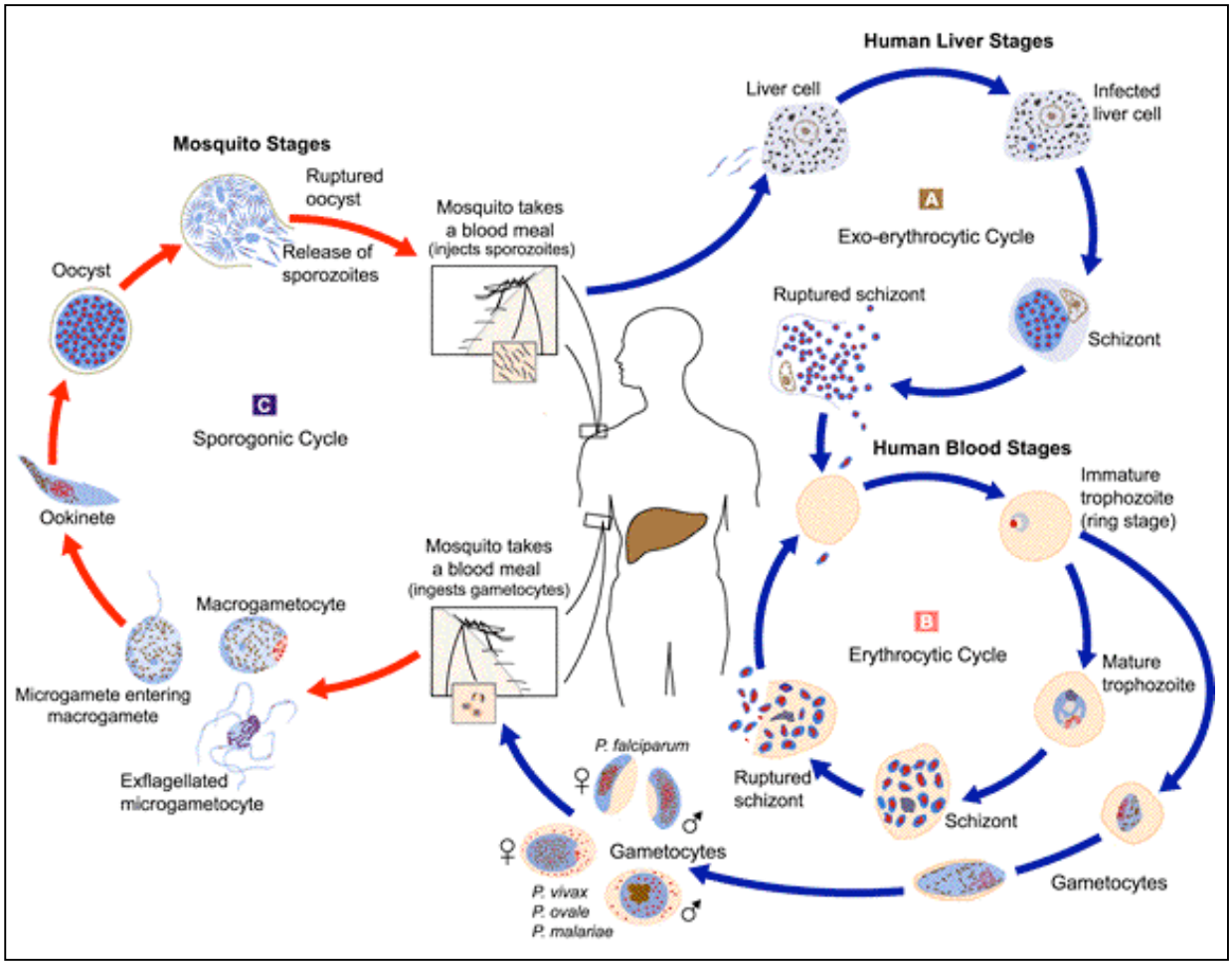


Figure 1. *Plasmodium falciparum* life cycle.

Schematic representation of the different stages of the life cycle of *Plasmodium falciparum*. In **A** the exo-erythrocytic cycle taking place in the hepatocytes is shown. In **B** the asexual replication in the erythrocytes and the development of gametocyes is shown. In **C** the cartoon shows the sexual reproduction in the mosquito and the

completion of the lifecycle by the inoculation of sporozoites into the vertebrate host upon a blood meal (Image modified from: Center for Disease Control and Prevention CDC, www.dpd.cdc.gov/dpdx).

Cytoadherence: Sequestration and rosetting make malaria severe

The clinical symptoms of malaria are exclusively caused by the asexual replication of *P.falciparum* in red blood cells. The pre-erythrocytic stages in the liver remain unnoticed. The common symptom of all malaria infections is high fever induced by rupture of infected red blood cells as termination of every cycle of asexual reproduction. The pyrogenic compounds released after ruption are grouped together as malaria toxins. Glycosylphosphatidylinositol (GPI) and haemozoin are the most discussed substances to act as pyrogens. Haemozoin induces endogenous pyrogens like TNF-α [8] and IL-1ß [9]. GPI can directly upregulate surface receptors like ICAM1 and VCAM1 and induce TNF and IL1 secretion of macrophages [10]. Cerebral malaria, a severe form of malaria, is thought to be induced by extensive TNF release [11].

The parasite is able to remodel the surface of the red blood cell and this remodeling enables the parasite to adhere to host endothelia. This causes blood clumping and oxygen deprivation of tissues and can lead to organ failure. Briefly, the integration of parasite proteins, especially Erythrocyte Membrane Protein 1 (*Pf*EMP1) into the erythrocyte plasma membrane mediates the interaction with a variety of host-cell receptors. This ability of the iRBC to bind to the vascular endothelium and to uninfected RBCs is called cytoadherence. Sequestration of parasites on the inner lining of the capillaries is essential for the parasite's survival, as iRBC would be cleared from the blood circulation during spleen passage. The effect for the host, however, is severe because the sequestered blood cells clog the thin blood vessels.

The host receptors which mediate binding to the iRBCs are numerous, including CD36, thrombospondin (TSP), VCAM-1, ICAM-1, PECAM/CD31, chondroitin sulfate A (CSA) and E-selectin [12-14]. The ICAM-1 receptor might play an important role in severe malaria as isolates from patients with cerebral malaria bind this receptor [13]. The presentation of ICAM-1 on endothelial cells is upregulated by TNF-α. TNF-α in turn is upregulated by the parasite itself as described above. However, the interaction between ICAM-1 with an iRBC is not strong enough to mediate binding

alone [15] For stable binding other receptors such as CD36 and TSP are needed simultaneously [16]. This is supported by experiments where binding of a parasite isolate to endothelial cells expressing both CD36 and ICAM-1 was only partially blocked by monoclonal antibodies against ICAM-1, but completely abolished with incubation of antibodies against both receptors [16].

A special case of infection is the pregnancy associated malaria (PAM). Semi-immune adult women, normally protected from severe disease, can develop a severe episode upon pregnancy and the fetus development can be impaired. The reason is the involvement of the placenta, which represents a new niche for the iRBC to adhere. The placenta is often heavily infected with sequestered parasites [17-19]. The high parasite load in the placenta is especially dangerous for the fetus, as it can lead to growth restriction, decreased birthweight or preterm delivery [20]. For the mother malaria infections during pregnancy are associated with severe anaemia [21]. This is perhaps induced by TNF release of the monocytes accumulated in the placenta. TNF is an inhibitor of erythropoiesis. Additionally oxidative stress by nitric oxide alters the erythrocytic membrane and leads to increased erythrocyte destruction [reviewed in 22].

The host receptor involved in placental iRBC sequestration is CSA. Parasites extracted from an infected placentas bind to CSA but not to other receptors commonly used by non-placental iRBC [18]. CSA in turn is not exploited by other iRBCs. The *Pf*EMP1 variant mediating binding to CSA is var2CSA [23]. Var2CSA is quite conserved even in isolates from geographically distinct areas. This could explain why antibodies against *var*2CSA can bind parasite isolates obtained from other regions than the antibodies [24].

Another ability of infected red blood cells is the binding to uninfected erythrocytes, called rosetting. It is thought that rosettes have a masking effect for the iRBC, as the iRBC is in the middle, and completely covered with RBCs so that no proteins (antibodies) or cells from the immune system can “see” or eliminate the pathogen. The proximity of RBCs to the bursting schizont might also be an advantage for the merozoites to more rapidly invade new cells. The aggregation of RBCs even enhances the negative effect of sequestration. Capillaries, which are already constricted by sequestered iRBCs, may be blocked completely by floating rosettes. This embolism-like obstruction of microcirculation also occurs in cerebral malaria

and is thought to be an underlying cause of coma [25]. It is thus not surprising that 50% of all wild type isolates show rosetting *in vitro* [26] and the rosetting rate is much higher in isolates from cerebral malaria compared with mild cases [27, 28]. The host receptors involved in rosetting are the blood group antigens A and B, complement receptor 1 (CR1), CD36 and glucosaminoglycans (GAGs) [29]. It is noteworthy that all of these receptors are glycosylated, which seems to be crucial for interaction. Additional serum factors are necessary as bridging molecules [30]. Luginbühl *et al.* showed that albumin, factor D and anti-band3 IgG are sufficient to restore the rosetting phenotype as in complete serum [31].

Clumping of iRBCs is mediated via platelets and the involved receptor is CD36. However, not all CD36 binding isolates show this phenotype, thus indicating the involvement of other receptors. The reason for the parasites to clump is yet unknown but has been shown to be associated with severe disease [32].

Association of host receptors with domains of *Pf*EMP1

The interaction of *Pf*EMP1 with the large number of different host receptors requires a large number of binding domains in the parasite protein. For a number of receptors the binding sites are already mapped (see Figure 3). The binding to the host receptor ICAM1 is mediated by the DBL2β-C2 region [33]. The binding to ICAM-1 is associated with cerebral malaria [34].

Another receptor which shows strong interaction with *Pf*EMP1 is CD36. The CIDR domain is the interaction partner for this receptor [35, 36]. CD36 is not abundant in the brain. The receptor is responsible for sequestration in organs other than the brain. In contrary to ICAM1 expression, the receptor is not sensitive to IFNγ or TNFα (reviewed in [25]).

The interaction with CSA, important for PAM, is mediated by DBL3γ [17, 37]. It seems that parasites expressing *Pf*EMP1 variants binding to CSA are rare and only have evolutionary advantages in pregnant women.

The DBL1α domain is exploited by CR1 for rosetting and by heparin sulfate for sequestration in the aorta [38, 39].

The binding of *Pf*EMP1 to different host receptors is extensively reviewed in [25, 40, 41].

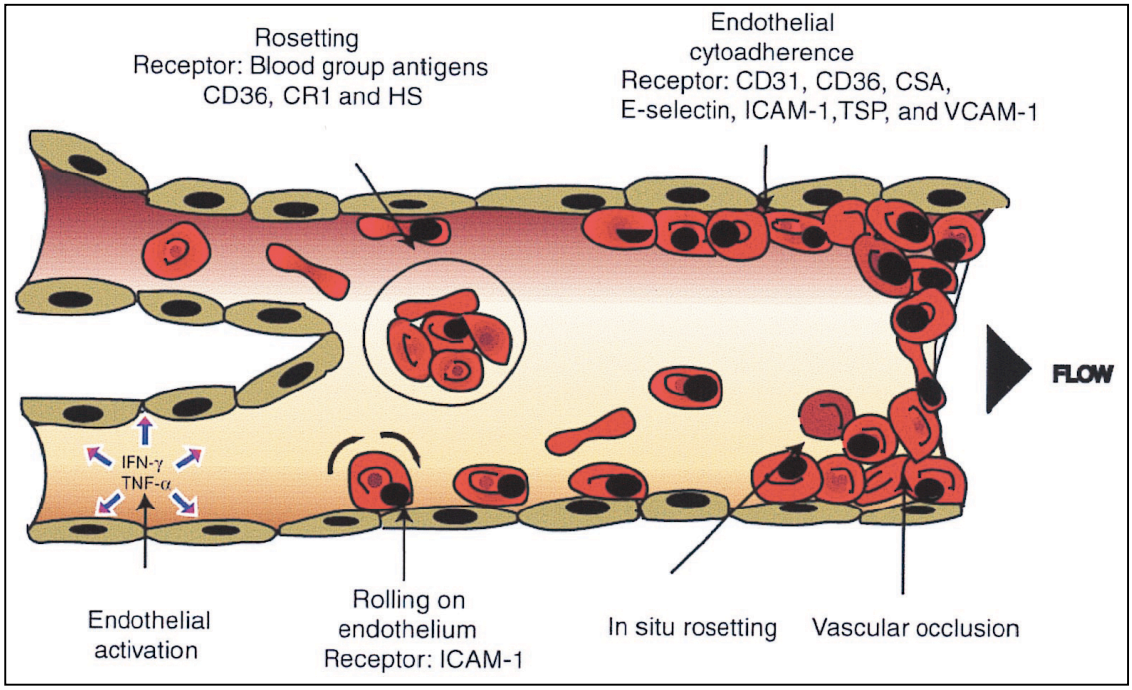


Figure 2. Schematic illustration of cytoadherence.

The cartoon shows the cellular basis of impaired microcirculation in the post-capillary venules due to sequestration of infected red blood cells and rosettes. The parasite induces the release of cytokines, which up-regulate receptors such as ICAM-1 necessary for endothelium binding (modified from [25]).

Structural details of *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (*Pf*EMP1)

P. falciparum expresses the large protein *Pf*EMP1, which is 200-350 kDa in size and is exported from the parasite to the erythrocyte surface. *Pf*EMP1 is encoded by the *var* gene family and is highly divers. About 60 different *var genes* are present in a haploid parasite genome. Only one *var* gene is expressed at a time in a mutually exclusive manner (*var* regulation: see next section). The architecture of *Pf*EMP1 is complex. It is a single-pass transmembrane protein. The extracellular part protruding from the erythrocyte membrane into the host's blood plasma is very variable. It is built from different blocks: the N-terminal segment (NTS), the Duffy binding like domain (DBL), the cysteine rich inter domain region (CIDR) and the C2

domain (see Figure 3). The NTS domain is semi-conserved and is located at the very N-terminal end of *Pf*EMP1. The DBL domain itself is classified into 5 sub classes indicated by a Greek letter (α-ε) [42], where DBL1-α represents the first domain after the NTS. The classification was done according to conserved sequence stretches and to conserved cysteins in the sequences (see Figure 4A). The CIDR consists of semi conserved stretches and is located between DBL domains and in special cases followed by a C2 domain. There are 3 different types of CIDR domains (α-γ) (see Figure 4B). The intracellular part, the acidic terminal sequence (ATS), is rather conserved and may function as an anchor by interaction with RBC skeleton proteins and additional parasite proteins such as KAHRP and *Pf*EMP3 [43, 44].

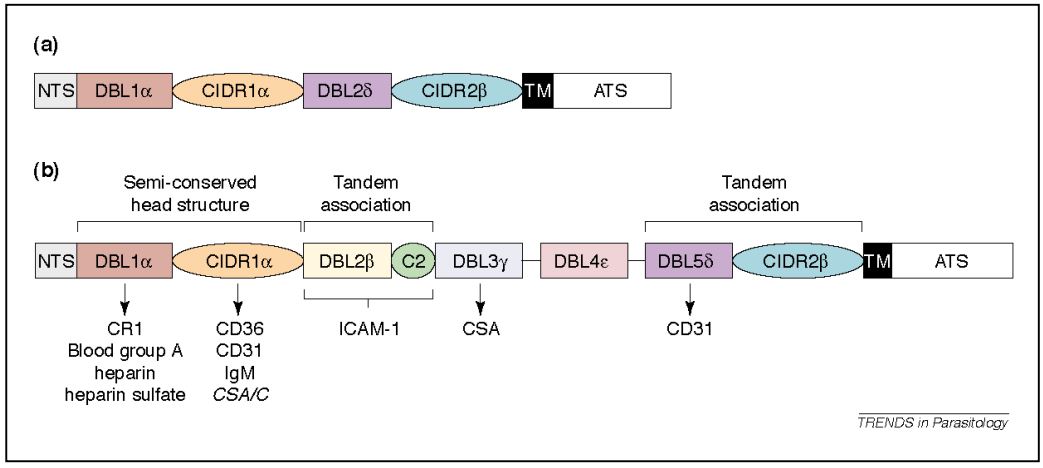


Figure 3. Illustration of *Pf*EMP1 domain structure.

(a) A small *Pf*EMP1 protein is shown consisting of the minimal arrangement i.e. the NTS domain, the DBL-CIDR tandem repeat and the ATS domain. In (b) a larger variant is shown. Here the C2 domain and higher order DBL domains are also included. The host cell receptors involved in binding at the respective domains are indicated (Figure from [41]).

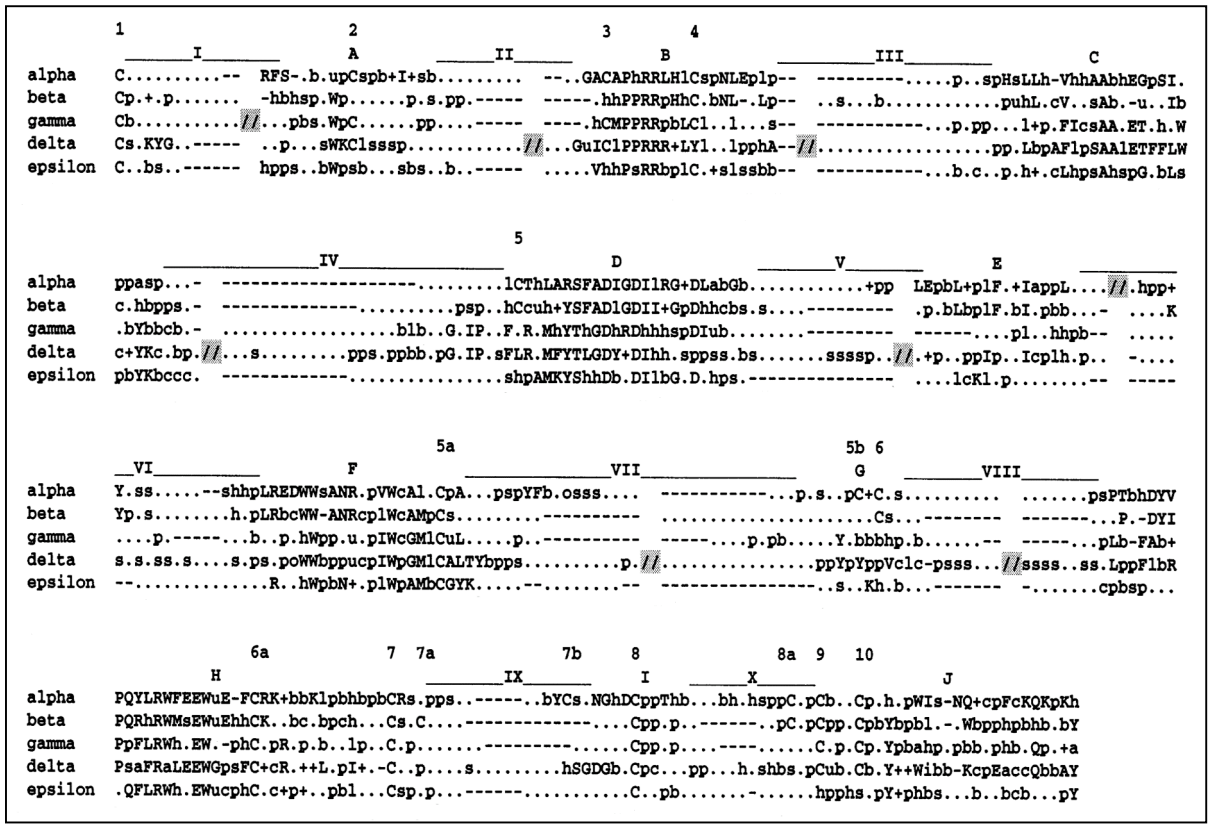


Figure 4A. Alignment and classification of DBL domains.

DBL domains have been grouped according to their conserved regions (capital letter A-J) and their conserved cysteins (arabic numbers 1-10). Variable domains are indicated with dots and lines (roman numbers). Capital letters in the sequence indicate amino acids, small letters amino acid types: c (charged: D, E, H, K, R), + (positive: H, K, R), h (hydrophobic: A, C, F, I, L, M, V, W, Y), p (polar: C, D, E, H, K, N, Q, R, S, T), s (small: A, C, D, G, N, P, S, T, V), u (tiny: A, G, S), b (big: E, K, R, I, L, N, S, Y, W) (Figure from [42])

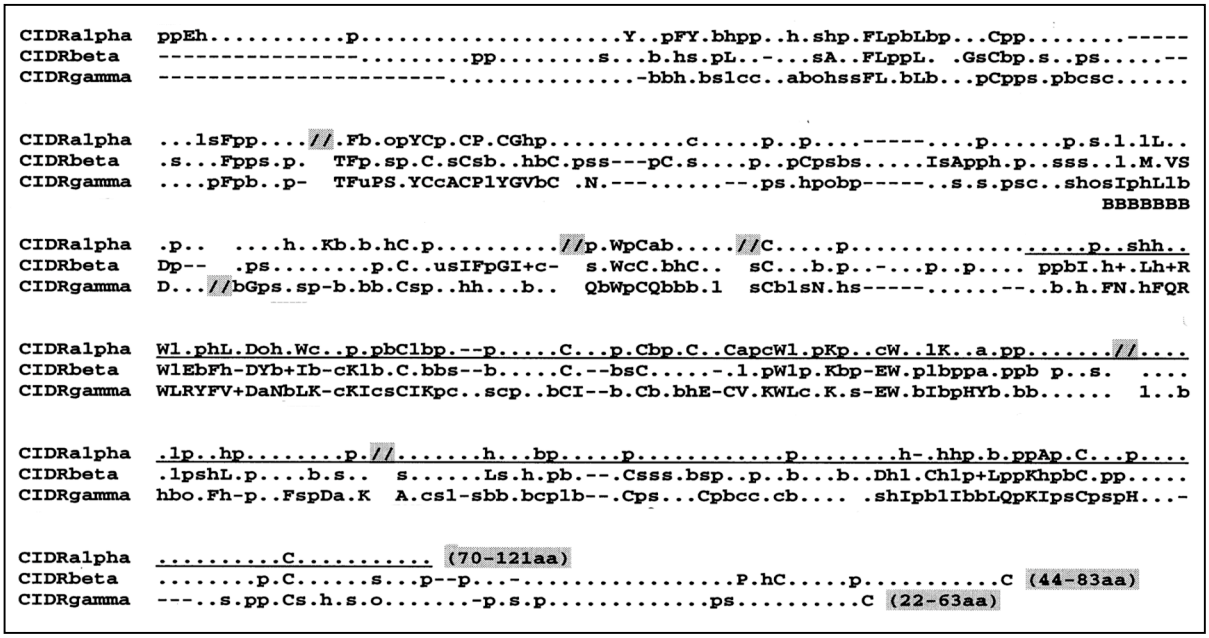


Figure 4B. Alignment and classification of CIDR domains.

CIDR domains classified according to semi conserved regions. Backslash indicates gaps in the sequence alignment. The amino acid code is the same as in Figure 4A (Figure from [42])

Knobs on the host cell surface

The insertion of *Pf*EMP1 on the RBC surface is not evenly distributed but instead packed into electron dens structures called knobs (Figure 5). These knobs are disributed over the surface of an infected RBC and visible by electron microscopy (see Figure 6) [45, Figure 6]. The knobs are the contact points in cell-cell interaction [25, 46]. Knob-less parasites are unable to adhere to endothelial cells under flow conditions [44]. Numerous parasite proteins are assembled in the inner face of the knobs including the knob associated histidine-rich protein (KAHRP) and *Pf*EMP3 [47]. It is thought that these proteins built up the knobs as it has been shown that KAHRP knock out parasites are knobless [43, 44] and anchor *Pf*EMP1 in the RBC. It has been shown that the ATS domain binds to spectrin, actin and KAHRP [48]. KAHRP in turn also binds to spectrin [49].

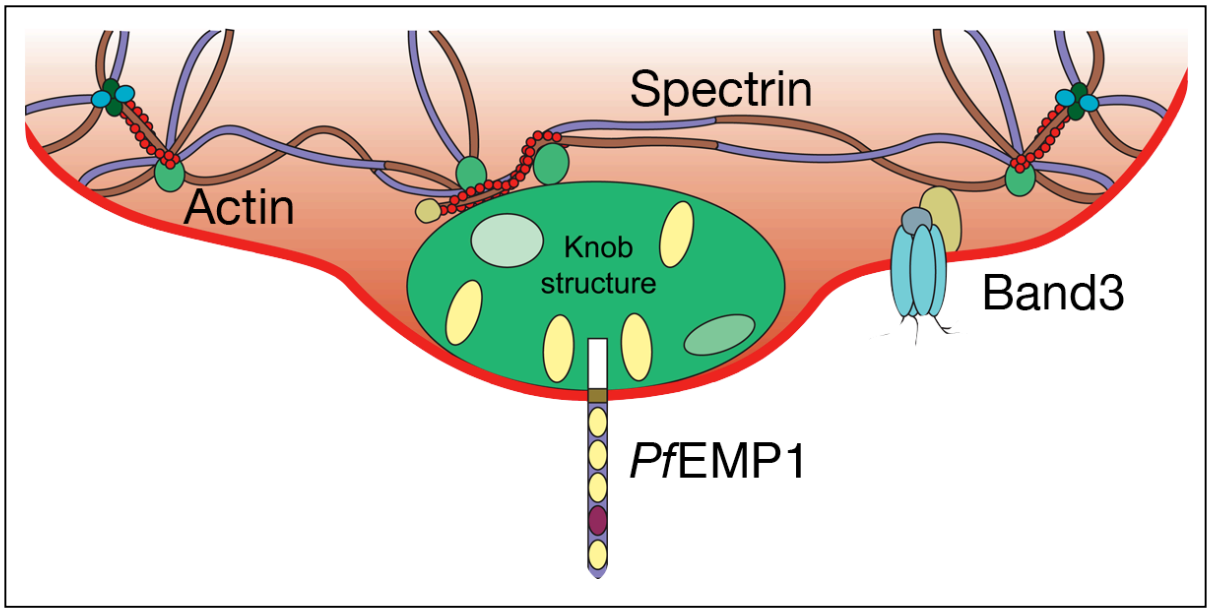


Figure 5. Schematic representation of a knob structure.

The cartoon shows the structure of a knob. The thick red line represents the erythrocyte plasma membrane. *Pf*EMP1 is inserted in the membrane and anchored probably via *Pf*EMP3 (yellow oval) and KAHRP (in green) and attached to the cytoskeleton at spectrin/actin junctions. The whole knob structure may contain additional proteins; this is indicated with the big green circle (knob structure).

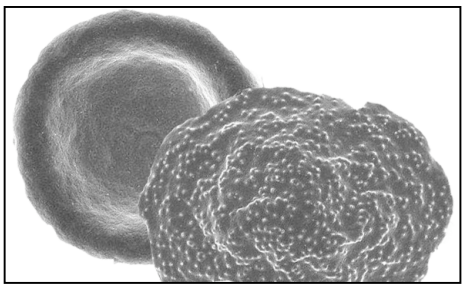


Figure 6. Electromicrograph images showing knobs on an infected erythrocyte.

Depicted are two erythrocytes where the backmost cell is uninfected and has a smooth surface, and the infected cell in front shows the knobby surface.

(Figure adapted from http://www.scidev.net/scidev_images/black-and-white.jpg)

Regulation of *var* gene expression

*Pf*EMP1 is encoded by the *var* multigene family. This gene family consists of approximately 60 genes per haploid genome but with an almost unlimited repertoire. Almost 70% of all *var genes* locate close to the telomeres at the end of the chromosomes, the rest is found near the centromers in the middle of the chromosome [50]. The *var* genes at the telomers are normally arranged in a tail-to-tail orientation, whereas the central *var*s are tandem repeats and thus show a head-to-tail arrangement. The direction of transcription and the location on the chromosome can be predicted by their 5` non-coding sequence [51]. The upstream sequences (Ups) are arranged in four groups UpsA, UpsB, UpsC and UpsE [52]. UpsA and UpsE *var* genes are subtelomeric and transcribed towards the telomere in contrast to UpsB *var* genes, which are subtelomeric and transcribed towards the centromer. UpsB *var* genes are also present in the central region together with UpsC (see Figure 7). The role of the different upstream regions is not yet completely clear. Voss *et al*. found conserved sequence elements in the promoter of upsB and upsC *var* genes. The subtelomeric *var* promoter element (SPE) is unique for upsB *var* genes and the chromosome-central *var* gene promoter element (CPE) for upsC genes. It was also found that transcriptional regulation of *var* genes is dependent on their chromosomal location. Sub telomeric *var* genes are only expressed up to 18 hours post infection whereas transcription of central *var* genes lasts 4-8 hours longer [53]. Expression of *Pf*EMP1 from different chromosomal located *var* genes was also correlated with morbidity. There is evidence that UpsA and UpsB *var*-gene expression (sub telomeric) is associated with severe disease in children in Tanzania [54] but this has only been proven for UpsB in samples from Papua new guinea PNG [55].

The regulation of *var*-gene expression is very complex. *var genes* are expressed in a mutually exclusive manner, with only one gene being expressed by an individual parasite at a given time [56]. This mechanism of antigenic variation helps the parasite to escape from the host's immune system. The switch from one *var* gene to another must be fast enough to evade the adaptation of the immune system but also as slow as possible to not exhaust the repertoire of *var genes* before being transmitted*. P.falciparum* does not undergo DNA rearrangement or gene conversion into an active expression site [57]. Transcription activation is restricted to a special

location in the nucleus and controlled by transcription initiation [56, 58, 59]. The mutual exclusive expression is not based on a negative feedback loop (e.g. *Pf*EMP1) but on the non-coding information in the 5` region [60]. This means that not the presence of the protein itself regulates the transcription, but factors including untranslated DNA sequences upstream of the translational start point. This was also proven by Voss *et al.* [51] by transfection of plasmids with a 5` region of a *var*-gene followed by a drug resistance gene. Upon drug pressure the parasites expressed the resistance gene under the control of the *var* promoter. These parasites did not express *Pf*EMP1 anymore; hence, the artificial promoter was filling the only transcription place for *var genes*. Proteins and transcription factors involved in this unique regulatory system are currently under investigation.

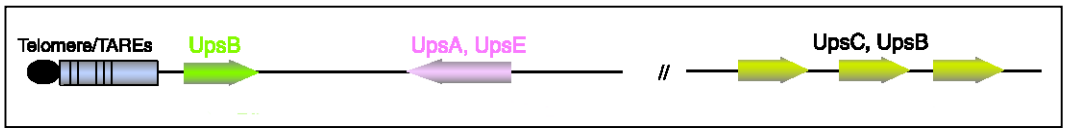


Figure 7. Chromosomal organisation of *var genes*.

The orientation of *var genes* at different locations within a chromosome are depicted. The black dot on the left is the telomere followed by the telomere associated repeat clusters (TARE). In green and in purple the subtelomeric *var genes* are shown and in yellow the central *var* genes are shown (Figure from [61])

Aims of this thesis

Improve recombinant protein expression of plamodial peptides in *E.coli*

Heterologous expression of proteins is a commonly used technique to produce antigens for molecular biological examinations. There are more and more heterologous expression systems available like yeast, mammalian cells, insect cells, or even *Dictyostelium discoideum* but the most widely used system is still *Escherichia coli*. For protein expression in *E.coli* a large number of cell lines and expression vectors are readily available. The transfection and cultivation of *E.coli* is simple, fast and cheap. Problems can arise when conformation and modification of recombinant proteins are important, because *E.coli* is a prokaryote and has different folding and modification patterns than eukaryotes. Misfolding can also lead to solubility problems and to increased toxicity for the bacteria, resulting in low expression levels. Recombinant expression of plasmodial proteins brings along additional problems, as the genome of *P.falciparum* has an AT content of over 80% and that of *E.coli* has about 50%. The translation machinery of *E.coli* uses other triplets to code for amino acids as *Plasmodium*. As codons used by *Plasmodium* are rare in *E.coli* there are bacterial cell lines containing additional plasmids coding for those rare tRNAs.

In my PhD I wanted to express differently sized fragments of *Pf*EMP1 in *E.coli*. I tested various vector systems as well as different cell lines and cultivation media. The expressed proteins have been used as antigens for serological studies and as antigens for the induction of antibodies in mice.

Screen for morbidity associated antigenic regions in *Pf*EMP1

Clinical immunity against *falciparum* malaria is conferred by a repertoire of antibodies. It is believed that the major protective effect is directed against infected erythrocytes and thus against *Pf*EMP1. This repertoire is acquired during childhood with repeated episodes of malaria. These episodes can be numerous and severe in young children but once the critical period of the incomplete antibody repertoire is overcome, the individual is mostly protected from clinical symptoms. However the number of infections needed to establish a protecting patchwork of antibodies seems to be small compared to the endlessness of possible surface antigen variants. That implies that it is not necessary to "see" all possible isoforms of *Pf*EMP1 to build up immunological protection. There is evidence that there is a relative small subset of variants, which are more virulent but also more frequent than others and after contact with these types the individual is partially protected.

In my PhD I was aiming to identify these variant *Pf*EMP1 domains responsible for pathology and severe disease. I used two different approaches to study differential recognition of sera from children with an incomplete antibody repertoire and sera from clinically immune adults. Firstly, I aimed to clone and express large fragments (above 1000 amino acids) of the 3d7 parasite line in *E.coli*. Recognition frequencies of these recombinant proteins should be recorded and compared between non-immune (incomplete) and immune (complete) sera in order to find differentially recognized variants. In the second approach I aimed to generate a random cDNA *E.coli* expression library. To ensure the expression of *Pf*EMP1 fragments only, selected full-length *var* mRNA was used as starting material for cDNA synthesis. The rational was that with this approach not only the N-terminal part of the large *Pf*EMP1 protein could be tested but any individually random fragment from anywhere in the protein, not restricted to boarders of domains. It was planned to detect any differential recognition directly on comparative colony plots of this library.

In addition to the random approach, we focused on DBL domains from different field isolates. DBLα domains are the most N-terminal domains in *Pf*EMP1 and are present in most variants. DBLs have conserved sequence stretches intermitted by highly polymorphic regions and are known to bind to CR1, blood group antigen A and heparin. The association of the DBL variant with disease is not yet completely

clear. However, Kirchgatter *et al.* correlated DBLs containing 2 cysteins (in a certain position) with severe disease [62].

We wanted to test for differences in recognition of DBL domains expressed in severe case malaria and in asymptomatic infections. First, we had isolated parasite RNA from malaria cases with different clinical manifestation. DBL domains from these cases were also sequenced. Sequence alignment showed no evidence of clustering of DBL sequences which would have revealed an association with certain clinical groups. As sequence differences were not obvious, we searched for differential recognition of recombinant DBL domains derived from severe cases or asymptomatic cases. We used adult sera from a cross sectional study and children sera from a longitudinal follow up study over 6 months with monthly intervals. We used samples from baseline and month 6. We wanted to test our hypothesis that DBL domains from severe cases are more frequently recognized in semi immune adults than DBL domains coming from asymptomatic patients. In the longitudinal study we were interested in the dynamics of recognition of DBL domains at baseline compared to samples from month 6.

Development of pan specific anti-*Pf*EMP1 antibodies

Work on *Pf*EMP1 is extremely hampered by the lack of specific non cross-reactive antibodies. Most of the available antibodies have strong cross-reactivity with human spectrin subunits, which can have a similar size as *Pf*EMP1. Thus it was another aim of my thesis to generate pan-specific antibodies against *Pf*EMP1 in mice, that are not cross-react with human proteins. We used different domains from various *Pf*EMP1 molecules to strive our aims. We selected the NTS and the ATS domain of strain FCR3 S1.2 to be recombinantly expressed. Furthermore, we designed and tested synthetic peptides (with modifications) from conserved regions in the ATS domain as antigens.

The lack of antibodies also delayed ongoing work in the analysis of the molecular interaction mechanisms in the formation of rosettes.

Chapter 2:

Objectives of this thesis

Objectives of this thesis are:

1 To optimize recombinant protein expression of plasmodial proteins in *E.coli.*

2 To screen for relevant antigenic regions in *Pf*EMP1 influencing morbidity.

3 To generate mouse polyclonal antiserum against *Pf*EMP1.

4 To perform localization studies on *Pf*EMP1.

5 To identify the cleaved fragment in rosetting and the role of *Pf*EMP1.

6 To assess recognition frequencies of DBL domains in different sera.

Chapter 3:

Protein expression and characterization of different mouse antisera

Introduction

Basic cell biology of *Plasmodium falciparum*

Invading merozoites adhere to the erythrocyte surface and reorient themselves so that the apical end points to the erythrocyte membrane [63]. During invasion the rhoptries, the dense granules and the micronemes release their contents, which mediates the invagination by tight junction formation [64] and red cell cytoskeleton disruption by proteases [65]. The invasion event encloses the merozoite in a parasitophorous vacuole (PV) delineated by a parasitophorous vacuolar membrane (PVM) which consist of host and parasite derived material [66] and persists during the complete intra-erythrocytic development. The parasitophorous vacuolar membrane is the interface and thus important interaction site between the parasite and the host. Within the PV the parasite develops from the small ring stage to the larger trophozoite stage and the host cell is considerably modified. The parasite feeds on haemoglobin and deposits its waste products (haemozoin) in the food vacuole. Additionally, new parasite derived membraneous structures form in the erythrocyte cytosol in close proximity to the host cell membrane. These organelles are called Maurer's clefts (as reviewed in [67]). Furthermore, the surface of the host cell membrane is extensively modified by insertion of parasite derived proteins and formation of protrusions visible by EM (electron microscopy). These protrusions are called knobs (see Figure 1) and it has been shown that *Pf*EMP1 (*Plasmodium falciparum* erythrocyte protein 1) is anchored in these knobs and thus knobs are important for cytoadherence [44]. The protein essential for knob formation is the knob associated histidine-rich protein (KAHRP) [43, 44].

As the parasite matures, the food vacuole with the haemozoin becomes visible in light microscopy. Visible brownish crystals consist of ferriprotoporphyrin dimers and are coloqually termed "malaria pigment". After the transition from trophozoite to schizont the parasite takes up the complete space in the red blood cell, the Maurer's clefts are pushed against the RBC membrane and grape like compartments become visible, which are the merozoites. The erythrocyte bursts and releases the merozoites.

For the remarkable restructuring of the host cell, the parasite has to transport proteins beyond its confines. This is a complex and not yet fully understood process, which will be discussed below.

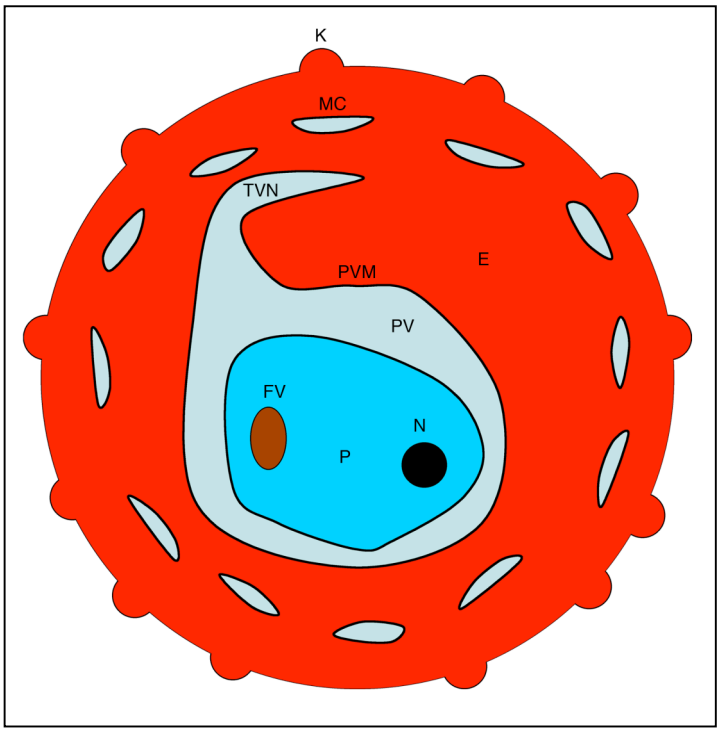


Figure 1. Schematic representation of an infected red blood cell.
The cartoon shows a simplified picture of a cross section of an infected erythrocyte. The parasite (P) contains the nucleus (N) and the food vacuole (FV) and is surrounded by the parasitophorous vacuole (PV). Beyond the parasitophorous vacuolar membrane (PVM) the Maurer's clefts (MC) are visible in the erythrocyte (E) cytosol. The protrusions on the erythrocyte surface are the knobs (K).

Protein trafficking in *Plasmodium falciparum*

During the asexual life cycle *P. falciparum* resides within a parasitophorous vacuole within the erythrocyte. Living in that special cell type implies advantages as well as disadvantages. The biggest benefit for the parasite is the protection from the host's immune system. The red blood cell contains no nucleus and protein synthesis machinery anymore and thus is not able to present antigens of an intracellular parasite on the surface via MHC I. The intracellular parasite remains invisible to cytotoxic T-cells. However, an infected erythrocyte looses its flexibility and is more

rigid. This and modifications of the RBCs surface lead to clearance by the spleen [68]. The parasite has to trade off some of its shelter to prevent spleen passage by cytoadherence. The iRBC adheres to the endothelial cells of the blood vessel by interaction between host cell receptors and the parasite protein *Pf*EMP1. The integration of *Pf*EMP1 into the RBC membrane is a complex procedure and leads directly to the negative aspects of living in a denucleated cell: the parasite has to build up completely new protein transport machinery.

This new transport machinery has to fulfill complex tasks. As the parasite resides in a parasitophorous vacuole, proteins targeted to the red cell plasma membrane must not only be transported through the parasites membrane but also through the PVM. Despite the discovery of the export signal sequences (the export element, PEXEL [69], or the vacuolar transportation signal VTS [70]), the mechanisms of this transport remain to be elucidated [71]. These signals consist of a short hydrophobic part in the very N terminal part followed by differently charged amino acids like +xϕx- i.e. RxLxE. To complicate a researchers life, there are some proteins transported beyond the confines of the PVM without carrying one of the mentioned signal sequences, among those is MAHRP1 (membrane associated histidine-rich protein 1) [45] and *Pf*EMP1 [71]. Parasite derived organelles which may play a role in protein transport are the Maurer's clefts (MCs). These vesicle-like membranous structures are located under the erythrocytes membrane. Many of the exported proteins are located at the MC either transiently like *Pf*EMP1 and the knob associated histidine-rich protein (KAHRP) [47] or terminally like MAHRP1 [45, 72] or the skeleton binding protein 1 (SBP1) [73]. The latter one seems to be important for the transport of *Pf*EMP1 from the MC to the erythrocyte surface. In a SBP1 knockout strain the *Pf*EMP1 transport is arrested at the MC [74], however in another study the point of arrest seems to be the PVM [75].

The transport of *Pf*EMP1 to the red cell surface includes different trafficking intermediates. It is described that *Pf*EMP1 is transported as soluble protein from the ER through the PM and PVM and then gets increasingly insoluble on its way to the MC clefts. It is inserted in the MC membrane with the C-terminal domain facing the erythrocyte cytoplasm. Here it complexes with KAHRP and is then transported to the erythrocyte membrane [76].

Since no specific and non-cross reactive antibody against *Pf*EMP1 exists, we tried to generate good pan specific anti-*Pf*EMP1 antibodies to further perform localization and trafficking studies on the major virulence factor *Pf*EMP1.

Mechanisms of rosetting of *Plasmodium falciparum*

The spontaneous binding of uninfected red blood cells to iRBC is called rosetting. Rosetting is associated with severe malaria and in Africa especially with cerebral malaria [27, 28]. In Papua New Guinea PNG no correlation of rosetting and severe disease was found [77] probably due to CR 1 receptor deficiency in 79% of the population [78]. The reason for the severe effects of rosetting is most likely the blocking of blood flow in the capillaries and the resulting oxygen lack in the tissue i.e. in the brain. Luginbühl *et al.* [31] showed that factors in the serum mediate rosette formation. Their work showed that after mechanical rosette disruption, rosetting of the culture strain FCR3 S1.2 could be completely restored by the addition of complement factor D, albumin and anti-band 3 NAbs (naturally occurring Antibodies). As the effect of these proteins is additive compared to the incubation with only one, it seems that there are different interaction partners on the iRBC and the RBC. The complement factor D is a serine protease and it was investigated if proteolytic effects are necessary for rosetting. It was very surprising that there was a 65kDa fragment cleaved from the iRBC surface since the only known substrate for factor D was factor B. However, the fragment has not yet been identified and remains to be elucidated from which parasite protein this peptide was cleaved off.

In order to identify potential candidates cleaved by factor D we aimed to generate anti-*Pf*EMP1 antibodies to further investigate if the processed protein is *Pf*EMP1.

Recombinant protein expression of plasmodial proteins

For the generation of antibodies it is crucial to express the antigen in reasonable large amounts and as pure as possible. We chose *E.coli* as a heterologous expression system for the expression of different protein domains from *Pf*EMP1. The difference of the two organisms *Plasmodium* and *E.coli* implies problems in protein expression. This typically results in lack of expression or in insoluble inclusion bodies [79]. In some cases hundreds of different refolding buffers had to be tested

to obtain a soluble protein from inclusion bodies [80]. Reasons for these expression problems are the high AT content of 80% in *Plasmodium* DNA and the resulting different codon usage compared to *E.coli*. Additionally, genes are often larger (50%) than their homologous in i.e. yeast [81] and possess long disordered regions [82]. The translational start sites of plasmodial proteins are also sometimes cryptic resulting in multiple truncated products in *E.coli* [83]. Because all these difficulties, the group of Mehlin *et al.* [83] cloned 1000 open reading frames to find a universal rule to predict the expressability and the solubility of proteins from *P.falciparum* expressed in *E.coli*. However, they concluded that there were no such general result, but they could align physical features of the protein with expression problems. Only 30% of all clones expressed a protein and only 63 were soluble. Increasing molecular weight, increasing pI (isoelectric point), greater protein disorder and lack of *E.coli* homology were all highly and individually correlated with expression problems. It was also reported that induction of an expression culture at post-log phase is advantageous compared to the usual induction at mid-log [79].

In this study we attempt to clone and recombinantly express protein domains of *Pf*EMP1 for subsequent serological studies. We attempted to generate a random *E.coli* expression library for *Pf*EMP1 fragments. Furthermore, we generated polyclonal mouse sera against different domains of *Pf*EMP1.

Chapter3

Materials and methods

In vitro cultivation of *Plasmodium falciparum*

Plasmodium falciparum strain 3D7 was cultivated in RPMI 1640 medium supplemented with 25mM HEPES, 0.5% Albumax II, 50mg/l hypoxanthine, 0.25% sodium bicarbonate, 10µg/ml neomycin sulphate and 0+ red blood cells at 5% haematocrit. Cultures were incubated at 37°C in an atmosphere of 3% oxygen, 4% carbon dioxide and 93% nitrogen as described previously [84].

Plasmodium falciparum strain FCR3 S1.2 was cultivated with 10% human AB+ serum instead of Albumax II.

Parasites were synchronized by 5% sorbitol treatment as described by Lambros and Vanderberg [85].

Enrichment of late stage parasites using a magnetic cell sorter

A MACS CS Column (Miltenyi Biotec) was assembled in the magnetic cell separator VarioMACS (Miltenyi Biotec) and flushed with 60ml of PBS according to the manufacturers protocol. A 22G hypodermic needle was used as flow resistor resulting in a flow rate of 3ml/min. 10 to 50ml resuspended late stage *Plasmodium* culture was run through the column. The column was washed with 3 culture volumes PBS. The flow through was discarded. The column was removed from the magnetic field, the flow resistor was removed from the column and the retained iRBCs were eluted from the steel wool by flushing with 50ml PBS. Eluted parasites were centrifuged at 4000g at 4°C for 10 min. The supernatant was discarded and the parasites stored at -20°C until use.

Plasmodium falciparum protein extraction

Saponin lysis

10ml of a *Plasmodium* culture with a parasitaemia of 3-7% were harvested by centrifugation at 1000g for 5 minutes at room temperature. Ice-cold PBS containing 0.05% saponin was added to the pellet and incubated on ice for 5 minutes. The lysed culture was centrifuged at 4000g for 10 min at 4°C and subsequently washed

with PBS until the supernatant was clear. The washed pellet was stored at -20°C until use.

Triton X-100 extraction

10ml of a *Plasmodium* culture with a parasitaemia of 3-7% was harvested by centrifugation at 1000g for 5 minutes at room temperature. 5ml ice-cold PBS containing 1% Triton X-100 and a protease inhibitor cocktail (Complete®, Roche) were added to the pellet and incubated on ice for 5 minutes. The lysed culture was centrifuged at 4000g for 10 min at 4°C and the pellet subsequently washed with PBS. The pellet was resuspended in PBS containing 2% SDS and centrifuged for 10 minutes at 15000g. The supernatant containing the Triton X-100 insoluble and SDS soluble protein fraction (integral membrane proteins) was stored at -20°C until use.

Genomic DNA isolation of *Plasmodium falciparum*

Saponin lysed parasite pellets were resuspended in 600µl TE buffer (10mM Tris, 1mM EDTA, pH 7,4). Parasites were disrupted by adding 18µl 20% SDS (final conc.: 0,6%) and 6µl 20mg/ml Proteinase K (final conc.: 200µg/ml) and incubated at 60°C over night. The DNA was extracted twice with 2 volumes of a 1:1 mixture of aqua-phenol:chloroform followed by an additional extraction with chloroform only. The aqueous phase was precipitated with 0.3M NaAcetat pH 5.2 and 2.5 volumes 100% ethanol at -20°C.

Plasmodial RNA extraction and complete cDNA synthesis

The pellet from synchronized parasites (minimum 5% parasites in late ring stage) was resuspended in 5 volumes Trizol® (Invitrogen). The RNA was extracted from the lysate with 0.2ml chloroform per ml Trizol® and precipitated with 3 volumes of isopropanol. To improve purity the pellet was again resuspended in Trizol® (0.5 original volumes), extracted with chloroform and then precipitated. The precipitated DNA was digested by two subsequent incubations with RQ1 RNAse free DNAse (Promega) according to the manufacturers protocol in the presence of RNAse inhibitors. After each DNAse digest the RNA was extracted and precipitated as described above. As gDNA is a notorious contaminant in RNA extractions, a control PCR was introduced prior to cDNA synthesis. The control PCR contained 1µl from

the RNA preparation as template and degenerated dbl1α_fwd and dbl1α_reverse primers. These primers amplify most DBL domains from *Plasmodium falciparum* (personal communication M. Kaestli). A positive PCR control was also included. After initial denaturation (5 min 96°C) the PCR cycle was as follows: 30 seconds annealing at 52°C, 45 seconds elongation at 72°C and 45 seconds melting at 94°C, 25 cycles. If PCR (visualized by gel electrophoresis) resulted negative, the extracted RNA was considered suitable for cDNA synthesis.

cDNA synthesis was performed using either Sensiscript® or M-MuLV reverse transcriptase with random hexamer primers, in accordance with the respective manufacturers protocol. RNA amounts used in a cDNA synthesis reaction varied from below 100ng to 1µg. A control reaction without reverse transcriptase was always included.

General RNA reverse transcription

RNA samples either from beads hybridisation or crude were mixed with 3.5µl 0.1M DTT, 2µl RNAse inhibitor, 7µl 10x RT-Buffer (Sensiscript/M-MuLV), 7µl 5mM dNTPs and RNAse free dH_2O to 70µl. The reaction mix was split in a 50µl and a 20µl aliquot. To the 50µl aliquot 2µl of reverse transcriptase was added, the other was left without enzyme as negative control. Both tubes were incubated at 37°C for 90 min. After the reaction the RNA:DNA duplex was melted at 93°C for 3 min and the RNA was digested with 1µl of RNAse A (1mg/ml) at 37°C for 20 min. The resulting single strand cDNA was stored at -20°C.

Full length *var* mRNA extraction

To extract only the full-length *var* mRNA transcripts from a total RNA preparation a hybridization technique was used as described in the following: 1pmol of a biotinylated oligonucleotide complementary to the ATS domain was incubated together with the total RNA in hot (65°C) binding buffer (0.5M LiCl, 1mM EDTA, 10mM Tris, pH7.5). After slow cooling from 65°C to 4°C over 30 minutes, 200µg of streptavidine coated magnetic beads (Dynal) were washed, dissolved in 5.5M LiCl and added to the RNA:DNA hybrid and incubated for 30 minutes at 37°C on an over-head shaker. Beads were collected on the wall of the reaction tube by a surrounding magnet and washed 3 times with wash buffer (10mM Tris, 1mM EDTA,

0.15M NaCl, pH 7.5). Reverse transcription was performed directly on the RNA hybridized to the beads as described previously (General RNA reverse transcription, Material and Methods Chapter3).

cDNA amplification

To amplify minute amounts of full-length cDNA a modified SMART® (Clontech) system was used. To start the synthesis, a modified SMART random primer was used. This primer contains a random part of 6 nucleotides followed by a known sequence that works as primer sequence in downstream experiments. The reaction also contains a SMARToligo, which has 6 guanidine residues on its 3` and a known sequence on the 5` end. At the 5` end of the RNA the reverse transcriptase includes several cytosine residues to the growing strand of the cDNA. The SMARToligo pairs with the extended cytosine rich cDNA tail and thus serves as a second template for the reverse transcriptase to switch to. The resulting first strand cDNA contains known sequences at both the 3` and the 5` end of the cDNA (see also Figure 1 for details). A PCR using the primer pair SMART_PCR_fwd and SMART_PCR_reverse amplified the cDNA.

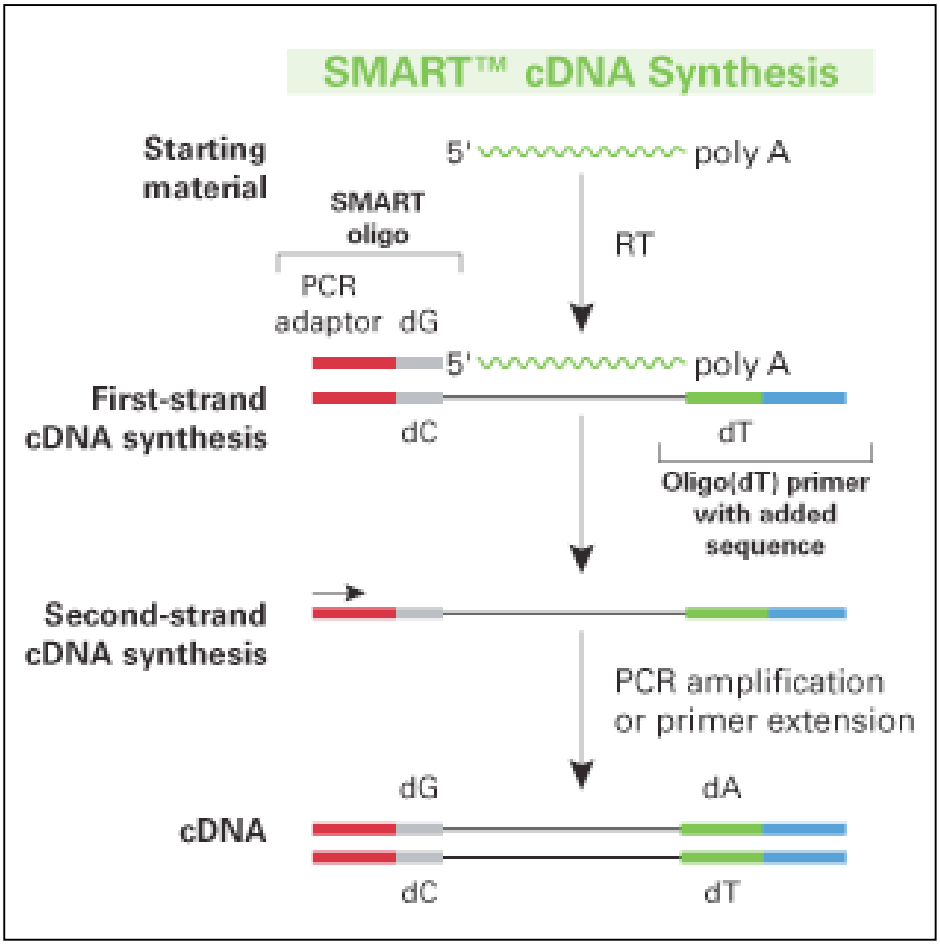


Figure 1. Schematic representation of SMART cDNA amplification.

(Source: www.clontech.com)

Polymerase chain reaction (PCR)

Regular PCR

A standard PCR reaction mix consisted of the following: 2U *Taq* polymerase, PCR Buffer BD (Solis Biodyne) containing 80mM Tris pH 9.4, 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, primer final concentration each 10µM, final concentration of each deoxynucleotide 2µM, and 10ng template DNA. The reaction volume was 50µl.

Long range PCR

As template for long range PCR, high molecular weight genomic DNA was used at a final concentration of approximately 50ng. A special reaction buffer containing 50mM Tris, 16mM ammonium sulfate, 2.5mM $MgCl_2$ and 150µg/ml BSA was used to maintain a constant pH throughout a wide temperature range. As polymerase a

mixture of 2.5U regular *Taq* and 0.5U proofreading *Pfu* was used. Primer concentration was 10µM and the final reaction volume was 50µl.

Agarose gel electrophoresis

0.8 to 2% agarose was boiled in 0.5x TBE buffer and poured into a gel chamber. DNA samples were loaded in 1 x blue juice (30% glycerol, a tip of spatula bromphenol blue and xylene cyanol, 70% TE) and run at 100V constant current for 1h and stained in ethidium bromide for visualization under a UV source.

Restriction digests and ligation

Restriction digests were performed in accordance with the manufacturers protocol using 10U of restriction enzyme in a final volume of 50µl.
For the preparation of the ligation mix, the following calculation was applied:

(10ng vector x size of insert in kb x 10) / size of vector in kb = ng insert.

The volumes of the insert and the vector were calculated according to their concentrations and mixed with 2µl ligation buffer and 1µl T4 ligase (Promega) to yield an end volume of 20µl. The reaction was incubated at room temperature for 30 minutes or over night at 16°C. After ligation the mixture was chlorofom:phenol extracted and precipitated for maximum purity.

Cloning of different expression constructs

(see also Figure 2 for cloning strategies)

Cloning of long range PCR products

A large set of different primers was tested for long-range PCR conditions. They consisted of seven forward (F1-F7) and four reverse primers (R1-R4) (see appendix: primer sequences). The best result was achieved with primer pair F4 and R4. All primers contained restriction sites for S*acI* and N*otI*. PCR products were ligated into pHIS parallel1 for recobinant protein expression.
(Figure 1A)

Random cloning of cDNA

cDNA from either preparations of complete cDNA or of full length *var* mRNA was subcloned via TOPO cloning kit (Invitrogen) into pTrcHIS2 expression vector without restriction digest using the A-overhangs from the PCR reaction in accordance with the manufacturers protocol.

(Figure 1A)

Vector modifications of pTrcHis2

A PCR product from the primer pair GST_fwd and MCS_reverse that contains the GST sequence as well as the multiple cloning site (MCS) of pGEX 4T1 was ligated into the pTrcHIS2 vector. A "gene of interest" cloned into the MCS of the new vector pTrcHis2_GST has two purification tags: an N-terminal GST and a C-terminal 6xHis.

(Figure1B)

*Cloning of acidic terminal segment (ATS) of Pf*EMP*1*

The ATS domain was amplified using the primer pair ATS_FCR_fwd and ATS_FCR_reverse, which contains the restriction sites E*co*RI and N*otI*. Genomic DNA from the *Plasmodium falciparum* strain FCR3 S1.2 was used as template. PCR products were ligated into the pTrcHis2_GST vector, which was cut with the same enzymes as the PCR product.

(Figure1B)

Cloning of ATS N-terminal and C-terminal fragments

The N-terminal part of the ATS was amplified from genomic DNA from the *Plasmodium falciparum* strain FCR3 S1.2 using primer pair ATS_FCR_2_fwd and ATS_FCR_nterm_reverse, that contain the restriction sites E*co*NI and B*gl*II. PCR products were cloned into the expression vector pQE-16. For the C-terminal part, the primer pair was ATS_FCR_cterm_fwd and ATS_FCR_2_reverse; the rest of the procedure was the same as for the N-terminal fragment.

(Figure1C)

Cloning of NTS (N-Terminal Segment) of FCR3 S1.2

The NTS domain was amplified using the primer pair NTS_FCR_fwd and NTS_FCR_reverse, which contains the restriction sites E*co*RI and N*ot*I. Genomic DNA from the *Plasmodium falciparum* strain FCR3 S1.2 was used as template and PCR products were ligated into the vector pTrcHis2_GST (Invitrogen).
(Figure 1B)

Preparation of electrocompetent cells and electroporation of E.*coli*

An over-night culture from a single colony was diluted 1:20 in LB medium, grown to OD_{600}= 0.8 harvested and washed 4 times in sterile ddH_2O at 4 °C to remove residual salts. The bacterial pellet was aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

For transformation, 40µl of concentrated electrocompetent cells were mixed with plasmid DNA in a precooled electroporation cuvette (gap 0.2cm). Electroporation settings were 2500V and 5ms single-pulse. For reconstitution, the transformed bacteria were immediately transferred into warm LB medium without antibiotics for 30 minutes. The suspension was spread on LB agar plates with the appropriate antibiotics and grown overnight at 37°C.

Plasmid preparation and sequencing

Plasmids were extracted from 2ml over-night culture with a MiniPrep kit (Qiagen). Plasmids were sequenced at Macrogen Inc. in South Korea.

Recombinant protein expression in *Escherichia coli*

Small-scale expression

For over-night cultures, 4ml LB-medium supplemented with 100µg/ml ampicillin was inoculated with a single colony containing the expression plasmid and incubated at 37°C, shaking at 220rpm. The overnight culture was diluted 1:100 in 5ml fresh LB-medium containing antibiotics and was further incubated at 37°C. The protein expression was induced by the addition of 1mM IPTG when OD_{600} reached 0.8. Bacteria were harvested 4 hours later by centrifugation at 4°C, 8000g for 15min. Pellets were stored at -20°C until further use.

Medium and large-scale expression

For medium and large-scale expression of recombinant protein, the volume of the expression culture was raised to 50 or 500ml.

E. coli expression strains used were: BL21 for pGEX plasmids, M15 for pQE plasmids and TOP10 for pTrcHIS2 plasmids. The LB medium was supplemented with 100µg/ml Kanamycin for M15 cells and with 1% Glucose for TOP10 cells.

E.coli cDNA library colony blots

Transformed *E.coli* were spread on LB agar plates and grown over night at 37°C. The next day colonies were printed directly from the plate onto a round nitrocellulose membrane. The membrane was then duplicated and re-grown on fresh plates (facing colonies up). One membrane was stored at 4°C as master. The other was transferred to a new agar plate containing 1mM IPTG and grown for additional 4h at 37°C. The membrane was gassed in chlorofom vapor for 15 minutes before it was transferred into lysis buffer containing 100mM Tris pH7.8, 150mM NaCl, 5mM $MgCl_2$, 15% BSA, 40µg/ml lysozym and 1µg/ml DNAse. Lysis was complete when no bacterial material was visible anymore, usually over-night. The membrane was developed similar to a Western blot, including blocking, primary and secondary antibody and stained with NBT/BCIP. Colonies that produced a recombinant protein left a black spot on the membrane. When the developed membrane was aligned with the master membrane positive clones were revealed and cultivated for further analysis (Sambrook and Russell, Molecular cloning, 3rd Ed, 14.14 Protocol 2).

Purification of 6xHIS tagged recombinant proteins

Purification of NTS

The bacterial pellet from the expression culture was thawed on ice. 5 pellet volumes of lysis buffer supplemented with 100µg/ml lysozyme and 1µg/ml DNAse were added and incubated for 30 minutes. The lysate was sonicated at 50% duty cycle with five, 10 seconds bursts with 10 seconds intervals in a microtip sonicator. Soluble proteins were separated from cellular debris by centrifugation at 10000g for 30 minutes at 4°C. The supernatant was mixed with 0.5ml of Ni-TA-Agarose beads (Invitrogen) and incubated for 1h. The protein was further purified according to the

manufactures protocol (Qiagen, the expressionist). Eluted proteins were stored at -20°C.

Purification of C-terminal ATS and N-terminal ATS

For the insoluble proteins ATS C-term and ATS N-term, the pellet was lysed in inclusion body lysis buffer (2% Triton X-100) supplemented with 100µg/ml lysozyme and 10µg/ml DNAse. Sonication conditions were as indicated above. The insoluble proteins were harvested by centrifugation at 10`000g for 30 minutes. The supernatant was discarded and the pellet resuspended in inclusion body lysis buffer. This step was repeated 3 times. For the last resuspension of the insoluble pellet, PBS was used to avoid Triton X-100 in the further purification procedure. The washed pellet was resuspended in 8M Urea buffer and mixed with 0.5ml Ni-TA-Agarose beads. The protein was further purified according to the manufactures protocol (Qiagen, the expressionist). Eluted proteins were stored at 4°C.

Purification of GST tagged recombinant proteins

The bacterial pellet was lysed as indicated in the "*Purification of NTS"* protocol. The supernatant was mixed with 0.5ml glutathione-sepharose 4B (Pharmacia) and incubated in an over-head shaker for 30 minutes at room temperature. The sepharose slurry was centrifuged, washed and eluted with 10mM reduced glutathione as described in the manufacturers protocol. Eluted proteins were stored at -20°C.

SDS-PAGE and Western blot analysis

Proteins were resuspended in Laemmli buffer, boiled for two minutes and separated on a 5 or 12% SDS polyacrylamide gel according to their size. Proteins were transferred to a nitrocellulose membrane (Hybond C, 0.45µm, GE Healthcare) in a semi-dry blotter. Tris-Glycine buffer containing 20% methanol was used as transfer medium. Membranes were blocked with TNT (Tris, NaCl, Tween-20 0.1%) containing 5% non-fat milk powder. Primary and secondary antibody incubation was done in TNT containing 1% non-fat milk powder. Primary antibody dilutions for mouse anti-NTS and mouse anti-ATS1-4 were 1:200 – 1:1000 and for mouse anti-6xHIS 1:5000. Membranes were incubated with the primary antibody for 2h on a

shaker at room temperature or over night at 4°C. Washing was performed with TNT 3 times for 10 minutes. Secondary antibody dilutions used were: goat anti-mouse AP 1:10000 and goat anti-human HRP 1:5000. Both were incubated for 1 hour at room temperature. Secondary antibodies were visualized depending on the liked enzyme: for alkaline phosphatase alkaline Tris-buffer containing 300µg/ml BCIP and 150µg/ml NBT was used and the color reaction was stopped with ddH_2O; for horseradish peroxidase a enhanced chemoluminescence detection kit (GE Healthcare) was used and the filters exposed to Kodak XE films for 10 seconds to 10 minutes.

Immunization of mice

Recombinant NTS

Six NMRI female mice were immunized with purified recombinant NTS. 3 mice were subcutaneously immunized with 10µg recombinant protein and MPL-TDM (Sigma) as adjuvant and 3 mice with ImmuneEasy (Invitrogen) as adjuvant. The mice were boosted twice with the same antigen concentration after 3 and 5 weeks. 10 days after the last boost the mice were sacrificed and the sera stored at -20°C for further analysis.

Synthetic peptides

Four different peptides ATS1 to ATS4 (Alta-Bioscience) (see appendix for sequence information) all derived from conserved ATS regions of *Pf*EMP1, were chosen for immunization. Two peptides were differently modified to enhance antigenicity. Peptide ATS1 was coupled to Keyhole limpet heamocyanin (KLH), peptide ATS2 was synthesized as Multi-Antigenic-Peptide (MAP) in which 8 peptides are coupled to a branched lysine core. Peptide ATS3, being the longest peptide, was not modified. Peptide ATS4 was chosen for its predicted coiled-coil domain and also not modified to avoid interference with the self-assembling of its 3-dimensional structure.

For mouse immunization a peptide solution was emulsified with TiterMax Gold® (Sigma) in a ratio of 1:1 containing 100µg antigen per injection. For each antigen 3 NMRI mice were immunized once and titers were check 5 weeks later. Boosting was

not necessary and mice were sacrificed 6 weeks after immunization and the sera stored at -20°C for further analysis.

Enzyme linked immuno adsorbed assay (ELISA)

MaxiSorb (Nunc) 96 well ELISA plates were coated with 10µg/ml peptide or protein in PBS at 4°C over-night. Plates were washed in an ELISA-plate-washer once and blocked with 5% non-fat milk powder on PBS-T (PBS, 0.01% Tween-20) at room temperature for one hour. Plates were then washed again and incubated with mouse sera in serial dilutions starting at 1:100 in PBS-T with 1% non-fat milk powder at room temperature for 2 hours. Plates were washed twice and incubate for another hour with goat anti-mouse alkaline-phosphatase labeled antibodies (Sigma) at a dilution of 1:10000. The washed plate was developed with 1mg/ml PNP (p-Nitrophenyl Phosphate) in alkaline buffer and measured in an ELISA reader at a wavelength of 405nm one to three hours later.

Immuno fluorescence assay (IFA)

Mixed-stage parasite cultures with at least 8% parasitaemia were thinly smeared on a microscopic glass slide. Cells were fixed in ice-cold acetone:methanol (1:1) for 5 minutes. The slides were blocked with sterile-filtered 5% BSA in PBS for one hour at room temperature in a humid chamber. Cells were washed and incubated with mouse sera in PBS-T with 1% BSA for 2 hours. Slides were washed again and incubated with goat anti-mouse Cy3 labeled antibodies for one hour. IFA slides were washed, dried and mounted in anti-fade solution containing DAPI (1µg/ml) for visualization of the nuclei.

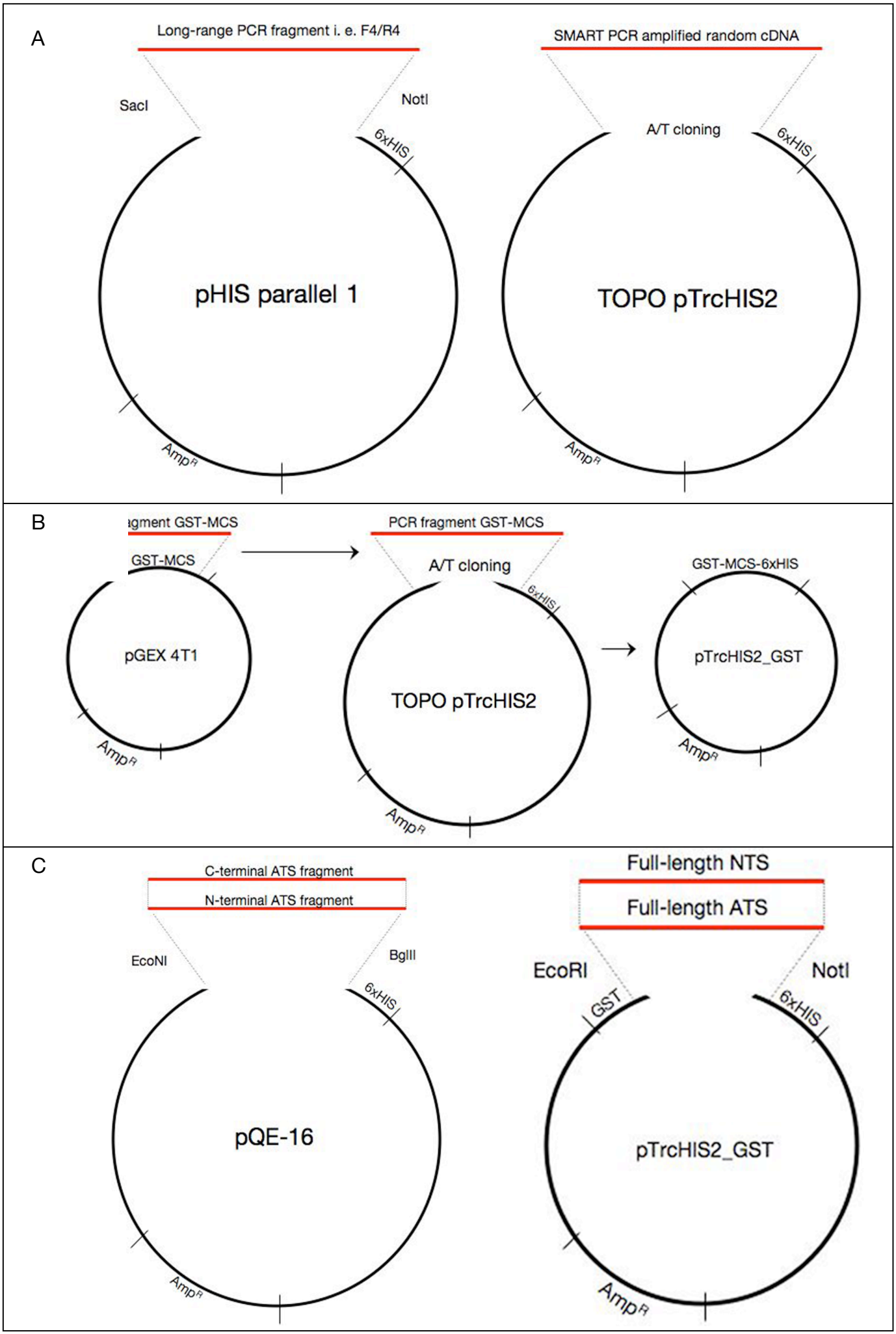

A
Long-range PCR fragment i. e. F4/R4
SacI
NotI
6xHIS
pHIS parallel 1
Amp^R
SMART PCR amplified random cDNA
A/T cloning
6xHIS
TOPO pTrcHIS2
Amp^R
B
GST-MCS
pGEX 4T1
Amp^R
PCR fragment GST-MCS
A/T cloning
6xHIS
TOPO pTrcHIS2
Amp^R
GST-MCS-6xHIS
pTrcHIS2_GST
Amp^R
C
C-terminal ATS fragment
N-terminal ATS fragment
EcoNI
BglII
6xHIS
pQE-16
Amp^R
Full-length NTS
Full-length ATS
EcoRI
NotI
GST
6xHIS
pTrcHIS2_GST
Amp^R

Figure 2. Vector maps of cloned expression constructs.

In **A** vectors and enzymes are depicted for cloning of long-range PCR and amplified cDNA cloning. In **B** the modification of the TOPO pTrcHIS2 vector with the GST expression cassette from pGEX 4T1 is shown. The new vector pTrcHIs2_GST was used for cloning of ATS and NTS domains. In **C** the cloning of ATS N- and C-terminal fragments is indicated.

Chapter 3

Results

To dissect the immuno-protective role of different domains in *Pf*EMP1, we attempted to generate a set of recombinant proteins against which we wanted to test sera from patients with different clinical history or age. Furthermore, we planed to generate specific antibodies against different domains in *Pf*EMP1.

We chose classical molecular biological methods to tackle our aims. We attempted expression of long *Pf*EMP1 fragments from a random cDNA library, from full lengths ATS and from full length NTS domains. We investigated immunization with recombinant NTS and synthetic peptides of the ATS domain. These multiple approaches and corresponding results are described in the following.

1. Long range PCR, cloning and expression

To obtain large *Pf*EMP1 PCR fragments for recombinant protein expression, a set of seven forward primers (F1-7) and four reverse primers (R1-4) were designed based on the *P. falciparum* 3d7 genome sequence (primer sequences listed in "Primers & Peptides"). The fragments reached from the NTS domain to the DBLγ domain and sized from 3 to 5 kb, depending on the domain structure of the *var* gene. Each forward primer was tested with each reverse primer and results are shown in Figure 1. To assess the limits of the long-range PCR (l-rPCR), an ATS reverse primer was also included. It was possible to amplify a small full-length *var* gene with a size of about 7kb (see Figure 1, Lane 10). As the primer sequences were chosen on relatively conserved DNA stretches each primer can anneal to a subset of *var* genes.

Various fragments were prepared for cloning into an expression vector, however the 3.8 kb fragment amplified with primer pair F4/R4 had the highest yield (Figure 1, lane 14) and was the only one, which was successfully cloned for protein expression in pHIS paralell1 (see Figure 2). Upon protein induction however, no recombinant protein expression was detectable neither in Coomassie blue stained polyacrylamide gels (Figure 3) nor in Western blots (data not shown). Alterations in temperature, induction time, different bacterial strains (M15, Bl21, Origami) or rich expression media did not improve protein expression conditions.

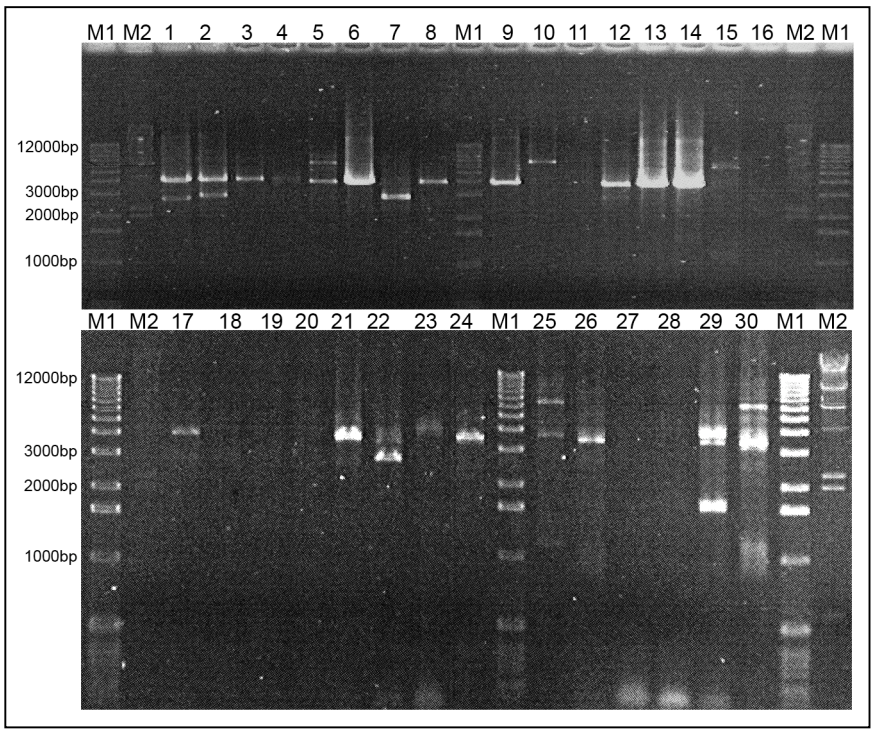


Figure1. Testing different primer combinations for *Pf*EMP1 long-range PCR.

PCR products of different primer combinations (see Table 1) were tested. 5 microliters of a 50 µl PCR reaction were loaded on a 0.7% agarose gel in lanes 1-30. M1, 1kb ladder; M2, lambda H*ind*III ladder.

Lane	5` primer	3` primer	Lane	5` primer	3` primer
1	F2	R1	16	F5	R1
2	F2	R2	17	F5	R2
3	F2	R3	18	F5	R3
4	F2	R4	19	F5	R4
5	F2	ATS	20	F5	ATS
6	F3	R1	21	F6	R1
7	F3	R2	22	F6	R2
8	F3	R3	23	F6	R3
9	F3	R4	24	F6	R4
10	F3	ATS	25	F6	ATS
11	F4	R1	26	F7	R1
12	F4	R2	27	F7	R2
13	F4	R3	28	F7	R3
14	F4	R4	29	F7	R4
15	F4	ATS	30	F7	ATS

Table 1. Different primer pair combinations applied for long-range PCR amplification of *Pf*EMP1.

The table shows all possible permutations of forward primers F2-F7 and reverse primers R1-R4 and reverse-ATS with its corresponding lane in figure 1. Primer F1 did not work and was excluded. Primer pair F4/R4 in grey was used for downstream experiments (for sequence information see: “Primers & Peptides”).

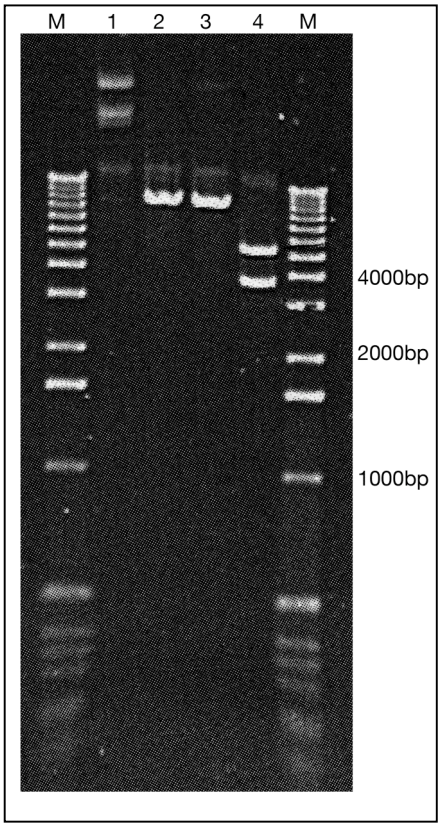


Figure 2. Control digest of cloned I-rPCR..

Restriction enzyme digests of plasmid preparation of pHIS1_F4/R4. Lane 1 undigested plasmid, lane 2 and 3 plasmid digested with either S*ac*I or N*ot*I, lane 4 plasmid digest with both enzymes. The F4/R4 fragment is visible at 3800 bp, the backbone at 5500 bp. M, 1kb marker.

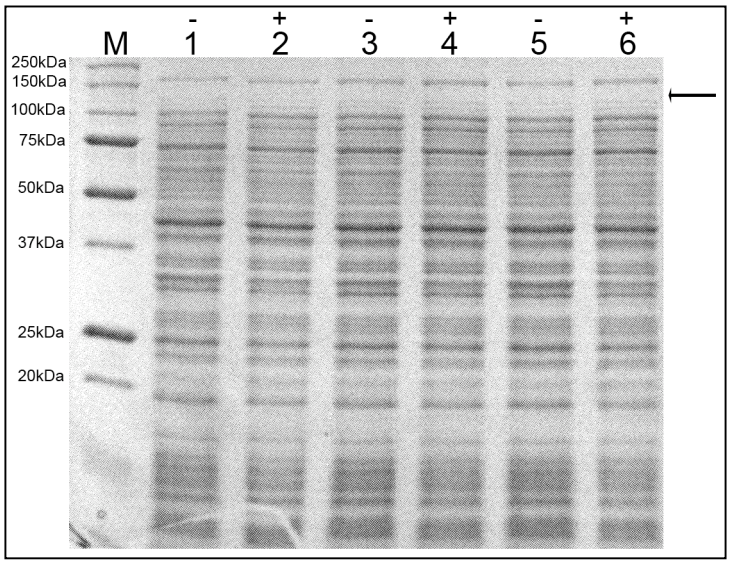


Figure 3. Small-scale expression of PCR product F4/R4 in pHIS parallel1.

Total protein extracts of induced(+) and noninduced(-) bacterial cultures harboring plasmid pHISparalell1_fragF4/R4 were separated on a 12,5% acrylamide gel stained with Coomassie blue.

Three different expression clones were tested: clone 1 (lane 1 and 2), clone 2 (lane 3 and 4), clone 3 (lane 5 and 6). Arrowhead is positioned at expected size of recombinant protein (135 kDa). M, Marker SeeAllBlue (Invitrogen).

2. Random cDNA cloning and protein expression

With the aim of testing the serological effect of different domains of *Pf*EMP1, we performed a random cloning approach. Total RNA was isolated from *Plasmodium falciparum* 3d7 and FCR3 S1.2 culture strains (see Figure 4A). The total RNA was reverse transcribed with random primers and the resulting cDNA was cloned into TOPO pTrcHIS2 plasmids for random expression. Sequencing analyses of these clones revealed that only rRNA had been reverse transcribed and thus is present as inserts in TOPO pTrcHIS2. There was no clone containing a reverse transcription product derived from an mRNA fragment. Therefore, a different approach was chosen to enrich for full-length *var* mRNA from the crude RNA preparation (see materials and methods "*var* mRNA extraction"). The isolated mRNA was transcribed into cDNA. Due to its low concentration, it was not possible to clone the cDNA obtained after mRNA purification.

Therefore an amplification cDNA step was added using the modified SMART® method (see Figure 4B). Although the amplification was successful the cloning of the amplified cDNA again only showed rRNA and tRNA. Neither an annotated mRNA fragment nor a *var* transcript was ever found in the cloned fragments.

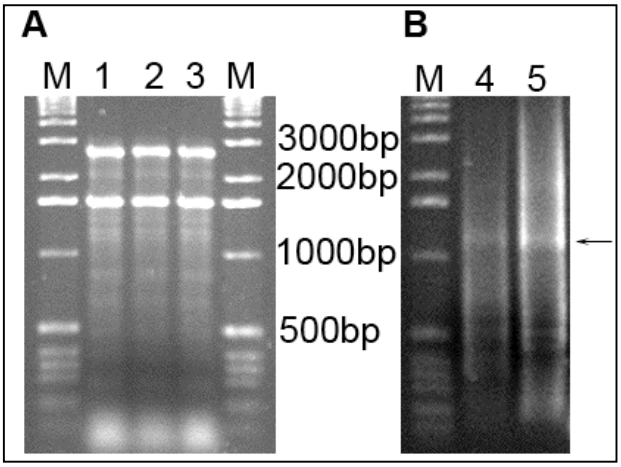


Figure 4. RNA preparation and SMART cDNA amplification.

A. RNA extractions from two different *Plasmodium falciparum* strains were separated on a non reducing agarose gel. The ribosomal RNA is visible as very strong bands at 1,6kb and 2,9kb. Lane 1: 3d7; lane 2+3 FCR3 S1.2. **B**. Agarose gel with two SMART cDNA amplification products from 3D7 (lane 4) and FCR3 S1.2 (lane 5). The dominant transcript length is around 1kb as indicated by the arrowhead.

3. Establishing the colony blot method for ATS expression from *Plasmodium falciparum*

The colony blot system was originally designed to find expressing and serological relevant clones in a complex cDNA library. As the cDNA library was notoriously contaminated with transcripts of rRNA, an expressing clone was never found. But when used as tool to screen for an expressing colony after cloning a specific PCR product amplified from genomic DNA, this problem should not occur. We successfully applied the method for screening for an expression clone of the complete FCR3 S1.2 ATS domain (see Figure 5A). Positive clones were picked from the master plate and analyzed by PCR, where 80% proofed to have a correct insert.

As depicted in Figure 5C, Western blots of small-scale expression cultures revealed a week expression of full length GST-ATS-HIS at 90 kDa, however the expected size was 72 kDa. Unfortunately, purification of the recombinant protein over a Ni-TA-column did not enable sufficient purity (Figure 5B). Purification using Glutathione-Sepharose was not possible, because the expressed protein was not soluble. Because of the unsatisfactory purity, the wrong size and the low expression level the protein was not used for further immunization of mice.

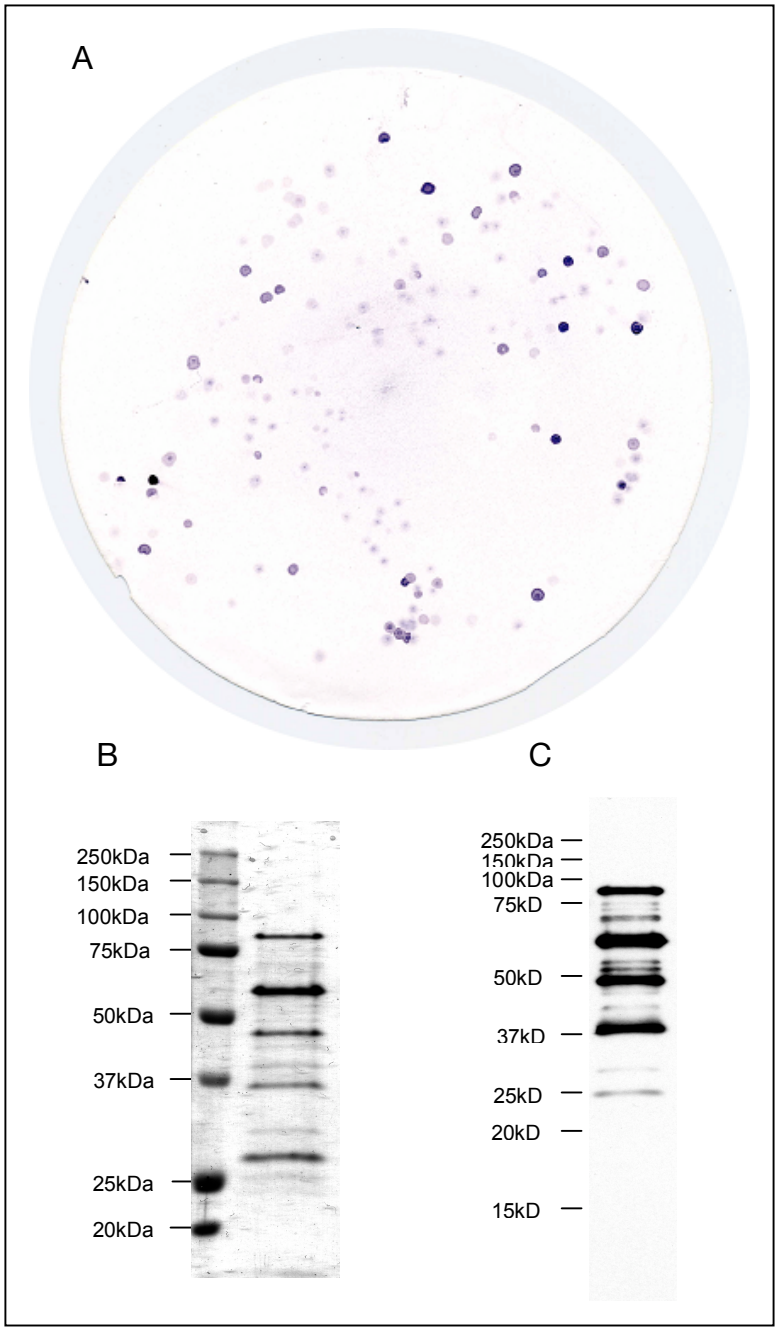


Figure 5. Colony blot and expression of ATS in pTrcHIS2_GST.

A. Colony blot with ATS-6xHIS expressing clones (dark black spots). The blot was probed with anti-6xHIS antibodies. **B**. Coomassie blue SDS page with purified recombinant ATS. **C**. Western blot of recombinant ATS probed with anti-6xHIS antibody.

4. Cloning and expression of the NTS of strain FCR3 S1.2

For performing localization studies and Western blots we generated a set of polyclonal mouse sera. Because immunization with recombinant ATS was not possible due to unsatisfying yield and purity, we decided to use the NTS domain instead. For this, the smaller NTS domain was cloned into the same expression vector as the ATS domain. The expressed recombinant protein has an expected size of 33.6 kDa and an apparent size of 40kDa (see Figure 6). It was purified sequentially over a glutathione-column first and then over a Ni-TA-column. The purity of the recombinant protein fraction met our standards and this recombinant protein was used to immunize NMRI mice.

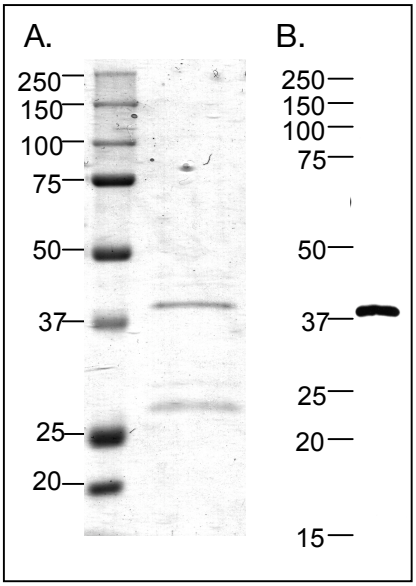


Figure 6. Polyacrylamide gel and Western blot of recombinant NTS.

The recombinant protein NTS is visible at approximately 40 KDa. **A**. Coomassie blue SDS PAGE with purified recombinant NTS, an additional band is visible at 30 kDa. **B**. Western blot of recombinant NTS probed with 6xHIS antibodies. The recombinant protein is visible at 40 kDa.

Immunization of mice and characterization of anti-NTS sera

When immunizing mice with recombinant GST-NTS-HIS from the FCR3 S1.2, two slightly different immunization protocols were used in order to optimize immunization. Briefly, 3 mice were immunized with MPL-TDM (Sigma) adjuvant (MPLM1-MPLM3) and 3 mice were immunized with Immune-Easy (Qiagen) adjuvant

(IEM1-IEM3). The sera were tested against recombinant protein and total parasite protein lysates. Figure 7 shows that sera tested in Western blots recognized the recombinant protein GST-NTS-HIS with a band at 37 kDa. There were also multiple lower bands, which may represent breakdown products of the recombinant protein. Serum from IEM1 showed the most specific interaction, but also the weakest. In all other sera background bands were visible. MPLM3 showed the strongest interaction.

The sera were also tested on parasite lysates to detect the full length *Pf*EMP1 (see Figure 8). The pattern obtained with the sera showed two bands above 250 kDa, which is in the size range of endogenous *Pf*EMP1. However, controls with pre-immune mice sera and with other anti-*Pf*EMP1 serum (against the ATS domain, kindly provided by M. Wahlgren), revealed that the recognized bands were not *Pf*EMP1. As the same bands also come up in lysates of uninfected red blood cells, it must be cross reaction with an erythrocyte protein, most likely alpha and beta spectrin.

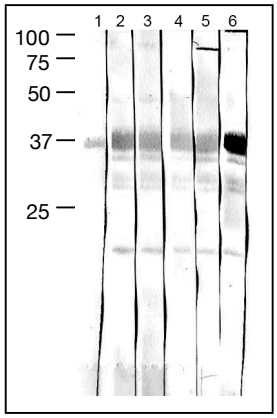


Figure 7. Western blots for testing six mouse anti-NTS sera.

Recombinant NTS was blotted and probed with 6 different mice sera. Lanes 1-3 represent sera from IEM1-IEM3, and lanes 4-6 are sera from MPLM1-MPLM3. The apparent size of the recombinant protein is about 40kDa.

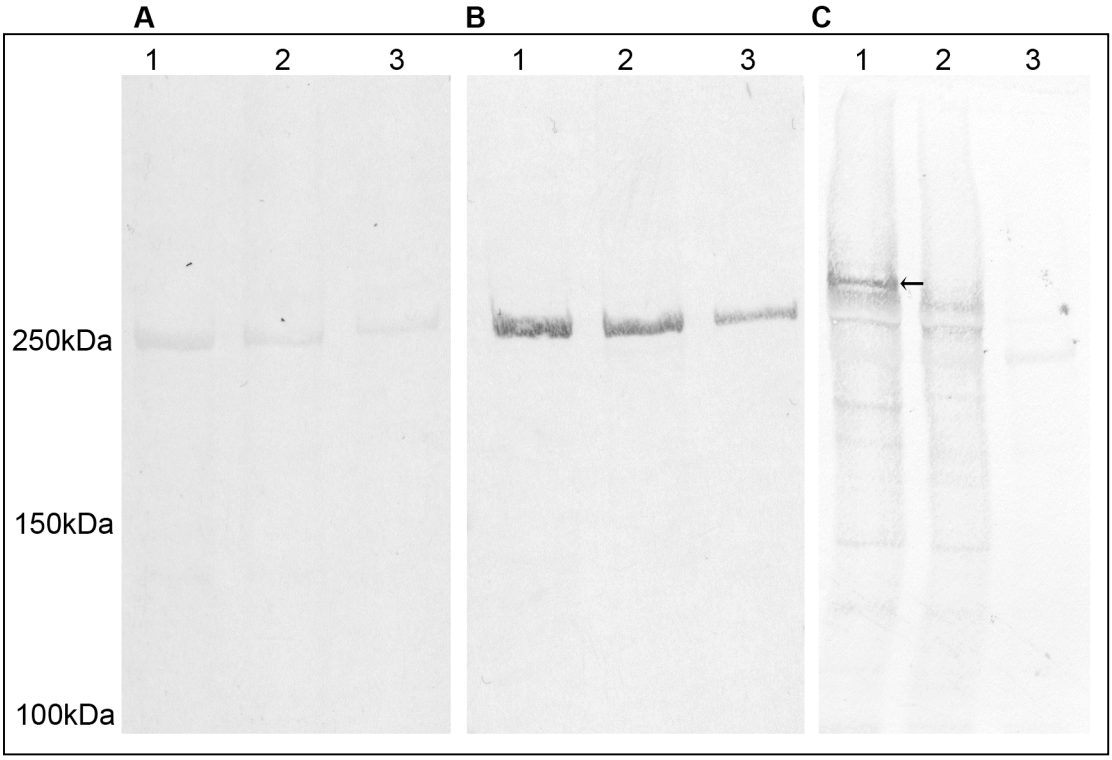


Figure 8. Western blots of parasite lysates probed with different anti-sera.
Lysates from parasite lines FCR3 S1.2 (lane1), 3d7 (lane 2) and red blood cells (lane 3) were tested with pre-immune mice sera pool (**A**), anti-NTS mice serum pool (**B**) and rat anti-ATS (**C**, provided by M. Wahlgren). All sera were used 1:200. The arrow indicates the band expected to be *Pf*EMP1.

Sera from NTS immunized mice tested in IFA

To rule out that epitope modification during protein electrophoresis and transfer was the reason for the unclear immunoblot results of total parasite protein preparations, all sera were additionally tested in immunofluorescence assays (IFAs). The sera were incubated with fixed (either acetone/methanol or glutaraldehyde/formaldehyde) smears on microscopic glass slides and investigated under a fluorescence microscope. Only 2 sera gave reasonable signals, namely IEM2 and MPLM3. All other sera gave no signal or showed elevated background levels, which made it impossible to discriminate any labeled structures. In glutaraldehyde/formaldehyde fixed infected red blood cells both IEM2 and MPLM3 sera showed the same fluorescent pattern: a sharp rim-like or tube-like structure surrounding the nucleus (see Figure 9) indicating localization within the endoplasmatic reticulum (ER) [86-88].

The dotted staining of the parasite and the red blood cell cytosol characteristic for *Pf*EMP1 [43, 74] was never observed.

Acetone/methanol fixed infected red blood cells showed a different picture and were only positive for mouse MPLM3 (see Figure 7B). With this fixation, the pattern was diffuse. A faint and fuzzy staining of the host cell cytosol was visible, but only with long exposure times.

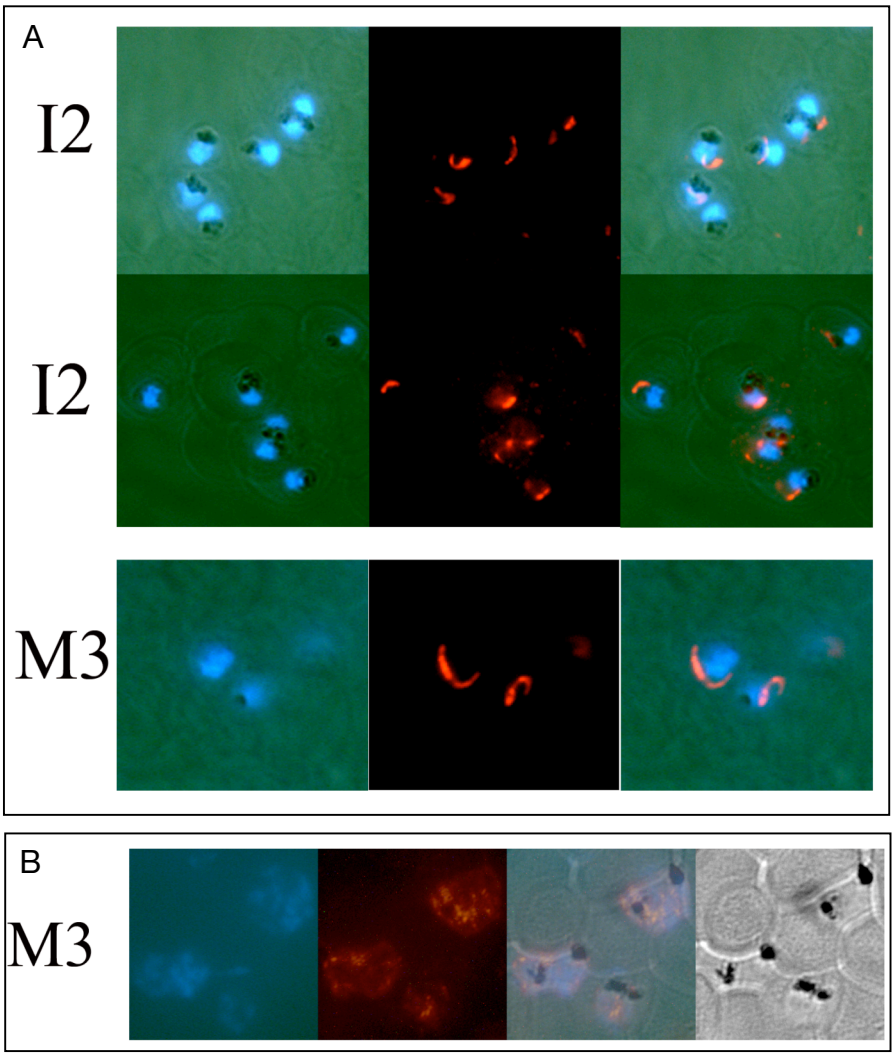


Figure 9. IFA of infected red blood cells probed with anti-NTS mice sera using two different fixation methods.

Sera MPLM3 and IEM2 were tested and the secondary antibody used was goat IgG anti-mouse-Cy3.

A. Formaldehyde/glutaraldehyde fixed FCR3 S1.2 parasites. In red the rim like structure of the anti-NTS serum is visible. In blue is the DAPI stained genomic DNA. Left column: brightfield image and DAPI overlay; middle column: anti-NTS signal (Cy3); right column: overlay of left and middle column. I2 is IEM2; M3 is MPLM3. **B.** Acetone/methanol fixed parasites. Serum from mouse 3 MPL on FCR3 S1.2. In red anti-NTS (Cy3), in blue DAPI. From left to right: DAPI, Cy3, overlay, bright field.

5. Cloning, expression and purification of C- and N-terminal ATS fragments

Sera raised against synthetic peptides (see section 6) can only be tested on Western blots with very sophisticated Western blot protocols for small peptides.

To circumvent establishment of a peptide Western blot protocol, we generated a pair of recombinant proteins covering the ATS. The ATS domain was split into two separate domains, ATS-N and ATS-C (see schematic representation in figure 9), to facilitate recombinant expression. The domains were cloned into a pQE-16 vector expressing the cloned gene as DHFR-fusion protein. The expected size for ATS-N was 50 kDa and for ATS-C 59 kDa. Both domains overlapped in the middle by 6 amino acids. Upon recombinant expression, both domains were not soluble but expressed in high levels and were successfully purified over Nickel column in 8 M urea (see Figure 10). Protein extracts were later used as controls to assess antibody levels and selectivity in Western blots of sera from ATS immunized mice.

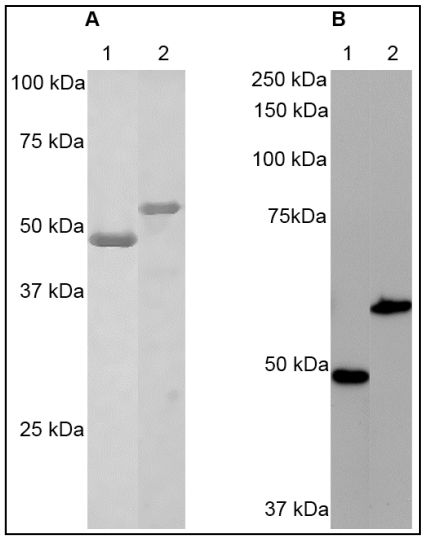


Figure 10. Coomassie blue stained SDS PAGE and Western blot of recombinant ATS-N and ATS-C.

Purified recombinant ATS fragments were separated by gel electrophoresis; lane 1, ATS-N; lane 2, ATS-C. **A.** Coomassie stained 12,5% Polyacrylamide gel. **B.** Western blot from 10% Polyacrylamide gel probed with anti-6xHIS antibodies.

6. *In silico* ATS analyses to select synthetic peptide candidates

Due to a persisting lack of specific anti-*Pf*EMP1 antibodies, we searched for new possible sequences to use as antigens for raising antibodies in mice. The ATS domain of *Pf*EMP1 which faces the erythrocyte cytosol is not under immunological pressure, hence is not as variable as the extracellular portion of *Pf*EMP1. We aligned all 3d7 ATS sequences to find conserved stretches (see appendix alignment 1). We chose three stretches (ATS1-3) within the ATS domain which have a homology greater than 80%. The peptide ATS1 (SDITSSESEYEELDINDIYVP) had a length of 21 amino acids (aa), peptide ATS2 (PKYKTLIEVVLEPS) had a length of 14 aa and peptide ATS3 (GIDLINDTLSSGNHIDIYDEVLKRKENELFG) 31 aa. In addition we selected a fourth peptide (ATS4) not because of its homology but because of its predicted coiled-coil structure. The peptide ATS4 (LDRHRDMCEKWKNKEDILNKLK EEWNKENINN) was 32 aa long (see Figure 11 for details). The peptides ATS1 and ATS2 were modified to enhance immunogenicity as described in materials and methods.

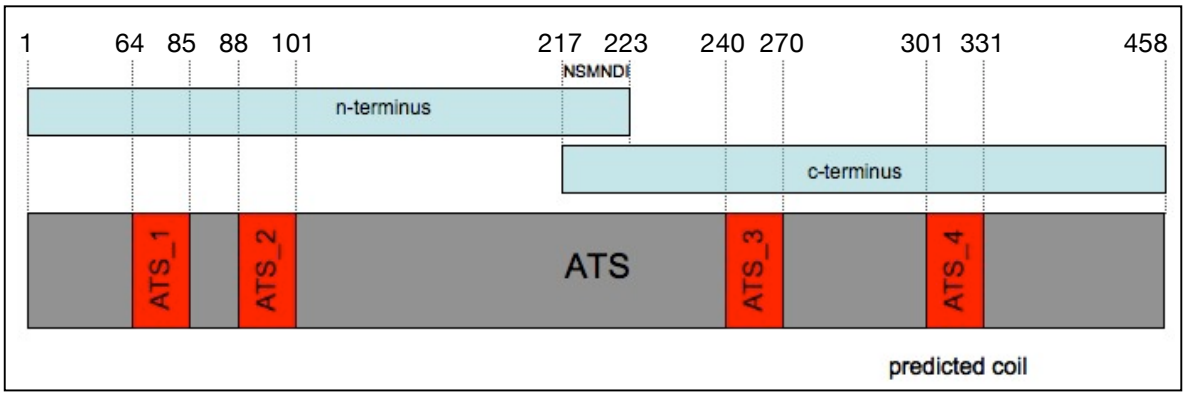


Figure 11. Schematic representation of the ATS domain of *Pf*EMP1 in the FCR3 S1.2 strain.

Depicted in grey is the full ATS domain of *Pf*EMP1. Blue bars represent the recombinantly expressed ATS-N and ATS-C domain, which overlap by the 6 amino acids NSMNDI. Synthetic peptides were chosen according to 3 regions with over 80% homology shown as red boxes (ATS1-ATS3). A fourth synthetic peptide (ATS4) was defined according to regions of predicted coiled-coil domain. The numbers indicate the amino acids starting after the transmembrane domain (numbers and amino acids are valid for FCR3 S1.2 ATS, AF003473).

Immunization with synthetic peptides and characterization of sera

A new adjuvant TiterMax Gold (Sigma) was used for immunization of mice with synthetic peptides. For every peptide, 3 mice were immunized, resulting in sera ATS1M1-ATS1M3, ATS2M1-ATS2M3, ATS3M1-ATS3M3, and ATS4M1-ATS4M3, Specificity of all sera was tested in ELISAs (see figure 10). Sera ATS1M1-3, ATS3M1 and ATS4M2-3 showed very high titers ranging from 1:3000 to 1:10000. All ELISA-positive sera were additionally tested in Western blots on recombinant ATS fragments (see Figure 13). All ATS1 immune sera recognized the recombinant N-terminal ATS fragment but not the C-terminal fragment. Non of the ATS2 immune sera gave a signal in Western blots. Only sera from mouse1 from immunization with ATS3 recognized the recombinant C-terminal and not the N-terminal fragment. Sera ATS1M1-3 and ATS3M1 have high antibody titers and are specific for their antigen. These sera were also tested on Western blots of total parasite lysates from lab strains 3d7 and E8B (see Figure 14). All sera recognized two strong bands above 250kDa. The same bands were also recognized in Western blots of uninfected red blood cells. As the recognized bands seem to be of erythrocytic origin, MACS enriched parasites were used for the preparation of protein lysates to improve the parasite:erythrocyte ratio. MACS is a magnetic cell sorter which can bind trophozoite and schizont stage iRBCs due to magnetic iron deposits in the food vacuole. Younger stages and RBC do not bind. The intensity of the recognized bands was reduced significantly in the MACS sample (Figure 14, lane 4), strengthening the evidence for erythrocytic origin of the recognized bands. With the exception of the MACS enriched sample there were no additional bands in immuno blots of infected red blood cells compared to blots of uninfected red blood cells. In the MACS purified sample there was an additional unidentified 150kDa band. Thus our Western blot analyses show, that none of the immune sera raised against 4 different domains of the ATS recognized native *Pf*EMP1 in Western blots. The additional band at 150 kDa in the MACS sample is not *Pf*EMP1 as this is expected to be bigger than 250kDa. In Figure 12 the Western blot of ATS1 M1 is shown, which is representative for all ELISA positive sera.

Furthermore, all sera were tested in IFAs on the parasite line FCR3 S1.2 to confirm the results from Western blots (see Figure 15). Only a very faint signal was detected and in accordance with Western blot results the signals was also present in

uninfected erythrocytes and is thus unspecific for *Pf*EMP1. IFAs from three different mice immunized with ATS1 giving the strongest signals are shown in Figure 12. M3 is shown here as an example and all other sera tested stained the RBCs as well.

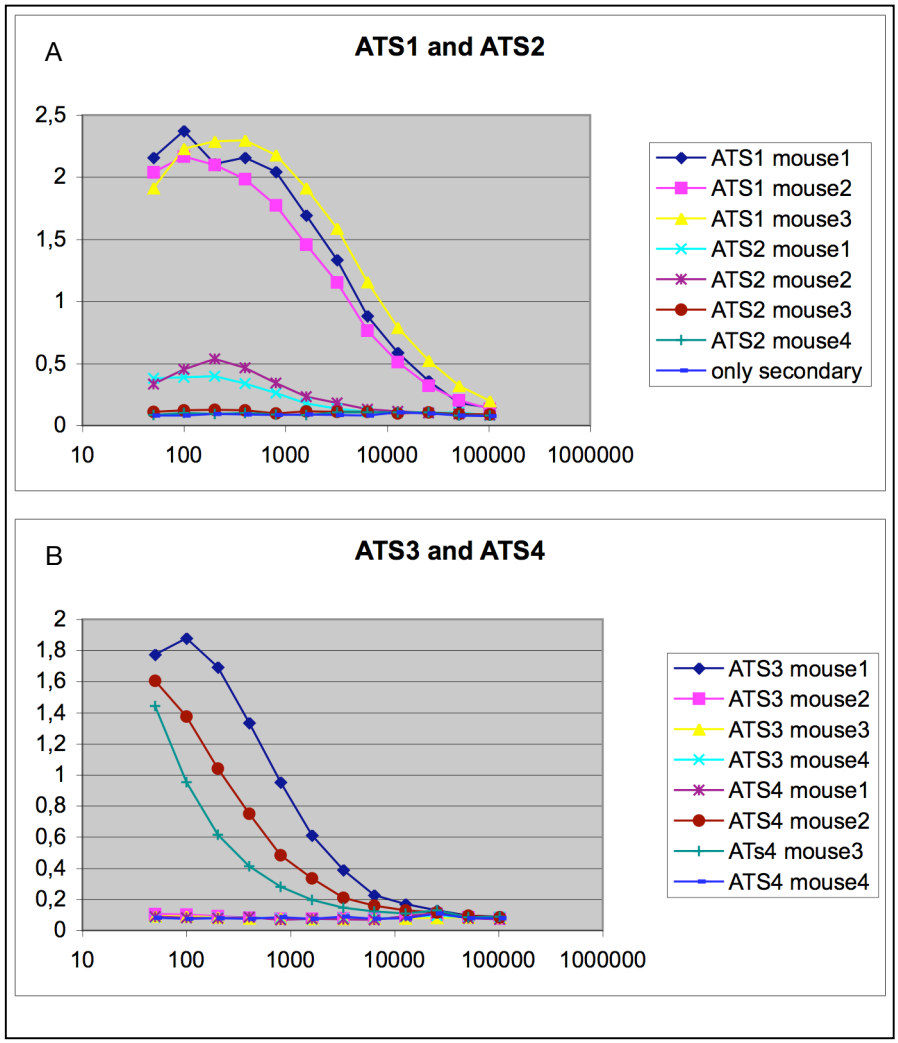


Figure 12. Enzyme linked immuno fluorescence assays of mice sera on synthetic ATS peptides.

A. ELISA results for ATS1 and ATS2. Mice immunized with ATS1 (ATS1M1-M3) had very high titers ranging between 1:5000 and 1:10000. For ATS2 only M1 and M2 show a (weak) response. In blue: negative control on ATS1 without primary antibody. **B.** Results from ATS3 and ATS4. ATS3 M1 and ATS4 M2-3 show high response with titers ranging between 1:1000 and 1:10000.

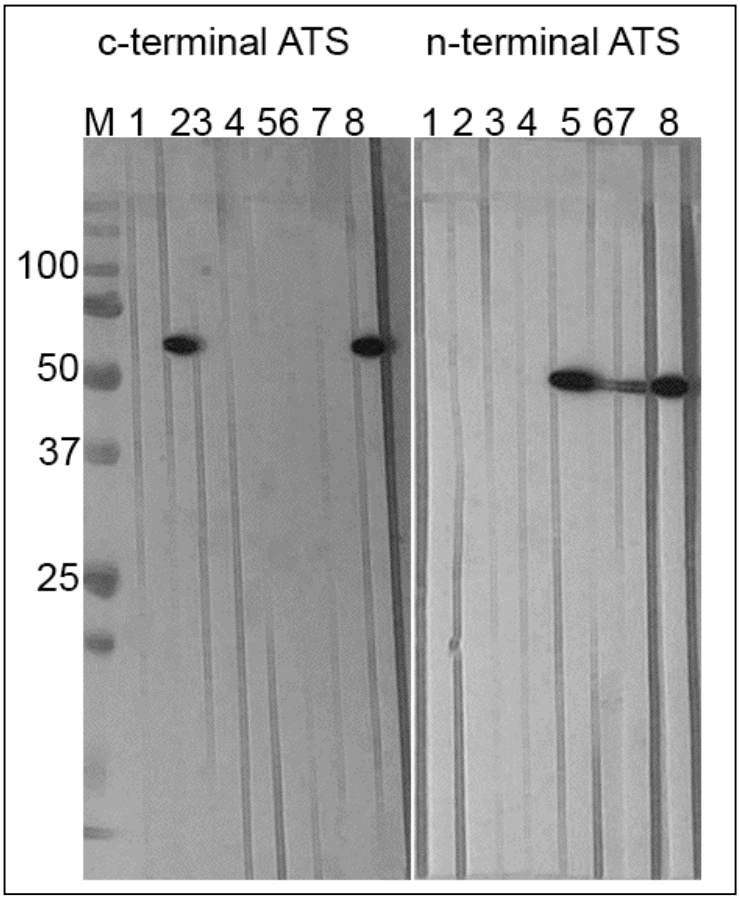


Figure 13. Western blot of different mice sera on recombinant ATS N-terminal and C-terminal fragments.

The C-terminal fragment (A) and the N-terminal (B) fragment of the ATS were blotted. The sera tested are: Lane 1,mouse 1 anti-ATS2 (ATS2M1); lane 2, ATS3M1; lane 3, ATS2 M2; lane 4, ATS4 M2; lane 5, ATS1 M1; lane 6, ATS1 M2; lane 7, ATS1 M3; lane 8, anti-6xHIS antibody.

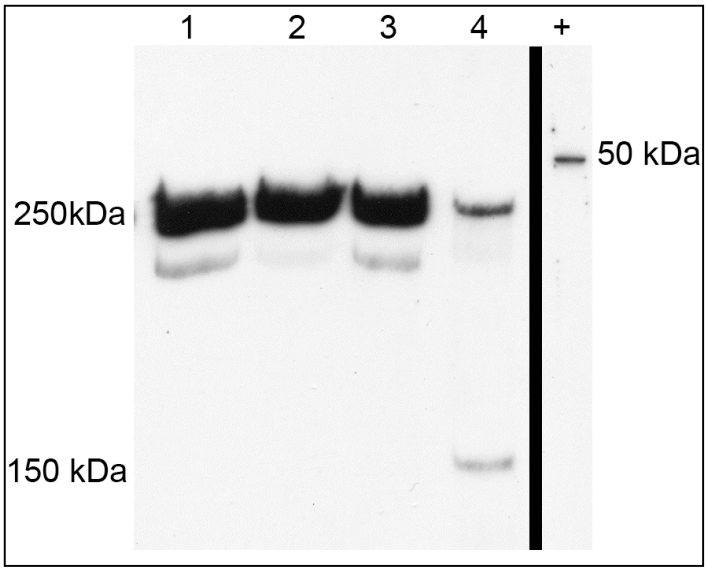


Figure 14. Western blot of parasite lysate probed with ATS1 M1 serum.

Serum (1:200) from mouse1 immunized with ATS1 was tested against different lysates. Strong recognition of a protein from the erythrocyte is visible around 250 kDa. Lane 1, erythrocyte lysate; lane 2, *Pf*3d7 lysate; lane 3, E8B lysate; lane 4, MACS purified *Pf*3d7 lysate. As positive control recombinant ATS-N was included (+).

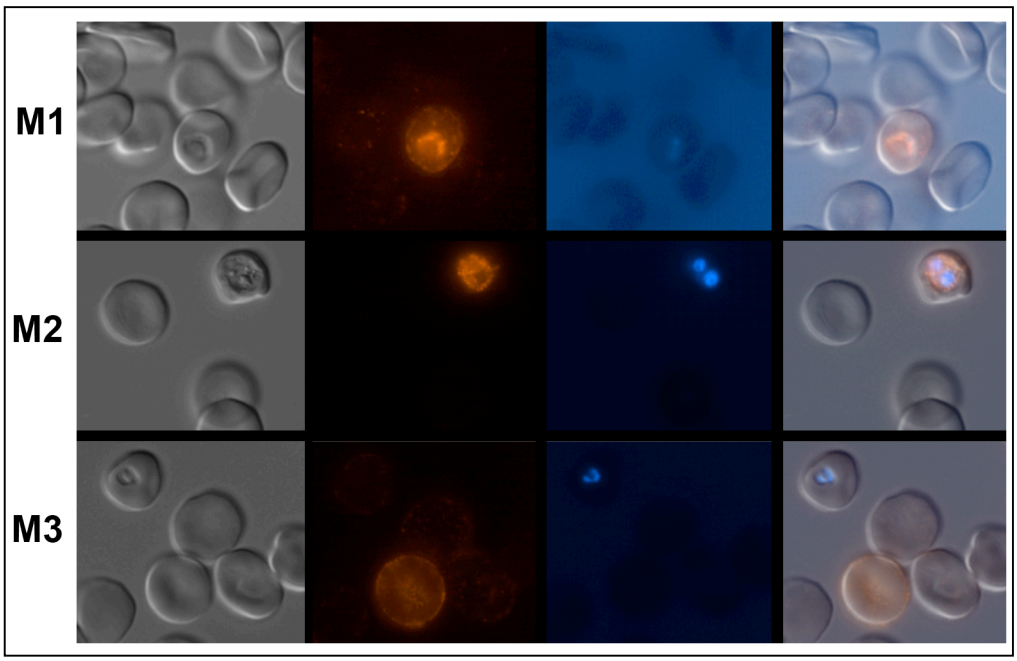


Figure 15. IFA of *Plasmodium falciparum* 3d7 infected red blood cells probed with sera from ATS1-immunized mice.

Formaldehyde fixed 3d7 cultures were stained with DAPI and probed with 3 different mouse sera ATS1 M1-M3. For M1 and M2 structures in the infected red blood cell were labelled. For serum from M3 an uninfected red cell was stained. Columns from left to right: DIC image, anti-ATS sera Cy3, DNA DAPI stain, overlay.

Chapter 3

Dicussion

Expression of large recombinant proteins

Investigations on recognition frequencies of *Pf*EMP1 are mainly based on the DBL domain. To obtain a broader picture of the differential recognition of *Pf*EMP1 variants, we tried to express large fragments of this protein from the sequenced strain *Plasmodium falciparum* 3d7. We established long range PCR and were able to amplify DNA up to 8kb long. The difficulties arose with cloning of the amplified fragments. The E.*coli* expression vector chosen for this experiment had a size of about 5 kDa. Despite the numerous long range PCR fragments we obtained, cloning efficiency was poor. This ineffective cloning strategy is most probably due to the size of the inserts, as the probability for re-ligation drops with increasing insert size. Nevertheless, we generated one construct with a 4 kb insert. However, the expression of this fragment was impossible although sequencing revealed that the insert was in the expected frame. Retransfection of the plasmid into a bacterial strain coding for rare tRNAs did not improve expression at all. The protein encoded on the plasmid had an expected size of almost 140kDa and we concluded that this size overstressed the translation machinery of *E.coli* [83]. This is supported by the fact that almost no *E.coli* protein visible on a SDS gel exceeds 100kDa (see Figure 3, Results).

Random cloning of cDNA

Because we could not solve the cloning and expression problems with large *Pf*EMP1 fragments we decided to move to a more random approach. Bull *et al.* showed by agglutination assays that parasite isolates, which cause severe disease, are frequently recognized by heterologous sera whereas parasites causing mild symptoms present uncommon antigens and are thus less frequently recognized [89]. Based on these findings we wanted to narrow down the differential recognition to certain domains of *Pf*EMP1. We decided to generate an E.*coli* expression library that expresses random peptides of plasmodial proteins. Two identical copies of the

library should be probed with either non-immune or immune sera. Differentially recognized clones would be analyzed subsequently.

We isolated RNA from cultured parasites and reverse transcribed it into cDNA. The library constructed from this cDNA was heavily contaminated with ribosomal rRNA, which made it impossible to go on with the serology. We tried to improve our library and reduce the complexity of the sample by isolating full length *var*mRNA from crude RNA samples [55]. For this the mRNA is hybridized to an immobilized probe specific for the ATS domain and subsequently transcribed. But the introduction of this step dramatically reduced the amount of cDNA obtained. Irrespective of the detection of *var* cDNA by PCR it was not possible to clone it. It was concluded that the low amount was the reason for negative cloning result. The amplification of the mRNA prior to cDNA transcription should overcome this problem. A method exploiting the template switching effect of reverse transcriptase was used to introduce linkers to the cDNA. With the known linker sequence the cDNA could be PCR amplified afterwards. Amplification and cloning of the modified cDNA worked, however the resulting library contained again only ribosomal and transfer RNA. Despite the enrichment of *var* mRNA, the contamination of other RNAs was so intense that the library could not be used. A more stringent washing step introduced after hybridization of the *var* mRNA resulted in total sample loss.

Too many drawbacks and snags let us terminate these approaches to identify new domains which are differentially recognized by adult and children sera. From there on we focused on DBL domains only and performed serological studies (see also Chapter 4).

Expression of short fragments from different domains of *Pf*EMP1 as antigens

Rosetting is a major virulence factor of a malaria infection. The dissection of the molecular basis of rosetting is still ongoing and not yet completely understood. Serum factors and host proteins play an important role in rosetting as they act as bridging molecules [30]. Luginbühl *et al*. found that only three proteins can restore a rosetting phenotype in an otherwise protein free medium. The host proteins implicated in rosette formation are albumin, factor D, and anti-Band3 antibodies. It is further known that CR1 on the erythrocytes surface is involved in rosette formation [78]. The parasite proteins conferring the rosetting phenotype, however, are not yet

clear. The only evidence, so far, is that DBLα domains bind to CR1 and antibodies directed against DBLα block rosetting [90]. In *in vitro* rosette re-formation a 65kDa fragment has been shown to be cleaved off the iRBC with factor D. This was not observed with RBCs only or without factor D [31]. Being a protease, factor D is suspect of modifying the iRBC surface inducing rosette formation. However, factor D is a highly specific protease with the only known substrate factor B. To clarify the origin of the cleaved fragment and because there is a possibility that *Pf*EMP1 is involved in rosetting, we planned to generate *Pf*EMP1 antibodies. First we focused on the N-terminal and the C-terminal domains of *Pf*EMP1. Therefore we recombinantly expressed the NTS and the ATS domain. The use of recombinant ATS was discontinued, as it was not possible to purify the insoluble protein satisfying purity. We could not identify expression conditions which led to soluble ATS or prevented the breakdown of the protein as visible on Western blot (see Figure 5B and C). Late induction times, reported to improve such problems [79], did not increase the yield.

The NTS domain, in contrast, was expressed as soluble protein and purified subsequently via the 6xHIS- and the GST-tag. This co-purification gave pure protein acceptable for mice immunization. Two different adjuvants were used to increase the probability of a good response the antigen. On Western blots all sera were positive on the recombinant protein (see Figure 7). However on blots of parasite lysate the anti-sera did not recognize *Pf*EMP1. The immunogenicity of the recombinant proteins may result from the recognition of GST, as GST was also present in the immunized antigen. When using the mouse sera in IFA, rim-like structures stained which seems to be the ER [86-88]. Most proteins destined for export are co-translated into the ER [91]. Even though *Pf*EMP1 contains no classical PEXEL motif, it contains a modified variant within the first DBL domain [69]. In some organisms signal sequences are cleaved off during the translocation in the ER an it is possible that the NTS is cleaved off together with the signal sequence in the ER. Although it is unknown whether signal sequences are cleaved off in *Plasmodium*, recently it was reported that the PEXEL motif is cleaved in the ER (HH. Chang, Woods Hole, unpublished). Such N-terminal processing of *Pf*EMP1 would explain the results obtained in IFA and Western blots. Therefore it cloud be possible that *Pf*EMP1 was present in the protein lysates used for Western blots but was already

trafficked through the ER and did not contain the NTS domain anymore. This would explain why Western blots with these sera on whole protein lysates were negative. In IFA, the signal obtained could represent signal sequence which has already been cleaved off and is in the ER or the complete protein on its way to the PVM. Further investigation is required to clarify if the mice anti-NTS sera generated really bind to the endogenous NTS domain or if the IFA results are an artifact. The plan of using this antibody to detect the processed fragment after rosette formation was abandoned, as sera characterized were not completely satisfying.

Protein expression in general – size matters

Recombinant protein expression of plasmodial proteins seems to be difficult in general. In a large experiment where 1000 ORFs were cloned for expression, only 337 expressed a protein at all; from those only 63 where soluble [83]. These numbers indicate that soluble expression of malarial proteins is a challenging task. In the same publication the expressability and the solubility were correlated with the isoelctric point, the size of the protein and the homology to *E.coli*. Authors concluded, that the smaller the protein, the more acetic the pI and the closer the homology to E*.coli*, the greater is the chance of soluble recombinant expression. The predicted expressability of proteins was not the criteria for the choice of the proteins expressed in our work. But in a retrospective analysis of all proteins expressed we can conclude the following: In our case the homology to *E.coli* could not be taken into account, as there is no homology of *Pf*EMP1 to the bacterial proteome. The acidic pI e.g. for the ATS did not enhance solubility in our work. The only factor that influenced the expressebilitiy (but not the solubility) was the size. The large *Pf*EMP1 fragments did not express at all and the ATS domain was better expressable when it was split into two smaller fragments.

The proteins expressed in this thesis were all but one insoluble. However, the expression of soluble fragments is generally possible, as it has been demonstarted for MAHRPs and ETRAMPS in our lab [72, 92]. A real determinant for the solubility of *plasmodial* proteins expressed in *E.coli* remains to be elucidated. Alternatively, more efforts on dialysis and refolding of expressed proteins could help to increase the solubility.

Synthetic peptides as antigens in mice

Since we were unable to generate antibodies against the NTS domain, we focused again on the ATS domain. The C-terminal domain of *Pf*EMP1 qualifies for the generation of pan-specific antibodies, as this part is intraerythrocytic and not subject to antigenic variation. The recombinantly expressed ATS was impossible to purify and was prone to degradation. The ATS was insoluble and the GST tag implicated for a second purification step was thus useless. We had expected the ATS to be in the soluble form as it is the domain with the highest prediction of solubility of all *Pf*EMP1 domains [93]. We changed our approach from recombinant protein expression to synthetic peptides. Synthetic peptides harbor a lot of advantages compared to recombinant proteins. They are pure, soluble and contain only the desired sequence against which antibodies should be raised, i.e. no tags and no multiple cloning sites. The poor antigenicity of synthetic peptides can be overcome by antigenicity increasing modifications such as conjugation to large antigenic proteins like the Keyhole Limpet Hemocyanin (KLH) or by connecting several peptides to a star-like structure. We chose 3 peptides based on a 3d7 sequence alignment of all ATS domains. In the conserved regions the consensus sequences reached similarities of over 90% and we selected 3 peptides from these areas. An additional fourth peptide was chosen from the FCR3 S1.2 ATS domain due to its predicted coiled-coil structure. The self-assembly of these structures is used to mimic a natural conformation of the otherwise linear peptide. Using Titer-Max-Gold which is an oil-in-water emulsion, a single dose of antigen is enough to elicit a boosted antibody response. Subsequent boosting is therefore not necessary and consequently requires less antigen.

To perform Western blots with peptides is a rather complicated procedure for several reasons as listed in the following. Firstly, as peptides are very small they often travel thru the nitrocellulose membrane. Furthermore, hydrophobic interactions with the membrane are weak also according to their size. This often leads to the loss of the antigen during washing steps. To overcome the problem of performing Western blots with the synthetic peptides to test the sera, we chose a different approach. In order to test whether the peptides would elicit antibodies that recognize the ATS domain we split the ATS domain into two and expressed each fragment in *E.coli*. Both parts were insoluble and expressed in high levels. We used

these proteins as antigens to test the mouse sera in Western blots. The immunization yielded 4 different sera, which were positive in Western blots and 6 sera that were positive in ELISA. The ELISA plates were coated with the peptide but not with recombinant protein. The sera which were positive in ELISA but not in Western blots therefore probably recognized specific conformations of the peptides or the ends of the peptides which are not present in recombinant proteins in Western blot. These sera were excluded from further analysis. The other 4 sera were tested on different parasite lysates. Unfortunately, all sera recognized a host protein also present in uninfected red blood cells, most probably it is human spectrin. This crossreactivity has been reported by other groups too [43]. Consequently, lysate conditions were modified to obtain protein fractions that are enriched for parasite proteins. The best possibility to achieve this was to purify iRBC over a magnetic cell sorter. The elution contained almost 100% parasitized cells. This cells were Triton-X-100 lysed, which enriches for integral membrane proteins. This reduced the complexity of the sample as was seen because the background recognition of spectrin was reduced compared to other methods (see Figure 14). Nevertheless, no endogenous *Pf*EMP1 was detected. It is not clear whether the epitope on the endogenous protein is somehow altered by the blotting procedure or whether the expression level of *Pf*EMP1 in cultured parasites is below the detection limit. However, others have shown perfectly stained *Pf*EMP1 with antiserum [76, 94]. But these antisera were either affinity purified or preadsorbed on ghosts. Our initial efforts on such purification steps ended with complete loss of reactivity of our sera (data not shown).

Also, none of the sera labeled *Pf*EMP1 in IFAs in a way previously shown [43, 74]. We observed occasional staining of uninfected RBCs, which is consistent with the results from our Western blots in which uninfected RBC also gave a signal.

For future work, further efforts in the development of pan-specific antiserum should include the generation of monoclonal antibodies to reduce cross-reactivity and the selection for a high affinity isotype. Additionally more efforts can be done on the affinity purification of the polyclonal sera.

Chapter 4:

Recombinant expression and serology of DBL domains isolated from patients with severe or asymptomatic malaria

Introduction

Switching of *var genes*

One way how *Plasmodium falciparum* escapes the immune system is by antigenic variation. *Pf*EMP1 is expressed on the surface of erythrocytes and mediates cytoadherence. It is encoded by the *var* multigene family. There are about 60 different *var* genes encoded in the parasites genome, however, only one is expressed at a time [56, 95, see also Chater1; Introduction]. From the parasite's perspective, this mutually exclusive expression is a "two edged" situation: On the one hand, the parasite has to maintain the expression of the same *var* gene through several cycles to not exhaust the repertoire to early. On the other hand however, it has to switch to a new variant as soon as the immune system starts to defend the currently expressed *Pf*EMP1 [96]. A switching rate of 2,4% per generation was calculated for *in vitro* cultures [97], but rates for *in vivo* infections seem to differ substantially. Artificial infection of non-immune adults resulted in a switching rate of 16% for the initial switching event, though switching rates of later events decreased and seemed to be dependent on various factors such as the *var* gene itself and host conditions [98]. It was also shown that the expression pattern of central *var* genes was more stable than the expression of subtelomerically located *var* genes, which switch faster to alternative *loci* [99]. But switching rates are not correlated with the 5` promoter group (ups) [96]. The regulation of this mutually exclusive transcription is not yet completely understood but involves histone modifications [100] and the location within the nucleus [101]. *var* gene regulation will however not be discussed in this chapter.

Correlation of *var* variant and disease outcome

var gene sequences are grouped in four different groups (A, B, C, E) based on their upstream sequences (ups). The regulatory differences between these groups remain to be elucidated. Different promoter elements have been found in upsB and upsC *var* genes. A subtelomeric *var* promoter element 1 (SPE 1) was locted in upsB *var* genes and the chromosome-central *var* gene promoter element (CPE) was only

found in ups *C var* genes [53]. This implies a differential regulation of these two types of *var* genes. What role they play in regulation and silencing is currently under investigation.

Malaria pathology however, has be associated with group specific expression. An upregulation of upsB *var* genes was found in severe and mild malaria cases in Africa and PNG, whereas upsC was correlated with asymptomatic cases [54, 102]. Bull *et al.* [103] used the DBL domain to classify different groups of *Pf*EMP1 variants. The basis of these classifications is the number of cysteine (cys) residues as well as positions of limited variability (PolV). The “Bull-groups” 1-3 represent the 2cysDBL group, which represents the upsA and small subgroup of upsB *var* genes, whereas the groups 4-6 with 4 cysteine residues cover upsB and upsC *var* genes [104]. 2cysDBL/upsA was associated with the rosetting phenotype [104, 105], cerebral malaria in children [104] and non-cerebral severe malaria in adults [62]. It was also shown that it is possible to select the laboratory strain 3d7 for *Pf*EMP1 variants normally expressed in severe cases [106].

The best understood correlation of disease and expressed *var* gene is that of *var*2CSA (reviewed in [22, 107]). Women who have previously acquired immunity against malaria can develop an episode upon pregnancy or have pregnancy complications as preterm delivery or stillbirth. The reason is that chondroitin sulfate A (CSA) is expressed in the placenta [108]. This low sulfated glucosaminocglycan (GAG) represents a new interaction possibility for surface molecules of the iRBC. After the first or second pregnancy women are protected from PAM (pregnancy associated malaria). The development of antibodies against special domains from that *var* gene seems to prevent binding of iRBCs to the placenta. Additionally, these domains seem to be not as divers as expected because sera from protected women bind to placental parasites from geographically distinct regions [24]. The phenomenon that antibody mediated protection against a very special *Pf*EMP1 variant is possible and even relatively long lasting, generates hope for the development of a vaccine. Even though it would not confer sterile immunity, it could prevent severe disease and pathology.

Rare and prevalent variants of *Pf*EMP1

The repertoire of *var* genes in one parasite is about 60, but the worldwide repertoire of all wild type *Pf*EMP1 is probably endless. It is known that the antibody pool in a semi-immune individual is sufficient to protect from a malaria episode, as is the case for semi-immune adults living in endemic areas in Africa. However, such individuals have never seen all *Pf*EMP1 variants because the worldwide *Pf*EMP1 repertoir is endless. Nevertheless, semi immune individuals are protected from malaria. Obviously, there seem to be antibodies against specific *Pf*EMP1, which contribute more to protection from severe disease than other anti-*Pf*EMP1 antibodies.

*Pf*EMP1 is considered to be the main interaction partner to host cell surface receptors, and antibodies against *Pf*EMP1 prevent cytoadherence and therefore severe malaria. Although *Pf*EMP1 must be very diverse to trick the immune system, it must also contain conserved domains which confer binding to the receptors. Modifications in binding domains will indeed account for antigenic variation but will reduce the binding efficiency to their receptor. Not all variants of *Pf*EMP1 will have the same binding affinity to the host endothelium, thus making some variants more virulent than others.

Bull *et al.* [89] used agglutination assays to show that host immunity develops against *Pf*EMP1. In their experiments they took serum samples from children during a severe malaria episode and after convalescence. They also cultivated the parasites causing this malaria episode. They found that patient's convalescence sera agglutinated the cultured parasites whereas the acute sera did not. This showed that the diversity of *Pf*EMP1 is reflected in the fact that normally only parasite isolates matching the sera will agglutinate, but this sera will not bind to parasite isolates from other patients [89]. Immunity against malaria is variant specific. The same authors found that parasite isolates from patients with severe disease are recognized frequently by different serum samples thus representing common antigen variants. Parasite isolates that are not widely recognized (expressing a rare type of *Pf*EMP1) are generally not obtained from severe case patients. This supports the hypothesis of a trade-off between variability vs binding

efficiency. An immune adult that exhibitis a patchwork antibody repertoire against the common isolates (good cytoadherence= severe disease) will not develop a severe episode since the rare isolates (bad cytoadherence = mild/no disease) can proliferate.

Organ specific expression of *Pf*EMP1

The capacity of *P.falciparum* to cause severe malaria is at least in part due to the ability to cytoadhere. As the host cell receptors are numerous and *Pf*EMP1 is diverse, the site of adherence can be distributed in different tissues. Montgomery *et al.* [109], investigated the genotypes (based on the *msp2* locus) sequestered in different organs as well as the *var* types expressed. They found that over 100 different *var* types were expressed in a single patient and up to 10 different per genotype and per organ. This is conflicting with other studies where less than 2 different *var* types were found per genotype [55]. They also investigated the correlation of genotype or *var* expression with specific tissues. Despite the random distribution of different genotypes in the body without genotypic preference in special organs [110], there was tissue specific expression of *var* types. The organ dependent *var* expression was obvious for brain and heart mirovasculatur [109]. Not only that the same *var* types were found in one organ in one patient but the same types were also found in the same organ in other patients from a geographically distinct location. These patients with fatal severe malaria, which shared some *var* types of parasites sequestered in the brain, were from different villages in Malawi.
In the same study they found that asymptomatic children who died of other causes than malaria had almost no sequestered parasites in the brain, regardless of extremely high peripheral parasitaemias with more than 300000 parasites per micro liter blood.

These findings support the feasibility of a vaccine containing *Pf*EMP1 domains, because within the endlessly large repertoire of worldwide *Pf*EMP1 variants, apparently not all *Pf*EMP1 variants are equally pathogenic. By tackling the disease-causing variants, the repertoire of *Pf*EMP1 candidates could be narrowed down to key candidates of high pathogenicity.

In conclusion, even though *Pf*EMP1 is very diverse it appears that only a relatively small subset of *var* types is responsible for severe disease. This has already been shown for PAM where also the same *var* type is dominant in different patients as described above.

In this work we have further investigated in the role of certain *Pf*EMP1 variants in severe and asymptomatic cases. We selected DBL domains from *var* genes expressed in severe episodes and asymptomatic infections. We recombinantely expressed them in *E.coli* and measured antibody levels against these DBL 1α domains in sera from semi-immune adults. We found that DBL domains from patients with severe malaria are more frequently recognized than DBL domains from patients with asymptomatic malaria. We used the same recombinant DBL domains and tested sera from children which were collected at two different timepoints within a longitudinal study in Tanzania. The children had no acute malaria episode in between the two timepoints.

Chapter 4

Materials and methods

Isolation of DBL domains from patients with severe and asymptomatic malaria

Blood samples were collected in Tanzania from children with severe malaria (8) and from asymptomatic children (7). Total RNA was extracted, full-length *var* mRNA isolated and reverse transcribed from each sample as described below. Subsequently the DBL domain was PCR amplified from the cDNA using primers dbl_fwd and dbl_reverse (see appendix for sequence information) and cloned into pGEM-T (Promega) vector for sequencing (performed by Paschal Mugasa). For each patient, plasmids were isolated from 50 clones and subsequently sequenced.

Plasmodial RNA extraction and complete cDNA synthesis

Blood samples from *Plasmodium falciparum* infected patients were resuspended in 5 volumes Trizol® (Invitrogen). RNA was extracted from the lysate with 0.2 ml chloroform per ml Trizol® and precipitated with 3 volumes of isopropanol. To improve purity the pellet was again resuspended in Trizol® (0.5 volumes, compared to the first extraction step), extracted with chloroform and then precipitated. The precipitated DNA was digested by two subsequent incubations with RQ1 RNAse free DNAse (Promega) according to the manufacturer's protocol, in the presence of RNAse inhibitors. After each DNAse digest, the RNA was extracted and precipitated as described above. cDNA synthesis was performed using sensiscript® reverse transcriptase with random hexamer primers, in accordance with the respective manufacturer's protocol. RNA amounts used in a cDNA synthesis reaction varied according to the parasitaemia of the blood sample. A control reaction without reverse transcriptase was always included.

Reverse transcription of RNA

RNA samples either from bead hybridisation (full length *var* mRNA) or crude were mixed with 3.5µl 0.1M DTT, 2µl RNAse inhibitor, 7µl 10x RT-Buffer (sensiscript/M-MuLV), 7µl 5mM dNTPs and RNAse free dH_2O to 70µl. The reaction mix was split into a 50µl and a 20µl aliquot. To the 50µl aliquot 2µl of reverse transcriptase was

added, the other was left as negative control without enzyme. Both tubes were incubated at 37°C for 90 min. After the reaction the RNA:DNA duplex was melted at 93°C for 3 min and the RNA was digested with 1µl of RNAse A (1mg/ml) at 37°C for 20 min. The single strand cDNA was stored at -20°C.

Full length *var* mRNA extraction

To extract only the full-length *var* mRNA transcripts from a total RNA preparation, a hybridization technique was used as described in the following: 1 pmol of a biotinylated oligonucleotide complementary to the ATS domain was incubated together with the total RNA in hot binding buffer (0.5M LiCl, 1mM EDTA, 10mM Tris, pH7.5). After slow cooling from 65°C to 4°C over 30 minutes, 200 µg of streptavidine coated magnetic beads (dynal) were washed, dissolved in 5.5 M LiCl and added to the RNA:DNA hybrid and incubated for 30 minutes at 37°C on an over-head shaker. Beads were collected on the wall of the reaction tube by a surrounding magnet and washed 3 times with wash buffer (10mM Tris, 1mM EDTA, 0.15M NaCl, pH 7.5)[55]. Reverse transcription was performed directly on the RNA hybridized to the beads as described previously.

Cloning of DBL domains

The DBL domains had to be amplified from the plasmid preparations for re-cloning in expression vector pQE-16 (Qiagen). The primer pair dbl1_fwd and dbl1_reverse (see appendix for sequence information) containing the restriction sites E*co*NI and B*gl*II were used. The PCR product was purified (PCR clean-up, ROCHE) and digested with E*co*NI and B*gl*II. The vector was digested with the same enzymes, purified and ligated with T4 ligase (NEB) according to the manufacturer's protocol. The ligation was phenol:chlorophorm extracted, precipitated and resuspended in 10µl dH_2O. Ice cold electrocompetent M15 *E. coli* (Qiagen) were mixed with the resolved ligation and electroporated (single pulse, 2500V, 0.2cm gap, 5ms). Bacteria were spread on agar plates and screened for correct inserts by PCR with primer pair pQE_fwd and pQE_reverse (see appendix for sequence information).

Recombinant expression of DBL domains

50ml bacterial culture medium terrific broth containing the antibiotics ampicilin (100µg/ml) and kanamycin (25µg/ml) were inoculated with 1ml over night culture of M15 *E. coli* harboring the pQE plasmid with the DBL domain. The culture was incubated at 37°C and induced with a final concentration of 1mM IPTG when OD_{600} was 0.8. Four hours later bacteria were harvested by centrifugation at 8000g for 15 minutes and stored at -20°C until further use.

Purification of recombinant DBL domains

The bacterial pellet was lysed in inclusion body lysis buffer (50mM Tris pH 8, 100mM NaCl, 5mM EDTA, 0,5% Triton-X-100, 0.1mM PMSF and 1mM DTT) supplemented with 100µg/ml lysozyme and 10µg/ml DNAse (adapted from [111]). The slurry was sonicated 5 times 10 seconds in 10 second intervals with a microtip sonicator (50% duty cycle, output control 5). The insoluble proteins were separated by centrifugation at 10000g for 30 minutes. The supernatant was discarded and the pellet resuspended in inclusion body lysis buffer again. This step was repeated 3 times. In the last resuspension step of the insoluble pellet, PBS was used instead to avoid Triton-X-100 in the further purification steps. The washed pellet was resuspended in Urea buffer (8M Urea, 300mM NaCl, 10mM Tris pH 8) and mixed with 0.5ml Ni-TA-Agarose beads (Qiagen). The protein was further purified according to the manufactures protocol (Qiagen, the expressionist). Proteins were eluted from the beads with low pH elution buffer (8M Urea, 300mM NaCl, 10mM Tris, pH 4,5). Eluted proteins were stored at 4°C.

Purity and quantity assessment

Purity and quantity of eluted proteins was checked on SDS PAGE and stained with Coomassie blue. Quantity was estimated from marker bands with known protein concentration. Fractions containing the pure protein were pooled and again measured in a spectrophotometer at 280nm (NanoDrop), and then stored until use at 4°C.

ELISA of DBL domains with human sera

Maxisorb 96 well ELISA plates (Nunc) were coated over night at 4°C with 10µg/ml recombinant protein in PBS. Plates were washed once in an ELISA washer and blocked for 1 hour at room temperature with 75µl 5% non-fat milk powder in PBS 0.01% tween 20 (PBS-T). Then, plates were washed again and incubated with serial dilutions of human sera starting from 1:200 in 1% non-fat milk powder in PBS-T (see table 2). After incubation for 2 hours at room temperature, the plates were washed twice in the ELISA washer and then incubated with the secondary antibody (goat anti-human IgG alkaline phosphatase labeled (1:5000)) in 1% non-fat milk powder in PBS-T for one hour at room temperature. After washing, the plate was developed with PNP (p-Nitrophenyl-Phophate 1mg/ml) in alkaline substrate buffer (160mM $NaHCO_3$,130mM Na_2CO_3, 1mM $MgCl_2$, pH 8,6) and measured in an ELISA reader at 405nm.

Dilution	Sera											
	positive control	negative control	serum1	serum2	serum3	serum4	serum5	serum6	serum7	serum8	serum9	serum10
1:200												
1:600												
1:1800												
1:5400												
1:16200												
1:48600												
1:145800												
1:1312200												

Table 1. ELISA plate layout.

In the column in yellow serum dilutions are indicated. In blue the different serum samples 1-10 tested in the experiment are shown. Positive and negative controls remain the same for all experiments. As positive control goat anti-6xHIS antibodies were used, and the negative control was a serum pool from European adult blood donors with no history of traveling in malaria endemic areas.

ELISA calculations

100 sera were tested on all different DBL domains (15 in total). As all recombinant proteins contain the DHFR tag, all sera were additionally tested on DHFR to assess the background. All OD_{405} values were recorded and were corrected for the DHFR recognition by subtraction: $OD405_{Sera1} - OD405_{DHFR}$. The corrected values were plotted against the logarithmic dilution. The mean of the negative values plus 2

standard deviations gave the threshold. The endpoint titer corresponded to the dilution were the curve of the plotted sera meets the threshold line. The threshold was calculated for each plate individually.

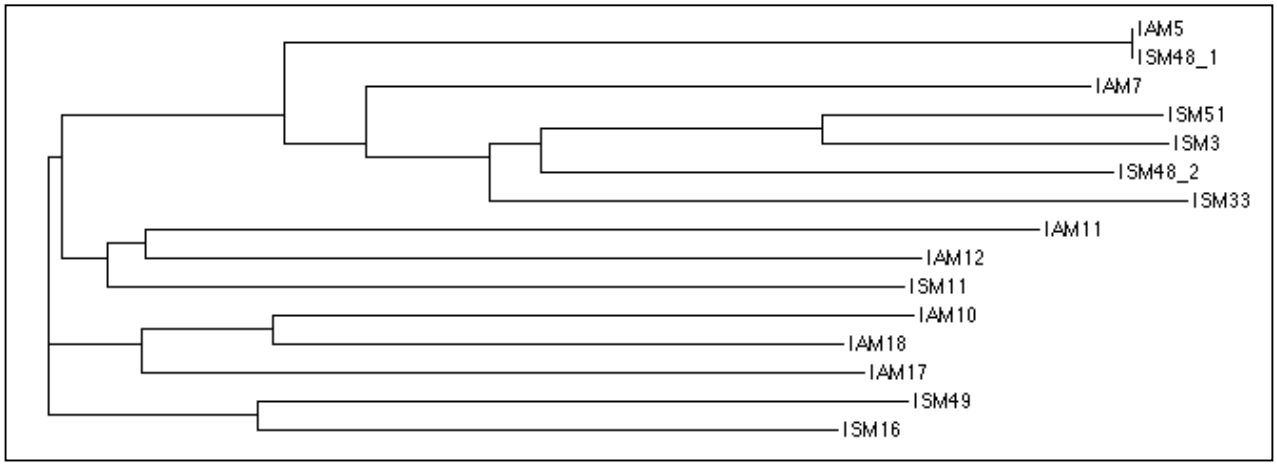
Results

There are approximately 60 plasmodial *var* genes coding for variants of *Pf*EMP1. There is several evidence indicating that *Pf*EMP1 plays a crucial role in host immune evasion and thus represents a key virulence factor (as reviewed in [25, 112]). However, it is to date unclear why this *Pf*EMP1 repertoire is so big and what differences might exist in terms of virulence/pathogenicity between the different *Pf*EMP1 proteins. In this work we wanted to test how recombinantly expressed DBL domains of *Pf*EMP1 from parasites of severely ill patients versus DBL domains from parasites in asymptomatic cases are recognized by sera of semi immune adults. We hypothesize that semi immune adults have higher titers against DBL domains deriving from severe cases than against asymptomatic cases. The rationale behind it is that adults who are semi-immune (thus not sick) are better protected because they recognize the repertoire of *Pf*EMP1-DBL domains causing severe pathology better than those causing asymptomatic malaria.

To test our hypothesis we have chosen a sero-epidemiological approach and assessed antibody titers of 100 semi immune adults from asymptomatic malaria cases of a cross-sectional study in Papua New Guinea. Sera titers were assessed against 15 different recombinantly expressed DBL sequences from *Pf*EMP1. Seven of those DBL domains were isolated from severe malaria patients and seven were from asymptomatic cases. One additional DBL domain was also expressed, but later excluded from analysis because it did not represent the dominant infection. These DBL domains were termed either “severe DBL domains” when derived from sequences of severely ill patients, or “asymptomatic DBL domains” when derived from patients with asymptomatic malaria.

We also tested sera from 34 children deriving from a longitudinal study for their DBL recognition at baseline and six month later. Importantly, these children did not suffer from a clinical episode between the two time points. By testing which DBL domains were recognized by these children at two different time points, we were able to study the dynamics of the antibody repertoire.

Eight recombinant DBL domains sequences were from severe case (ISM) patients and 7 from age matched asymptomatic children (IAM). We did a ClustalW alignment to test if clusters were identifiable. As shown in Alignment 1, there was no clustering of severe and asymptomatic DBLs in an amino acid sequence alignment



Alignment 1. Alignment of 15 DBL protein sequences of *Plasmodium falciparum* erythrocyte membrane protein 1.

Amino acid sequences from DBL sequences derived from parasites infecting severe ill malaria patients (ISM) and asymptomatic samples (IAM) were aligned. Numbers correspond to the different isolate IDs (clustalW, default settings).

Expression of DBL domains

15 DBL domains were successfully expressed as DHFR fusion proteins with a 6xHIS tag (Figure 1). Additionally, recombinant DHFR-6xHIS was expressed as control. All proteins were expressed in *E. coli* in high levels but were insoluble. Purification of recombinant proteins resulted in very pure highly concentrated recombinant proteins. Concentration was at best 3mg/ml in a volume of 2.5 ml from a 50ml *E. coli* culture.

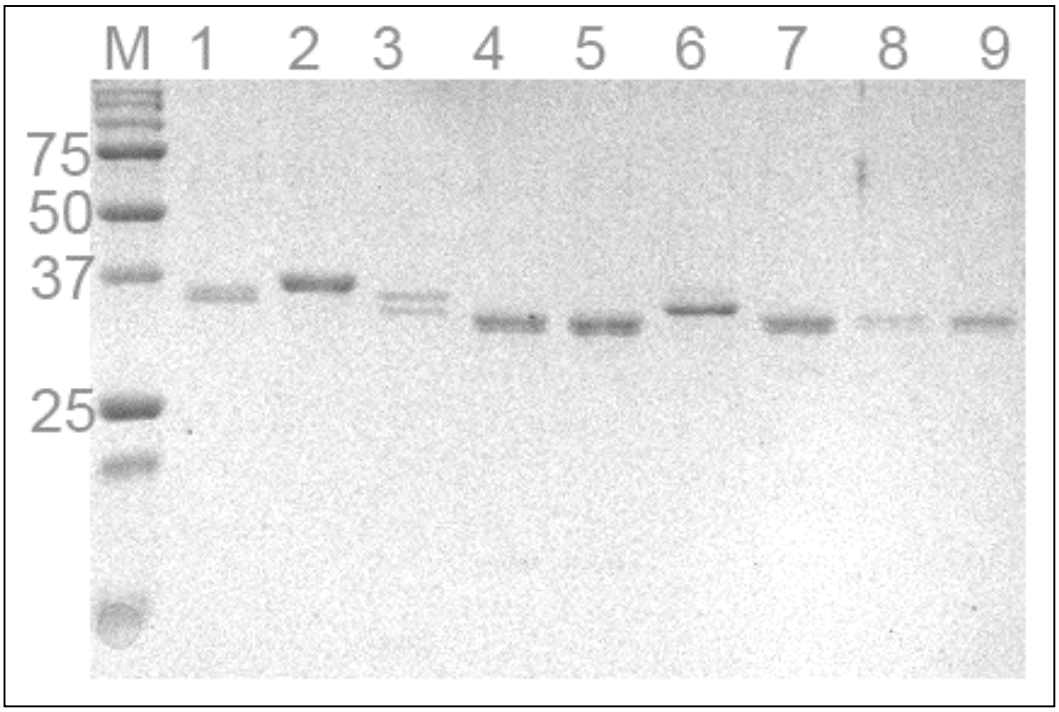


Figure 1. Coomassie stained polyacrylamide gel of recombinant expressed DBL domains.

Nine different DBL domains purified over a Nickel column were size-separated on a 12% SDS PAGE and stained with Coomassie blue. Samples were diluted 1:100 from original elution. M, marker in kDa; lanes 1-9 show the first 9 expressed DBL domains, in detail: ISM51, ISM16, ISM3, ISM33, IAM10, IAM11, IAM17, IAM12, IAM18.

Western Blot of DBL domains with a positive serum pool

To test for contaminations not visible in Coomassie stained gels, all purified proteins were blotted and probed with a positive serum pool from semi immune adults from Papua New Guinea as shown in Figure 2. All DBL domains were recognized at least by one serum, thus were immunogenic. The DHFR recombinant protein was also weakly positive. The blot was over exposed to amplify even weak signals.

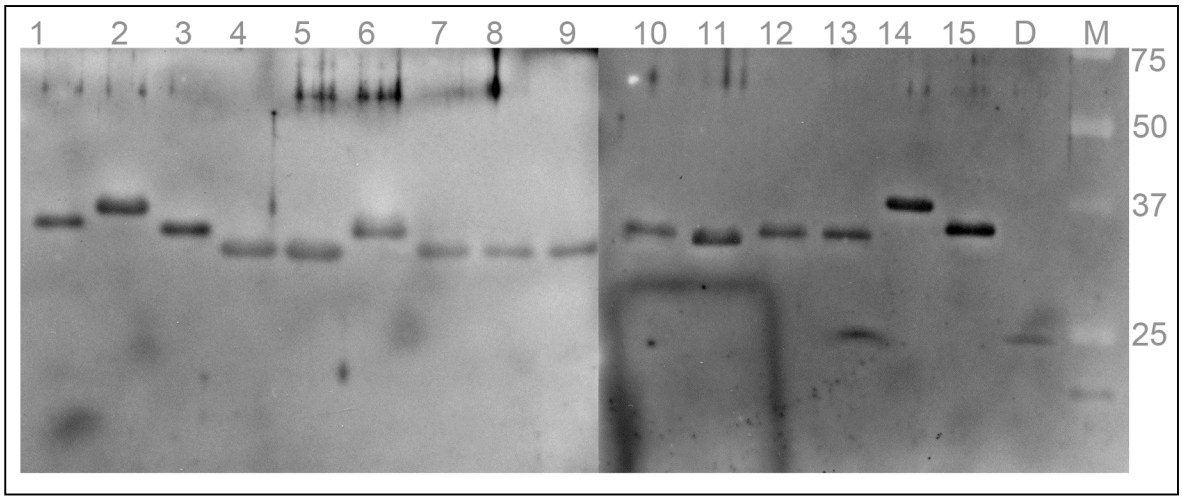


Figure 2. Immunoblot analysis of recombinantly expressed DBL domains.
20 ng of 15 different recombinantly expressed DBL domains were probed in Western blot with serum from Papua New Guinea. DBL domains are visible as clear bands at expected sizes between 35 and 40 kDa. DHFR (D) runs at an expected size of 25 kDa. Lane 1-15: DBL domains 1-15; D, DHFR; M marker in kDa. DBL domains in detail: ISM51, ISM16, ISM3, ISM33, IAM10, IAM11, IAM17, IAM12, IAM18, IAM7, ISM48_1 (minor), ISM48_2, ISM49, ISM51. The DBL ISM48_1 was the minor clone and was excluded from analysis.

ELISA with adult sera show that severe DBLs are more often recognized

To investigate frequencies of recognition of the different sera, we performed enzyme-linked-immunosorbent-assays (ELISAs). Briefly, recombinantly expressed antigens were immobilized in 96 well plates and sera binding was tested. Endpoint titers were measured from 100 adult sera from asymptomatic malaria cases of a cross-sectional study in Papua New Guinea. Data sets were assessed as shown in Table 3. All 100 sera recognized at least one recombinant DBL domain. There were two sera samples that recognized all 15 domains tested (see Table 1, SUK52, SUK93). There were striking differences in the recognition of different DBL domains. The three most frequently recognized domains were IAM11 with 72%, ISM3 with 73% and ISM51 79%. Two of those derived from the severe DBL group and one from the asymptomatic group. The three least frequently recognized antigens were IAM12 in 15%, IAM10 in 16% and ISM 48 in 16% of all sera tested. Two of these DBL sequences were from the asymptomatic group and one was a severe case.

To obtain a cleverer picture of the recognition of different frequencies for severe and non-severe DBL domains, the endpoint titer table was condensed to show only “recognition” or “no recognition”. For each of the 100 sera from semi immune adults, the number of recognized antigens (severe DBL vs asymptomatic DBL domains) was counted. The 7 severe DBL domains were recognized 295 times by 100 sera and the 7 asymptomatic DBL domains were recognized 213 times (see Graph 1A). Thus, severe DBL domains were recognized 1,4 times more often than asymptomatic DBL domains. Furthermore, all sera were grouped depending on their frequency of recognition. Groups from 0 (no DBL domain recognized) to 7 (all DBL domains tested recognized) were plotted in a histogram (Graph 1B). The number of sera recognizing asymptomatic DBL domains is high only for the “0 and 1 DBL recognized group”. In the case of severe DBL domains the recognition frequency peak is shifted towards higher numbers and peaks at 2 DBL domains recognized. Two or more DBL domains were recognized more often from the same sera when they were severe DBL domains. The results of all ELISA experiments are shown in the following Tables 1 and 2.

Table 1. ELISA endpoint titers of semi immune adult sera tested on different DBL domains

	IAM 11	IAM 5	IAM 7	IAM 12	IAM 17	IAM 18	IAM 10		ISM 3	ISM 51	ISM 16	ISM 48 2	ISM 33	ISM 48	ISM 11	ISM 49
SUK 4	200	600	200							400	1000					
SUK 8	1000	500							2000	2000		800		300		
SUK 11	500								300	400						
SUK 12	1000	500	500						800	2000		400			300	600
SUK 13	200															
SUK 14	3000															
SUK 16	500		2000						400	600		200				
SUK 21										300						
SUK 22									200	2000						
SUK 24									2000	10000						
SUK 25																
SUK 26											1000					
SUK 27	4000								2000	1000	500					
SUK 28	300								200	400	1000					300
SUK 29	500	400				200			400	4000	600	300			200	
SUK 30	900	400							1000	3000		1000		800		
SUK 31	300	300							5000			700				
SUK 32									2000							
SUK 35	500								900	2000	700	500		400		
SUK 38	10000								900	1000	700	1000				
SUK 39	200								800		800	400		200		
SUK 40	500	200							400	4000		300				
SUK 41				1000	1000	1000	1000		2000		800		1000			1000
SUK 43	1000									1000						
SUK 44	3000	500							600	1000	1000	400		200	500	
SUK 45	3000								600	1000					3000	
SUK 46	3000									300						
SUK 47	400	1000		300			300		200	1000	1000	600		400		
SUK 48	2000	600	2000	400	700	500	400		4000	10000	4000	500	400		1000	700
SUK 49	2000	1000	2000						2000	900	2000	1000		600	300	400

SUK 50		600		200					1000	2000	200	200		200		
SUK 51	3000	300	200						1000	2000	200	400		200	600	300
SUK 52	7000	5000	3000	4000	2000	3000	2000		8000	4000	5000	4000	1000	3000	4000	3000
SUK 53	2000								10000	10000						
SUK 55	200								400	200					600	
SUK 56	1000								400	600	1000		200			
SUK 58	400								200	200	300					
SUK 59									200		300					
SUK 60	500															
SUK 61										400	2000					
SUK 62	2000	500		400		400			2000	1000			300			
SUK 64	9000								600							
SUK 65	1000								200	200		200		200	200	
SUK 66	900	400	300						600	600		600		300		
SUK 67	1000	700	200						600	1000		1000		1000		4000
SUK 68	1000	1000	200	400	300	200			1000	11000	600	2000		1000		300
SUK 72									600							
SUK 73	1000								900	2000	200					
SUK 76	2000	2000		1000	1000	1000	1000		1000	2000	1000	1000	2000	500		600
SUK 79	300	400							1000	700						
SUK 80	3000	300							2000	1000	500					300
SUK 81	900								300	400						
SUK 83	600								300							
SUK 85		400	900	900	600	800	600		3000	13000	600		500	300	1000	500
SUK 86	900	800	200						900	1000	800	600	200			
SUK 87			300													
SUK 88	1000		200						1000	1000	800					
SUK 89	300								300	2000						
SUK 90	900	300							1000	1000	2000					600
SUK 93	3000	1000	1000	1000	1000	1000	1000		5000	4000	700	1000	1000	400	900	1000
SUK 94	4000	2000	900	3000		2000	2000		4000	4000	1000	300	3000	2000	1000	900
SUK 95											1000					

SUK 98	4000		200						900	5000						
SUK 102																
SUK 103	200									600						
SUK 105	1000		200						900	2000						
SUK 108	200		600			300				400						
SUK 109	300		300						2000	900				200		
SUK 110	300		400	200												
SUK 113	1000								500	4000						
SUK 115										600	3000					
SUK 116	300								300	500					300	
SUK 118	400	800			400	200	200		2000	2000		400				
SUK 119		2000	1000	900		800	200		2000	4000	3000	5000		2000	1000	2000
SUK 120																
SUK 122			500						1000	200						
SUK 127	900								300	2000	300	300				
SUK 128				2000	1000	2000	2000		2000	2000			3000			6000
SUK 130																
SUK 131	600								800	600		500				
SUK 134	700	400								500	600	500			2000	300
SUK 135			900				1000		400	900		400				
SUK 136	1000									6000	900					
SUK 136			1000								200					
SUK 137	700									200	7000					
SUK 139	2000	700		600	200	400			2000	2000	600	1000			200	400
SUK 142			800							400						
SUK 143	2000								1000	3000	200					
SUK 144		200	400	200	300				600	600	600					300
SUK 147		500	200			700	3000			1000		500		300		300
SUK 150	1000	200	200	300		400			600	500	200	300	200	300	200	200
SUK 151	1000	2000	300	1000	600	700	2000		1000	1000	1000	1000	900	2000	300	300
SUK 152	1000	2000			600				1000	700	300	400		1000	600	
SUK 159	500	200							300	600	3000					

SUK 185	1000	1000							2000	1000	3000			800		
SUK 190	1000				400	700	400		600	400				500		
SUK 195	1000	500								600		800		500		
SUK 196		500		1000	2000	1000	3000		200				3000	500		
SUK 199	4000			1000	2000				9000	2000			3000	500		
SUK 210										200	400					

Table 1. ELISA endpoint titers of semi immune adult sera tested on different DBL domains.

Adult sera tested on 15 different DBL domains. Left column: serum sample names. Top row: isolate names IAM (Ifakara Asymptomatic malaria) ISM (Ifakara Severe Malaria). Numbers in the table indicate the reciprocal endpoint titers. No number indicates that the serum is negative for the DBL domain tested. Colors: white, negative serum; yellow, endpoint titer 200-600; orange 700-2000; red over 2000.

Table 2. ELISA endpoint titers of children sera tested on different DBL domains.

	IAM11	IAM5	IAm7	IAM12	IAm17	IAM18	IAM10	ISM3	ISM51	ISM16	ISM48 2	ISM33	ISM48	ISM11	ISM49
BC583															
BP123	3000	1000	1000	1000	1000	1000	1000	5000	4000	700	1000	1000	400	900	1000
BC626															
BP125															
BC623				1000	700	1000	700	4000				1000			
BP146				2000	1000	2000	1000					1000			
BC619															
BP162															
BC640															
BP182															
BC582															
BP096															
BC568															
BP076										200					
BC600															
BP161															
BC598															
BP071										200					
BC569															
BP083	600							300							
BC609	200														
BP074	900	800	200					900	1000	800	600	200			
BC572			200								200			300	
BP131	900							300	400						
BC612	200	400													
BP159										300					
BC561															

BP157																
BC552	900								200							
BP177																
BC575	200									300						
BP091	300								300	2000						
BC648	600										300					
BP145									400							
BC574	300								200	200						
BP073	3000	400							2000	1000	500					300
BC642																
BP114																
BC599																
BP166																
BC565	200															
BP116																
BC559				10000	5000	1000	5000						9000			
BP107				500	600		200				600		1000			
BC646																
BP140	5000															
BC617																
BP108																
BC548																
BP078																
BC554	1000															
BP120																
BC624									200		700					
BP127																
BC578																
BP085	2000	2000		1000	1000	1000	1000		900	2000	1000	1000	2000	500		600
BC573																

BP075	1000								900	2000	300					
BC586																
BP165			200						1000	1000	700					
BC555												300		800		
BP128																
BC566																
BP070						800	200					400	300			
BC645															200	
BP134																
BC643			400													
BP170										200						

Table 2. ELISA endpoint titers of children sera tested on different DBL domains.

Children sera were tested on 15 different DBL domains. Left column: serum sample names of matched samples: BCXXX (baseline sample), BPXXX (6 months sample). Top row: isolate names IAM (Ifakara Asymptomatic Malaria) ISM (Ifakara Severe Malaria). Numbers in the table indicate the reciprocal endpoint titers. No number indicates that the serum is negative for the DBL domain tested. Colors: white, negative serum; yellow, endpoint titer 200-600; orange 700-2000; red over 2000.

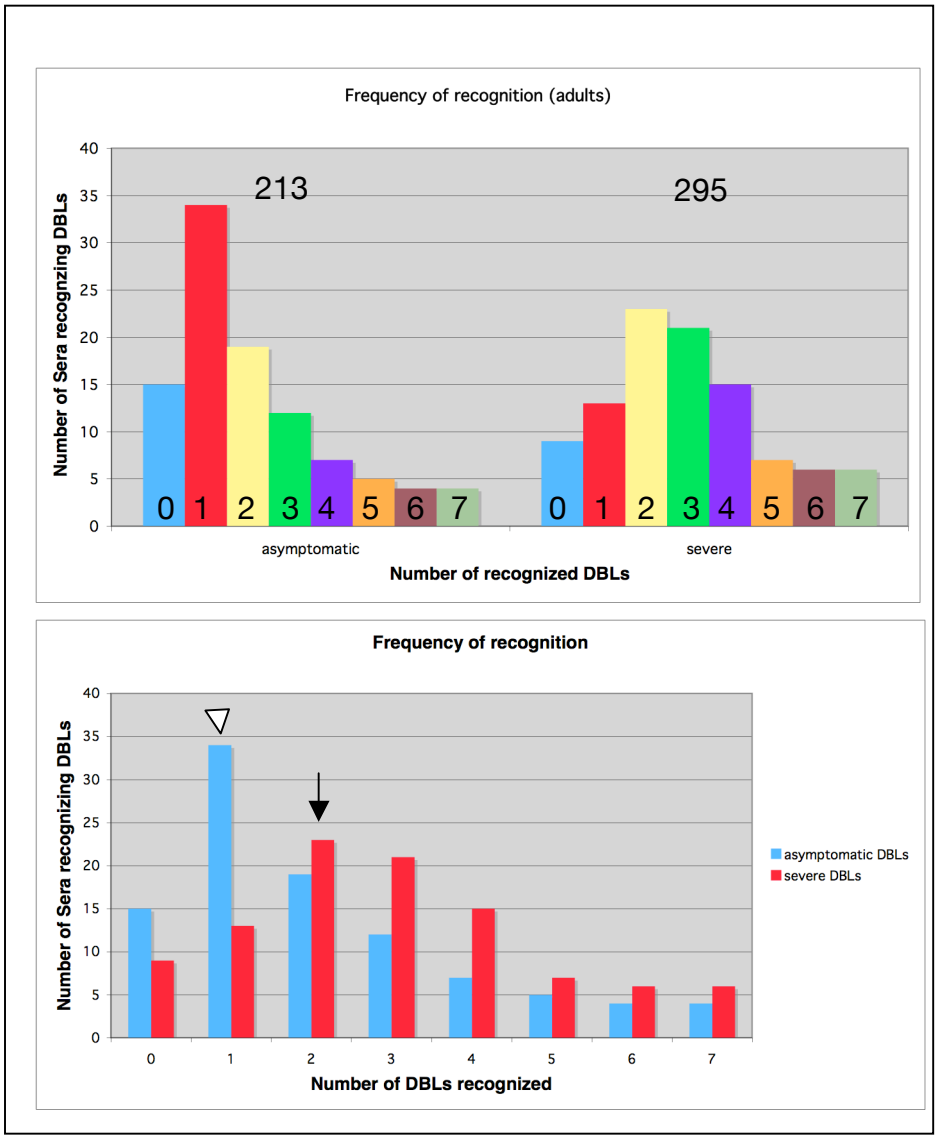


Figure 3. Graphic representation of differential recognition of severe and asymptomatic DBL domains.

TOP. Left: recognition frequencies for asymptomatic DBL domains. Right: recognition frequencies for severe DBL domains. **Bottom**. Frequency of recognition depending on their origin. The number of sera recognizing asymptomatic DBL domains is highest for "1 DBL recognized group" (arrowhead). For the case of severe DBLS the recognition frequency peak is shifted and peaks at 2 DBL domains recognized (arrow).

The difference in recognition frequencies between DBL domains from severe cases and asymptomatic cases is highly significant (*Score Test for Trend*s equivalent to chi-squared test for trend, p=0.002). This test only calculates with total numbers. Using the Wilcoxon signed-rank test, which takes pairing into account (ie each

individual has a value for severe and a value for asymptomatic) leads to the same conclusion (Prob > |z| = 0.0002). (Figure 3).

Recognition of severe DBLS does not correlate with age

To investigate whether frequency of recognition was correlated with age, we tested this correlation using the Spearman's Rank test. The age of the enrolled adults from which the serum samples were collected varied from 11 to 64 and the average was 32,9 years. As shown in Figure 4, there is no evidence of correlation between total number of recognitions and age (p-value 0.8825) using Spearman's Rank correlation coefficient.

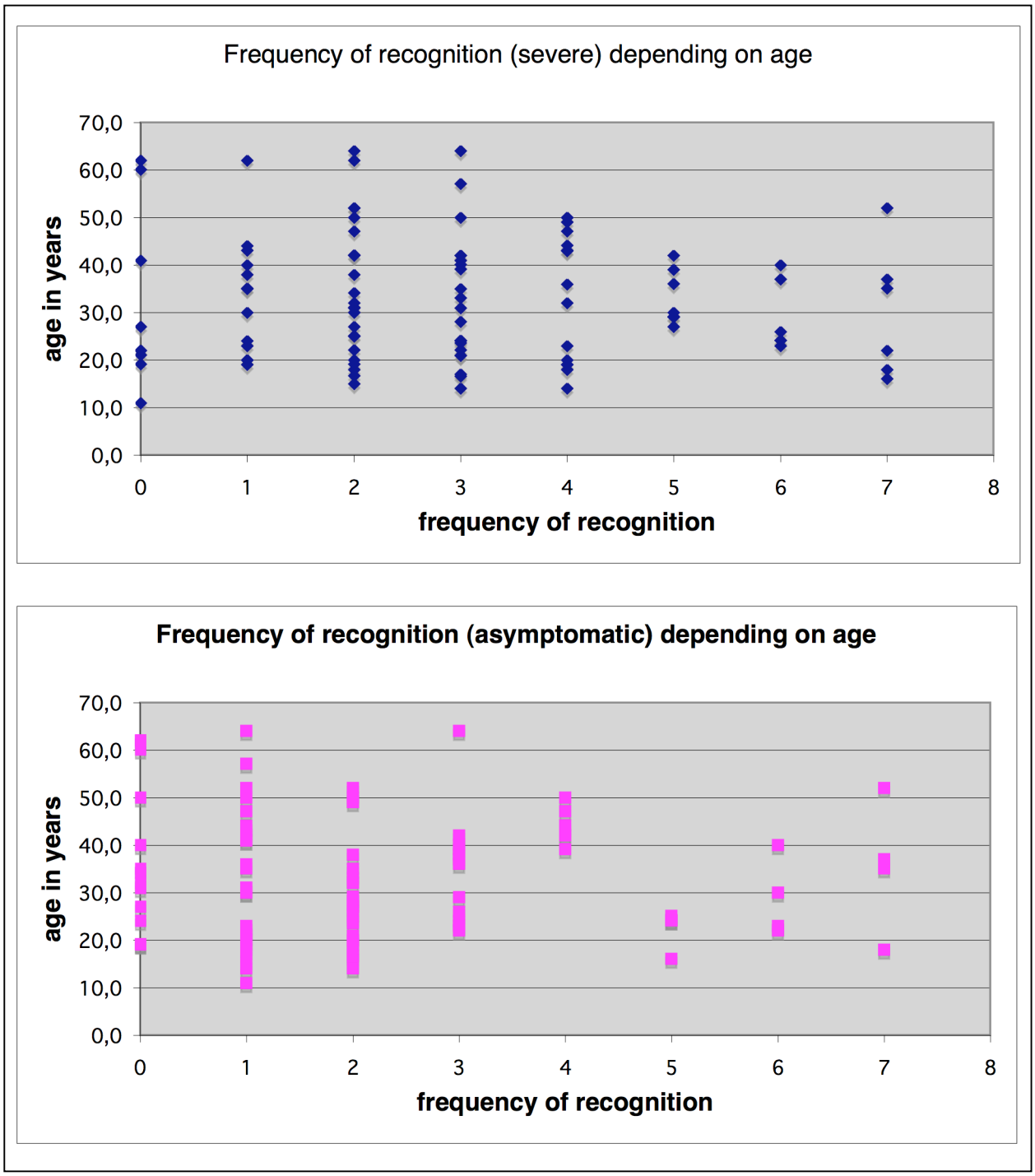


Figure 4. Age dependence of frequency of recognition.

In **A** the results for asymptomatic DBL domains are shown and in **B** the frequency of recognition for severe DBL domains are shown. There was no correlation of age and frequency of recognition.

Borderline evidence for different recognition between the sexes

From the 100 sera samples from semi immune adults, 48 sera were from female and 52 sera from male adults. To test if recognition frequency of DBLs was sex dependant, data were further analysed to investigate if there is a correlation between the sex of the semi immune adult and the recognition frequency. Using the score test for trends, we calculated that there is some borderline evidence that the overall frequency of recognition was different between the sexes (p-value 0.0669). This indicates an inhomogenous distribution of recognition frequencies depending on sex. However an obvious trend is not visible (Figure 5).

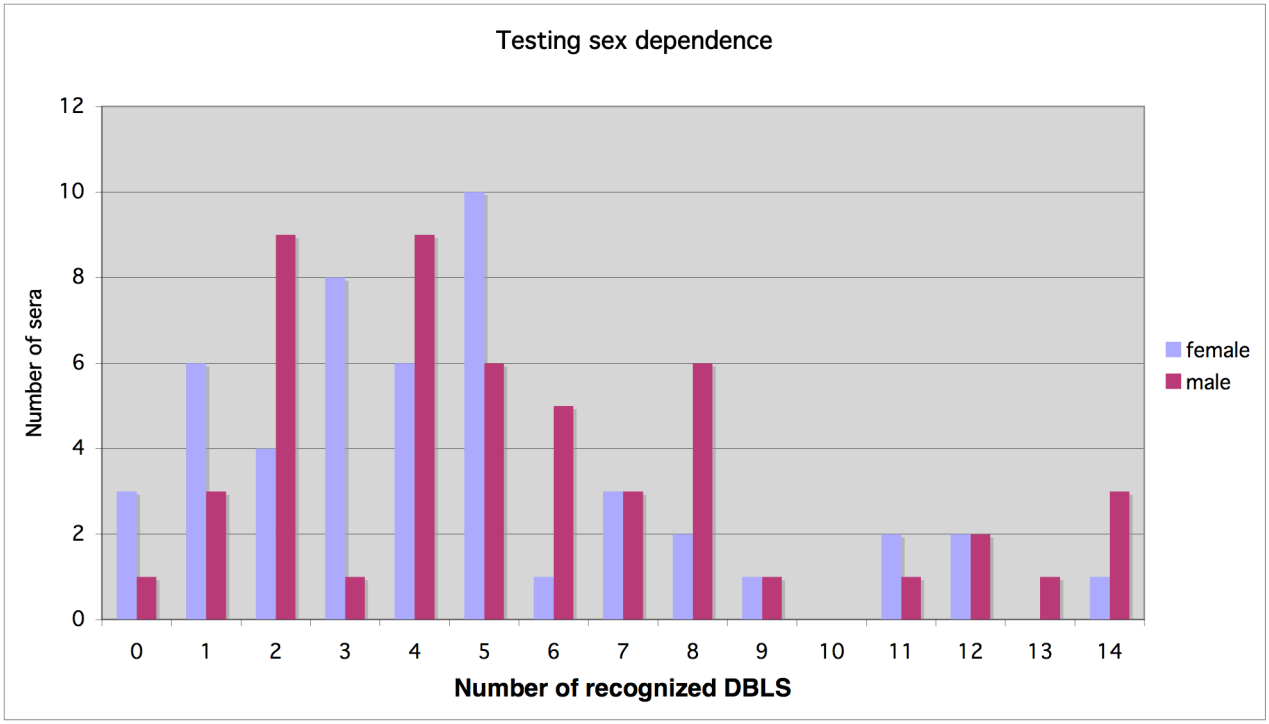


Figure 5. Testing sex dependence of recognition frequencies.

The X-axis represents the number of times a sera recognized DBL domains (sum of severe and symptomatic). The non-homogenous distribution in some recognition groups is obvious. I.e. group 2 and 8 are represented by more males than females and in groups 1 and 5 it is vice versa.

ELISA results from children sera

Sera samples from children from Africa (Tanzania, Ifakara) were collected in a logitudinal study in 1996. 34 sera samples for two time points (baseline and 6 month) were tested. No children in this study had a malaria episode in between the two time points. The dynamics of antibody titers against different DBL domains was measured in ELISA tests (see Table 2). In general, the frequency of positive sera and the endpoint titers of children sera were lower compared to those of adult sera. At the baseline, 25 sera did not recognize a single DBL domain, six month later only 18 samples were completely negative. Only one serum (BP123) recognized all DBLs tested. The most often recognized DBL domain was IAM11 with 25%. The three most rarely recognized antigens were ISM48, ISM11, ISM49 (4.5% each). It was calculated that for the baseline samples 34 recognitions occurred out of 510 tests (34 sera x 15 antigens= 510), which is 6.6%. Among those positives were 15 positive for severe DBL domains and 19 for asymptomatic DBLs.

Six months later 78 positive tests were counted, which is 15.3%. Those 78 positive tests split into 45 positive for severe DBL domains and 33 for asymptomatic DBL domains. Not only the number of positive sera increased overall. It is noteworthy that the increase of recognized “asymptomatic DBL” domains is only 14 (+73.7%), whereas for the “severe DBL” domains it is 30 (+200%).

Recognition frequencies were calculated similar to adult sera and represented in Figure 6. The 34 paired sera from baseline and 6 month later were tested on 7 asymptomatic and 7 severe DBLs. The recognition frequencies for asymptomatic DBL domains did not change in this 6 months period as shown by the blue bars in Figure 6. In contrast, the frequency of severe DBL recognition increased significantly ($p<0.0001$, Fisher's Exact test). This is shown by the red bars in Figure 6.

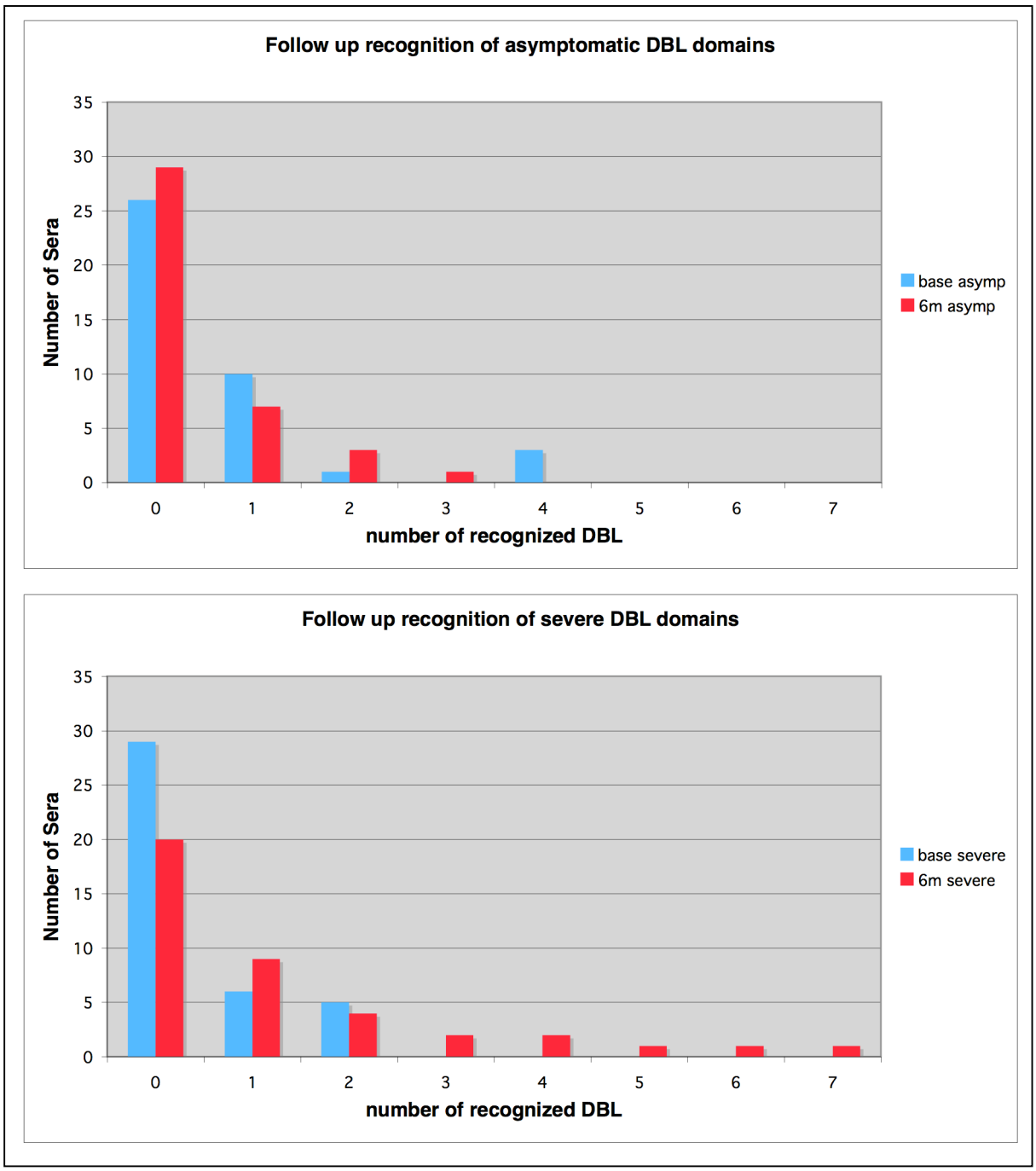


Figure 6. Follow up sera from children tested on severe and asymptomatic DBL domains.

Frequencies of recognition of different DBL domains by children are shown as bar graphs. **4A:** ELISAs on asymptomatic DBL domains tested with sera at baseline and 6 months later. **4B**: Severe DBL recognition frequencies are shown at the baseline and after 6 month. The children sera recognized more severe DBLs after 6 months than on baseline, as can be seen by the increase of the high frequency recognition groups (3-7), which come up only for severe DBLs after 6 month. (40 Sera are shown here, the 6 additional are unpaired sera)

(In blue sera from baseline, in red sera from 6 month later.)

Chapter 4

Discussion

Previously we have isolated full length *var* mRNA from blood samples from patients with severe malaria episodes and from age matched asymptomatic children. The DBL 1α domain of *Pf*EMP1 was amplified from cDNA, cloned and sequenced.

Here we have recombinantly expressed 14 dominant and 1 minor DBL domain from 14 patients in E.*coli*. Seven dominant DBL domains for each group (severe/asymptomatic) and one minor from the severe group. We have measured antibody titers of 100 semi-immune adult sera from PNG against these 15 recombinant DBL domains by ELISA. With this method we calculated recognition frequencies of the sera against the severe versus the asymptomatic DBL domains.

Briefly, we found that DBL domains derived from severe isolates were more frequently recognized than DBLs from asymptomatic infections (see graph 1).

Furthermore, we tested children sera from a longitudinal study at baseline and 6 months later. We found that the general recognition frequency was dramatically lower compared with the adults (graph 4). Additionally we found that the antibodies aquired by the children during the 6 months period were stronger directed against the severe DBL domains than against the asymptomatic DBL domains.

The choice of DBL domains

From a previous study we had a set of cloned DBL domains isolated from severe and asymptomatic children from Tanzania (unpublished, Paschal Mugasa). These sequences were cloned from full length *var* mRNA as described in [55]. We have sequenced 50 clones per isolate. We chose the most frequently cloned DBL domain for expression. Difficulties occurred for very complex samples where a prominent clone was not obvious. This was the case for ISM49, ISM16, IAM10 and IAM18. In these isolates about 20 different sequence groups (98% homology) were found per isolate. For example the most prevalent sequence in ISM49 was found 9 times and the next frequent was found 8 times. Even though the prominent clone in these samples was not significantly more numerous than others, we always chose the most frequent. In other less complex samples like IAM12 the difference was more obvious: 19 occurrences of the most prominent sequence and 4 of the second most

frequent. We also included a minor clone from ISM48 with only 3 findings (compared to 13 for the prominent). No obvious difference in titers was found for the minor clone compared with the major clones (see table 3). The minor clone was excluded for further statistical analyses because the role of the minor clones in an infection is not clear. However, we isolated parasites from peripheral blood, which *per se* do not necessarily seem to reflect the whole genotypical parasite load in an infected individual but certainly does not reflect the phenotypical parasite load [109, 110].

Difference in proteins and difference in recognition

All DBL domains in this experiment contain conserved sequences at the N and C terminal end (N terminal: ARSFADIGDII and C terminal: WFEEWAEDL). These short conserved stretches do not seem to be good epitopes, as titers among DBLs are dramatically different. When comparing relationships in sequence alignments with recognition frequency in ELISA we found differences. For DBL ISM3 and ISM51, which were the most frequently recognized antigens, alignments revealed that these two isolate were closely related. This was reflected in the ELISA results. However, in the case of ISM48 (recognized 39 times) and IAM5 (recognized 19 times), the alignment revealed a close relationship (see alignment 1, results, chapter4), however the ELISA showed differential recognition. It seems that even small differences in amino acid composition can be enough to change the recognition pattern.

Cloning and protein expression in E.*coli*

We cloned the DBL domains in a pQE16 plasmid. This plasmid expresses the cloned gene as a 5` fusion to a mouse DHFR (dihydrofolate reductase) gene. The recombinant protein has the domain structure NH_2-DHFR-DBL-HIS-COOH and had a calculated mass of 36kDa. It was necessary to express the DBL domain as DHFR fusion protein because expression with a single HIS-tag resulted in very low expression levels. The DHFR fusion was originally designed for expression of small peptides or toxic proteins, however in our case it enhanced the expression dramatically. This might be due to the greater stability of the mRNA or because the DHFR masks toxic domains or structures of the recombinant DBL domain. Neither the rare *E.coli* tRNA codons used by *Plasmodium* nor the high AT content seemed

to be important for high expression levels, as this was not altered with the DHFR fusion. We reached expression levels ranging from 0.5-4mg of recombinant protein from a 50ml culture. Beside the expression vector we found that rich medium (i. e. Terrific broth) is favored compared to normal LB, as the bacteria can grow to higher densities.

The recombinant protein was totally insoluble. That made it necessary to apply two purification steps. First, all soluble and Triton-X-100 soluble proteins were eliminated. This resulted in a very pure fraction of inclusion bodies, which was then purified further using a Nickel column. These modifications resulted in very pure highly concentrated (up to 2mg/ml) recombinant protein. It was stated earlier that successful expression of *plasmodial* proteins in *E.coli* was dependent on the isoelectric point (pI) and size of the protein [83]. In our case neither of those had an influence on protein solubility, pre-termination nor expression levels. Also late induction time in the early stationary phase, which favors solubility [79] had no effect.

The DHFR fusion protein was also expressed without DBL domain as a negative control for downstream experiments. Recombinant DHFR is mostly insoluble. About 90% of the expressed protein is in inclusion bodies, the rest can be purified under native conditions. Here we purified the insoluble fraction to follow the same conditions as for the DBL domains. The ODs obtained in ELISA for DHFR were generally around 0.2 (the positive control was 2 -3). The mouse DHFR used in this experiment is closely related to human DHFR with only little point mutations, thus it was expected that the titers against DFHR are very low (see Figure 2, lane D).

ELISA on recombinant proteins

Using recombinantly expressed proteins in ELISA experiments harbors intrinsic questions. The use of the same protein modifications system of the heterologue expression environment is more than doubt full. A lot of cell surface proteins are glycosylated, and this is also true for *Plasmodium* (as reviewed in [113] and commented in [114]). In *Plasmodium* proteins can be N- or O- linked glycosylated or GPI (glycosylphophatidylinositol) anchored. If the native DBL domain is glycosylated is unknown. It is also unknown to what extend the recombinant DBL domain is modified. However, glycosylation of proteins accumulating in inclusion bodies is

very unlikely as glycosylation occurs in the endoplasmatic reticulum (ER) lumen and insoluble protein aggregates have never entered the ER. It is further known that disulfide bonds stabilize the structure of the DBL domain. In the recombinant protein there is no formation of disulfide bonds as this is impossible in the reducing environment of the *E.coli* cytoplasm (reviewed in [115]), however disulfide bridges can be introduced by dialyzing in buffers containing red/ox components. We were aware of the improper folding of our recombinant protein and thus we do not claim that the antibodies binding our antigens have necessarily a role *in vivo*, as the antibodies detect most probably only linear epitopes. However, if recognition of the rDBL domians would be random due to their linear structure, we would not have found significantly different recognition patterns between severe and asymptomatic DBL domains. It is possible that important domains within the DBL are unstructured in the native protein as predicted for many *plasmodial* proteins [116].

Calculation of titers

Equal coating of different proteins was measured with a commercial monoclonal mouse anti-6xHIS tag IgG. Maximal titers of the positive controls were very high ranging from OD 2-3.5. Calculated half maximal titers of the mAB were around 1:10`000. Coating efficiencies varied merely between experiments. As each sera was tested on all DBLs in the same experiment the variation between experiments is negligible for the comparison of titers between antigens.

The negative control, a pool from 5 European blood donors, was approximately OD 0.2 for the lowest dilution. The threshold was calculated from the mean of the negative titers plus 3 standard deviations. Titers measured for the recognition of rDHFR were subtracted from the titers obtained for the rDBL domains.

Antibodies against a subset of *Pf*EMP1 variants prevent disease

In graph 1 we showed that sera from semi immune adults recognize severe DBL domains more frequently than asymptomatic DBLs. As it is known that these semi-immune adults are hardly suffering from severe malaria, we can conclude that their repertoire against severe DBL domains seems to contribute to protection against severe malaria.

Our findings with adult sera support the general hypothesis that different *Pf*EMP1s with different functions exist and thus antibodies against different *Pf*EMP1s can have more or less protective effects against severe malaria. If a human has an antibody repertoire against a subset of *var* genes coding for *Pf*EMP1 variants that mediate cytoadherence in deep tissue e.g. brain, heart and lung, this human might be protected from severe malaria. Consequently, the library of antibodies against *Pf*EMP1s that do not or to a lower extend (or in not as sensible tissues as the brain) mediate cytoadherence can be deficient without resulting in disease. Organs from fatal severe malaria patients are heavily infected with late stage parasites whereas organs from asymptomatic subjects contain almost no late stage parasites even though their parasitaemia were very high in peripheral blood samples [110].

Recognition of severe DBL domains does not correlate with age

To investigate whether frequency of recognition was correlated with age, we tested this correlation as shown in Graph 2. There is no evidence of correlation between total number of recognitions and age (p-value 0.8825) using Spearman's Rank correlation coefficient. We included age in our analysis because it can have a confounding effect in statistical analysis and because the antibody repertoire increases with age. This is especially pronounced in children when they develop their immunological protection. The correlation between age and protection from malaria has been already described by Ross and Koch over 100 years ago [117]. The age correlation is extensively reviewed in [118]. Recent chemoprophylaxis trials found that young children (2-12 month) treated prophylactically with anti malarials for a year and followed up for four years were at higher risk of a severe episode than the placebo group [119]. The reason might be the missing contact with the *Plasmodial* antigens during the time of treatment. At the time when chemoprophylaxis was stopped, the placebo group had already established a considerable protection against severe disease. However, in our adult group this phenomenon was not expected to contribute to the differential recognition of antigens as the median age is 32 and the youngest individual was 11 years old. All these individual are considered to be clinically immune.

Borderline evidence for different recognition between the sexes

To test if recognition frequency of DBLs was sex dependant, data were further analysed to investigate if there is a correlation between the sex of the semi immune adult and the recognition frequency. We found that there is some borderline evidence that the overall frequency of recognition was different between the sexes (p-value 0.0669). Among our 100 sera tested 52 were from men and 48 from women. There are reports that men are generally more susceptible to parasitic infections than females [120]. Although the incidence of infection is similar the intensity and the parasitaemias are higher in men [121]. The only confirmed major sex difference in malaria is PAM in which parasites show a differential recognition pattern to placental CSA. Sera from multigravid women bind to parasites isolated from placentas whereas no male sera recognize these parasites [24]. In our experiments we did not expect gender-dependant difference in. The DBL domains tested here are isolated from children and we do not have a PAM associated *Pf*EMP1-variant (which would be DBLγ). The inhomogeneous distribution of recognition frequency maybe comes from the sample number not being high enough. A trend for a differential recognition among males and females is not obvious (see graph 3).

ELISA results from children sera

Sera samples from 34 children from Africa (Tanzania, Ifakara) were collected in a follow up study in 1996. 34 sera samples for two time points (baseline and 6 month) were tested. In general, the frequency of positive sera and the endpoint titers of children sera were lower compared to those of adult sera. This what we have expected as children have lower IgG levels. The comparison of the two time points showed an increase of recognition with time. The increased recognition is not distributed equally between asymptomatic and severe antigens. The DBL domains derived from severe samples show a higher increase of recognition than the asymptomatic. This goes together with the results from the adult sera where the recognition of severe antigens is also higher. It is also postulated that parasites responsible for severe disease are the “common” parasites. Bull *et al.* showed by serum agglutination that parasites isolated from severe cases are readily agglutinated by heterologous sera [89]. Parasites from asymptomatic infections are

less frequently recognized. Thus it is conceivable that the children acquired some infections (from “common” parasites) during the six months period against which they developed antibodies. This theory is highly contrary to the assumption that the “common” parasites induce severe malaria in an unprotected individual, however non of the kids had a episode within the study. But reality might be more complicated and complex because not every child falls severely sick with an infection of a common variant. Otherwise there would be many more severe cases. The results obtained from the children follow up sera fit into the picture of the acquisition of clinical immunity to severe malaria, but the development of the antibody response cannot be explained satisfactorily and remains to be elucidated.

Outlook

It is still not obvious which modifications in the DBL domain are necessary to enable the parasite to prevent eradication by the immune system but remain cytoadherent. At the same time it is not clear if antibody levels against special DBL domains are enough to acquire semi-immunity. The DBL domain is often used to monitor expression variants of *Pf*EMP1 [102, 109] but it is only a small part (130aa) of the large *Pf*EMP1 (3000 aa) protein. The critical binding domain can easily be somewhere else in this or even another protein. It clearly needs further work, maybe including the whole protein in the analysis. As sequence analysis of the linear amino acids did not reveal conserved domains, it must be expanded into structural analysis.

Chapter 5:

General discussion and conclusions of this thesis

General discussion and conclusions of this thesis

The core objective of the present thesis was the investigation of conserved domains in *Pf*EMP1 responsible for the pathology of severe malaria. Despite the growing body of knowledge the decisive point what makes some variants of *Pf*EMP1 more virulent than other remains unknown. We expanded our experiments from long recombinant proteins over a very complex random *Pf*EMP1 cDNA library to a more goal-orientated DBL-serology approach. The difficulties in recombinant expression of plasmodial proteins and the minute amounts of material obtained from field samples forced us to reconsider our approaches over and over. The final assessment of titers against severe and asymptomatic DBL domains however is a new way of investigating host parasite interaction. In previous experiments the influences of sera were tested on whole iRBC thus never revealed the sole role of *Pf*EMP1. In our approach we used a new way to test antigens derived from different clinical cases on the same sera rather then different sera on the same antigen. To our knowledge this is the first time that the antigen was the basis of differential recognition. We could show that adults recognize more frequently DBL domains coming from severely sick patients compared to antigens coming from asymptomatic infections. This may indicate that even the DBL1α domain is a crucial epitope for the parasite to manage cytoadherence and to be the target of antibodies protecting from disease. Of course, questions concerning the choice of the right DBL domain extracted from very complex infections can be asked. Recombinant protein expression in heterologous systems like *E.coli* implicates doubts in conformation and modification of the peptide. But these complicacies account for both the severe and the asymptomatic DBL domains. With our experiments we show that recombinant protein expression can be useful in the assessment of titers against various antigens. These and other findings (like var2CSA in PAM) concerning protection from severe malaria by the recognition of specific domains from a variable antigen give hope to the possibility of the development of a disease preventing vaccine. Considering the pathological significance of *Pf*EMP1, intervention in sequestration, cytoadherence and rosetting represent ideal targets for a morbidity reducing vaccine.

The way to such a vaccine however is still long and rough. Recombination of the variant antigens must be considered. We currently believe that the repertoire of *var* genes is endless and is not static. The blocking of certain domains by vaccine induced antibodies can easily induce the rise of new variants. More knowledge is needed on sequence information of the whole protein and on the mechanisms of antigenic variation. And *Pf*EMP1 is not the only protein on the surface of an iRBC. Other proteins like the riffins or stevors may also account for cytoadherence and must be included in future analyses.

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Appendix 1

Sequences of recombinant DBL domains

DBL domains were expressed as DHFR-DBL-6xHIS fusion proteins. The recombinant DHFR protein contains also the 6xHIS tag. Numbers in brackets give the calculated mass and the isoelectric point of the fusion protein (kDa;pI)

>DHFR-6xHIS
MRGSGIMVRPLNSIVAVSQNMGIGKNGDLPWPPLRNEFKYFQRMTTTSSVEGKQNLV
IMGRKTWFSIPEKNRPLKDRINIVLSRELKEPPRGAHFLAKSLDDALRLIEQPELASKVD
MVWIVGGSSVYQEAMNQPGHLRLFVTRIMQEFESDTFFPEIDLGKYKLLPEYPGVLSE
VQEEKGIKYKFEVYEKKGSRSHHHHHH (23;9.34)

The following dbl domains replace the red sequence:

>IAM5
ARSFADIGDIVRGTDMFLGSNKEKEKIENSLQNIFKNIKKNNKKLKDLTDKQIREYWWA
LNRKEVWKALTCSVPYEAYYFTYKSDNFRTFSGYWCGHYEGAPPTNLDYVPQFLRWF
EEWAEDL (35;8.97)

>IAM7
ARSFADIGDIVRGKDLFLGHKQGKQKLEASLKTMFQNIQSTIDQLKRLSIDAVREYWWE
INRQEVWKAITCSAGEDDTYSKYLGDRTTGVSHGQCGHMDENVPTYFDYVPQFLRWF
EEWAEDL (35;6.84)

>IAM10
ARSFADIGDIIRGKDLFIGYDEKDRKEKKQLQQNLKNIFGKIHSEVTNGSNAEAAKARYK
DTTDFYQLREDWWDANRETVWEAITCGAAGGTYFRATCSDEENKSTLASNKCRCAG
KNADQVPTNFDYVPQYLRWFEEWAEDL
(37;7.13)

>IAM11

ARSFADIGDIIRGKDLYRGNSKEKDNLEKKLIEYFQKIHGGLTGDAQTHYNDKSGNFFK
LREDWWDANRQEIRNAIICDVPEDAKYLEQSDGSQSGSHQTKCRCHSGSVLTNFDYV
PQYLRWFEEWAEDL

(36;6.87)

>IAM17

ARSFADIGDIVRGKDLFYGNPQEKKQRKELDKKLKEVFGKIHEGLKNGKAKERYKDTT
NYYQLREDWWTANRETVWKAITCAAKVGDTYFMESRTNSYKFSGDKCGHNDDNVPT
NLDYVPQYLRWFEEWAEDL

(36;8.91)

>IAM12

ARSFADIGDIIRGKDLYLGDNRKDREQKVKLENKLKEIFAKIHENLGTQDAIGHYEDAKK
NYYKLREDWWTANRGTVWKAITCGAGKHDKYFRKTCNGGSPTKGYCRCNGDQPND
HKANIDPPTHFDYVPQFLRWFEEWAEDL

(37;8.95)

>IAM18

ARSFADIGDIVRGKDLYIGNRKEKEKEELQKNLKSIFKKIYGELKNGKTNGEAAKVHYQE
DGQNYYKLREDWWTANRETVWEAITCNAGGGTYFRGTCGKNDTWTREDCRCDGSN
WPTYFDYVPQYLRWFEEWAEDL

(37;8.20)

>ISM49

ARS FAD I GD I VRGRDLFLGNT YE S AQRDQLDKKLKE I F TQ I YND VTTNGKKP
ALQKRYKKDGKDPDFFKLREDWWYANRQEIWKAITCKVENAQYFKDTCSTGGHYEK
CRCNGDQPKSGKGGDVNIVPTYFDYVPQYLRWFEEWAEDL

(38;8.81)

>ISM51

ARSFADIGDIIRGKDLYLDHEPGKQHLEERLERIFANIQKENGDINTLKPEEVREYWWAL
NRVQVWKAITCRAEEKDIYSRIAGDTTIWNDNCGHHVNQDVPTNLDYVPQYLRWFEE
WAEDL

(35;6.19)

>ISM16

ARSFADIGDIIRGRDLF
YGNTQEKTKRKQLDKKLKDIFGDIYKELRKNGKKGELQKRYQKDGDKDFFQLREDW
WEENRETVWKAITCDAPPDAQYFRGTCGDNEKTATQTPSQCRCNDDQVPTYFDYVP
QYLRWFEEWAEDL

(38;8.20)

>ISM48_1

ARSFADIGDIVRGTDMFLGSNKEKEKIENSLQNIFKNIKKNNKKLKDLTDKQIREYWWA
LNRKEVWKALTCSVPYEAYYFTYKSDNFRTFSGYWCGHYEGAPPTNLDYVPQFLRWF
EEWAEDL

(35;8.97)

>ISM48_2 DEL

ARSFADIGDIIRGKDLYLDHEPGKQHLEERLETMFQNIQYNNTELKNIPLPKVREYWWA
LNRGQVWKAITCHAGKDDAYFRNSSGGEYKFTSGYCGRNEGKVPTNLDYVPQHLRW
FEEWAEDL

(35;7.82)

ISM11

ARSFADIGDIIRGKDLFIGNNKRDKLEKQLKEYFKNIYDNLNGAQKHYSDDDKGTKN Y
YQLREDWWALNRQEIWKALTCESGGGRYFRETCAGGTSRTQDDCRCRTNDVPTYFD
YVPQYLRWFEEWAEDL

(36;8.21)

>ISM3

ARSFADIGDIIRGKDLYLDHEPGKQHLEERLERIFENIKKKNNNNELNNLSLDKFREYW
WALNRVQVWKAITCRAEEKDIYSKTTDNGKLLLWNYNCGHHVNKDVPTNLDYVPQFL
RWFEEWAEDL

(36;7.81)

>ISM33

ARSFADIGDIIRGKDLFLGHEQRKKYLEARLEAMFDNIKKNNKKQLGELSTAQVREYW
WALNRGQVWKAITCGATMNDISFKNIGNGKLLLWNEKCGRGDYNLLTNLDYVPQFLR
WFEEWAEDL

(35;9.16)

Appendix 2

Peptide sequences:

ATS1: SDITSSESEYEELDINDIYVP

ATS2: PKYKTLIEVVLEPS

ATS3: GIDLINDTLSSGNHIDIYDEVLKRKENELFG

ATS4: LDRHRDMCEKWKNKEDILNKLKEEWNKENINN

Primer sequences 5 --> 3

pGEX_fwd: gggctggcaagccacgtttggtggt

pGEX-reverse_MCS: atgcggccgctcgagtcg

pQE_fwd: cccgaaaagtgccacctg

pQE_reverse: gttctgaggtattactgg

pTrcHis2_reverse: gatttaatctgtatcagg

pTrcHis2_fwd: gaggtatatattaatgtatcg

dbl1α_fwd: GCGTCCTCTCTGAGGCACGAAGTTTTGCAGATATAGG
dbl1α_reverse: catGGATCCaAAGTCTTCGGCCCATTCCTCGAACCA

ats beads:

TCHTCMGAAAGTGARTATGAAGAATTGGATATTAATGATATATATGYACCAGGRAT

WGAAGTRGTAYTWGAACC-Biotin

NTS_FCR_fwd: gaattcgcgacttcaggaggtagtgg

NTS_FCR_reverse: gcgaagcttgccacggattccttttgctg

ATS_FCR3_fwd: gaattcaagaaaaaaccawakcatctgttg

ATS_FCR3_reverse: nnnnnngcggccgcnngatattccatatatctgatatagg

SMART_PCR_primer: aagcagtggtatcaacgcagagt

SMARToligo: ggtatcaacgcagagtacgcggg

SMART_random: ggtatcaacgcagagtacnnnnnnnnn

SMART_polyT: ggtatcaacgcagagtacttttttttttt

F1: ggtgtgtgtatgcctccaagaag

F2: acgagctcaaatggtgaggacaggaggtagcgg

F3: acgagctcaaatgacatcatgtagtccggag

F4: acgagctcatggcggctgcaggaagtggagg

F5: acgagctcaaatggtacggtcgtcacgcg

F6: acgagctcaaatggcgaggccaggtagcgg

F7: acgagctcaaatggcgactggtagtgggggcg

R1
ttttccttttgcggccgcttagttagttacttctaggwggcatacatgctcc

R2
ttttccttttgcggccgcttagttagttactt ctg ggt ggc aca cac gca cc

R3
ttttccttttgcggccgcttagttagttactt ctt gga ggc ata cam rma cc

R4
ttttccttttgcggccgcttagttagttactt ctt gga ggc ata yak kca

Abbreviations

ATS	Acidic terminal sequece
BSA	Bovine Serum Albumin
CIDR	Cysteine rich inter domains region
CPE	chromosom central var promotor element
CR1	Complement receptor 1
CSA	Chondroitinsulfate A
DBL	Duffy binding like
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DTT	Diithiotreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked immuno fluorescence assay
ER	Endoplasmatic reticulum
FoR	Frequency of recognition
GPI	Glycophosphatidylinositol
GST	Glutathione S-transferase
IAM	Ifakara asymptomatic Malaria
IFA	Immuno flouresence assay
IL	inter leukine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
iRBC	Infected red blood cell
ISM	Ifakara severe Malaria
KAHRP	Knob associsated histidine-rich protein
kDa	kilo Dalton
KLH	Keyhole Limpet Haemocyanin
LB	Luria Bertani (lysogeny broth)
MACS	Magnetic cell sorter
MAHRP	Membrane associated histidine-rich protein
NTS	N-terminal segment
PAGE	Polyacrylamide Gel electrophoresis
PAM	Pregnancy associated malaria

PBS	Phosphate buffered saline
PCR	Polymeras chain reaction
PEXEL	Plasmodium export element
*Pf*EMP1	Plasmodium falciparum Erythrocyte Protein 1
PMSF	phenylmethanesulphonylfluoride
PNG	Papua New Guinea
RBC	Red blood cell
RNA	ribonucleic acid
SDS	Sodiumdodecylsulfate
SPE1	Subtelomeric var promotor element
STI	Swiss Tropical Institute
TBE	Tris Borate EDTA
TE	Tris EDTA
TNF	Tumor necroses factor
UV	Ultra Violet
vs	versus

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2007	Joint Meeting of the French Society of Parasitology, the German Society of Parasitology and the Swiss Society of Tropical Medicine and Parasitology in Strasbourg, France. Poster: Generation of mouse polyclonal antibodies targeting the ATS domain of *Pf*EMP1.
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Recombinant protein expression and purification
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Cultivation of *Plasmodium falciparum* blood stage forms
PCR set up and long range PCR

Computer skills

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