Immunological characteristics of a C-terminal fragment of the *Plasmodium falciparum* blood-stage antigen Pf332

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To my precious Wura and a rare gem

........... L.A.B
Summary

Till date, there are no effective control strategies against the deadly disease of malaria, and millions of children across Africa, Oceania, Asia, and Latin America are at the mercy of this long term enemy of man every second that passes by. Other control measures combined with vaccination might help improve control strategy against malaria, but the development of vaccines face various challenges as well, due to the complexity of the parasites’ life cycle and other host factors. The asexual blood stage antigen Pf332 of *Plasmodium falciparum*, is expressed during the trophozoite stage, and transported from the parasitophorous membrane to the outer erythrocyte membrane during schizogony. Previous studies have suggested this antigen as a potential vaccine candidate, because Pf332-reactive human monoclonal antibody (mAb 33G2) inhibits parasite growth and cytoadherence *in vitro*. Elucidating and understanding the immunological capabilities of antigen Pf332, as a vaccine candidate was the aim of the studies presented in this thesis.

In our first study we identified and characterized the immunogenicity of a non-repeat fragment of antigen Pf332, termed Pf332-C231, a 231 amino acids long fragment corresponding to 13 percent of the total protein. Various analyses carried out with this fragment reveal that recombinant C231 was immunogenic in rabbits. In addition, anti-C231 antibodies have *in vitro* inhibitory capabilities. In immunofluorescence and immunoblot assays, rabbit anti-C231 antibodies were able to recognize the native protein. In the other study, we examined the distribution of antibodies regarding recombinant C231 and crude *P. falciparum* extract in a malaria endemic area of Senegal. IgG antibody reactivity with crude *P. falciparum* antigen was detected in the sera of all the donors while many of the children lacked or had low levels of such antibodies against C231. The distribution of the anti-C231 antibodies in the different IgG subclasses differed from that shown by crude *P. falciparum* antigen. The crude *P. falciparum* antigen gives a higher IgG3 response than IgG2 for all age-groups, while C231 gave similar levels of IgG2 and IgG3. Correlation studies showed that the levels of anti-C231 antibodies were associated with protection from clinical malaria, but this only reached significance with IgE. These findings further emphasize the inclusion of antigen Pf332 as a subunit vaccine candidate against *P. falciparum* malaria.
This Licenciate thesis is based on the following original papers, which are referred to in the text by their roman numerals:


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ABBREVIATIONS

ADCI  Antibody dependent cellular inhibition
AMA  Apical membrane antigen
APC  Antigen presenting cell
CD  Cluster of differenciation
GLURP  Glutamate rich protein
GPI  Glycosylphosphatidylinositol
His-tag  Histidine tag
HZ  Haemozoin
ICAM-1  Intercellular adhesion molecule-1
IFN  Interferon
Ig  Immunoglobulin
IL  Interleukin
MHC  Major histocompatibility complex
NK  Natural killer
MSP  Merozoite surface protein
PDC  Plasmacytoid dendritic cell
RESA  Ring-infected erythrocyte surface antigen
SERA  Serine repeat antigen
TCR  T cell receptor
Th  T helper
TLR  Toll like receptor
TNF  Tumor necrosis factor
TRAP  Thrombospondin related anonymous protein
INTRODUCTION

Infectious diseases, among which *Plasmodium falciparum* malaria is one, has been ravaging the lives of its victims across Africa, Oceania, Asia, and Latin America, among whom are mainly children under the age of 5 and pregnant women. An estimated 2.2 billion people worldwide are exposed to *P. falciparum* malaria, resulting in about 500 million episodes yearly and in reality, the actual figure could be greater than this. The malaria situation has aggravated since time immemorial, due to insecticide resistance of the vector, the anopheline mosquito, which transmits the parasite, and as well because of resistance of the parasites to antimalarial drugs. Thus, the focus of research on the development of malaria vaccines is highly important, as it is one of the potential control measures to curb this long term enemy of man.

The Parasite and life cycle

*P. falciparum*, the most severe and life threatening form of this mosquito borne hemoparasitic disease, belongs to the Kingdom *Protista*, phylum *Apicomplexa*, class *Sporozoa*, order *Eucoccidia* and genus *Plasmodium*. There are 172 species of *Plasmodium* that infect birds, reptiles, and mammals, but only four cause malaria in humans, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Infection by *P. falciparum* is complex as shown by the life cycle, including asexual reproduction in the human host, and sexual reproduction in the mosquito host. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host. Sporozoites infect hepatocytes and mature into schizonts, which rupture and release merozoites. Of note, in *P. vivax* and *P. ovale* a dormant stage hypnozoites can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later. After this initial replication in the liver (exo-erythrocytic schizogony), merozoites infect red blood cells and the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony) (Fig. 1). The ring stage trophozoites mature into schizonts, which rupture, releasing merozoites. The asexual blood stage parasites are responsible for the clinical
manifestations of the disease. Some parasites differentiate into sexual erythrocytic stages (gametocytes). The gametocytes, male (microgametocytes) and female (macrogametocytes) are ingested by an Anopheles mosquito during a blood meal. The parasites’ multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes), which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

Figure 1. Denotes the erythrocytic cycle of P. falciparum malaria and the location of antigens of interest involved in parasite neutralizing immune responses ².
The Disease and symptoms

The pathogenic process in malaria occurs only during the erythrocytic stage. Cyclical fever is the hallmark of malaria, and typically occurs shortly before or at the time of red blood cell lysis as schizonts rupture to release new infective merozoites. In humans, the manifestations of severe and complicated malaria vary geographically, but are associated mainly with *P. falciparum* infection. Intense fever is accompanied by nausea, headaches and muscular pain, metabolic acidosis, hypoglycemia, anemia. These malaria associated symptoms are believed to be caused by a parasite released toxin, glycosyl-phosphatidyl inositol (GPI)\(^3\), that induces macrophages to secrete cytokines such as TNF-\(\alpha\) and IL 1\(^4\).

Cerebral malaria, a syndrome of unarguable coma, is often associated with fits and other neurological abnormalities, such as seizures, increased intramuscular muscle tone, and elevated intracranial pressure\(^5\). It is commonly found in low endemicity areas, and has been attributed in part to the unique ability of the parasites to alter the surface of the infected red blood cells, so that they bind to endothelial surfaces, using various adhesion molecules in the host, such as: CD36, ICAM-1, thrombospondin and E-selectin\(^6\). Parasite induced TNF-\(\alpha\) may be involved in the up regulation of ICAM-1 in cerebral vessels, which also leads to increased cytoa dherence of infected erythrocytes\(^7\), hence, causing obstruction of the cerebral blood flow. Recent observations suggest that pro-inflammatory cytokines and nitric oxide, induced by parasite material, also contribute to the pathogenesis of cerebral malaria.

Control of Malaria

Strategies against malaria infection and disease require an integrated approach, including control of the mosquito vector, use of chemotherapeutic and chemoprophylaxis agents and prompt, appropriate case management\(^8\)-\(^10\). The use of chemicals has not been successful across Africa and Latin America\(^11\), due to the emergence of mosquito strains resistant to the commonly used insecticides. One of the greatest challenges facing the fight against malaria today is drug resistance. In most malaria endemic areas, the disease is treated using anti-malarial drugs as additional control measure, and the administration of these drugs has either a prophylactic or curative effect. Increasing resistance of *P.
*falciparum* malaria to antimalarial drugs poses a major threat to the global effort to Roll Back Malaria. Chloroquine and sulfadoxine-pyrimethamine (SP) are being rendered increasingly ineffective, resulting in increasing morbidity, mortality, economic and social costs. One strategy, advocated for delaying the development of resistance to the remaining armory of effective drugs, is the wide-scale deployment of artemisinin-based combination therapy in most malaria endemic countries. However, resistance to most of these drugs has been documented, also with the new artemisinin based drugs. In a recent study carried out in Tanzania, it was shown that in an area of high drug resistance, there was evidence that in using Arthemether Lumefantrin and Amodiaquine + Artesunate combination therapy rather than monotherapy, were the most cost-effective drugs. Despite being the most expensive, these combinations are significantly more effective than other options and therefore reduces the need for further treatment. Due to increased mosquito resistance to insecticides, the global malaria control strategy adopted by governments and W.H.O. emphasized the need for early diagnosis, appropriate treatment with anti-malarial drugs, and selected use of preventive measures, including mosquito control where it is effective can lead to sustainable impact. In respect to the existing hindrances in these control measures, combination with vaccination might help improve control strategies against malaria.
Malaria and the human immune system

Unlike many acute viral diseases, which produce long lasting immunity to re-infection, immunity to malaria is a function of age, transmission intensity and continuity to exposure to parasite. Adults residing in malaria endemic areas are semi-immune, implying that individuals harbour parasites without experiencing acute illness \(^{15}\). Newborns do not contract the disease during the first months of life, probably due to the maternal antibodies that crossed the placenta. However, the role of maternal antibodies in this context has been questioned \(^{16}\). As the level of the passively acquired antibodies wane around 6-9 months of age, infants in malaria endemic areas experience their first clinical episode \(^{17}\).

Innate immunity

The innate immune mechanisms involved in parasite growth inhibition in the human host, are probably the reason for the low parasitemia seen during acute \(P. falciparum\) infection \(^{18}\). Within the last years, the family of toll-like receptors (TLRs) has been identified as comprising key host molecules in the induction of innate immune responses to microbial ligands \(^{19,20}\). The TLRs can recognize and orchestrate an early defense, largely dependent on activation of nuclear factor kappa \(\beta\) (NF-\(\kappa\) \(\beta\)), which often leads to the production of pro-inflammatory cytokines. In protozoans, TLR-2 has been shown to recognize GPI of \(Trypanosoma cruzi\) \(^{21}\). The GPI anchor of \(P. falciparum\) is thought to function as a critical toxin, which contributes to severe malarial pathogenesis by eliciting the production of pro-inflammatory responses by the innate immune system of mammalian hosts. Analysis of the fine structure of \(P. falciparum\) GPI, suggests a requirement for the presence of both core glycan and lipid moieties in the recognition and signaling of parasite glycolipids by host immune cells. Recently, \(P. falciparum\) GPI was reported to induce signaling via both TLR-2 and -4 \(^{22}\). However, a recent study on TLRs and malaria in humans, indicated that common \(TLR-4\) mutations in African children increase the risk of severe malaria. \(^{23}\).
Malaria parasites within red blood cells digest host hemoglobin into a hydrophobic heme polymer, known as hemozoin (HZ), which is subsequently released into the blood stream and then captured by and concentrated in the reticulo-endothelial system. Accumulating evidence suggests that HZ is immunologically active, but the molecular mechanism(s) through which HZ modulates the innate immune system has not been elucidated. However, studies have demonstrated that HZ purified from *P. falciparum* is a novel non-DNA ligand for TLR-9 24, 25.

Cells of the innate immune system, as well as host genetic factors, contribute to innate immunity against malaria. These cells include NK, gamma delta (γδ) and NK T cells. Polymorphonuclear cells such as neutrophils and eosinophils, soluble factors, such as interferons and complement factors, are also involved in the innate immune response against malaria 26. The innate immune mechanisms have been shown to operate when parasite density crosses a predefined threshold 27. NK cells have been shown to be the first cells to respond to *P. falciparum* infection by increasing in number and the ability to lyse infected RBC *in vitro* 28. In *P. falciparum* infection, direct contact between parasitized RBCs and NK cells leads to IL-12 and 18 production, which further leads to IFN-γ production 29. Thus, the IFN-γ produced by the NK cells activates macrophages to eliminate parasitized RBCs. Evidence for the role of macrophages in the innate immunity is their ability to phagocytose infected erythrocytes in the absence of cytophilic or opsonizing malaria-specific antibodies 30, 31, and thereby contributing to the reduction of initial parasitemia. The role of NKT cells in malaria infection could be speculated from their simultaneous production of high levels of both IFN-γ and IL-4 upon primary TCR stimulation, as shown in other systems 32. Plasmacytoid dendritic cells (PDC) have been investigated in malaria, and it has been shown that soluble products of the late stages of the parasite can activate PDC in a TLR 9-dependent manner 25. During malaria infection, γδ T cells are more expanded in the circulation than other T cell subsets, and they have been shown to directly inhibit the growth of blood-stage parasites 33.

The influence of the genetic make-up of the host on susceptibility to malaria infection endemic areas has been established, especially in the genetic red cell disorders, including
sickle cell trait, thalassemia, enzyme deficiencies, ovalocytosis and ABO blood groups. These host factors may confer natural protection against malaria infection. Other innate factors, such as polymorphisms in ICAM-1, a putative receptor for erythrocyte binding to the brain endothelium, and a polymorphism in the promoter region of TNF-α, appear related to the frequency of severe disease\textsuperscript{34,35}.

**Adaptive Immunity**

**Humoral responses to malaria**

During *P. falciparum* infection, the functional role of antibodies regarding protective immunity is unclear. However, a number of evidence has shown that antibodies are important for clearance of parasite loads in both animal and human blood stage infections\textsuperscript{36}. In most malaria endemic countries, malaria infection induces humoral immune responses, involving production of predominantly IgM and IgG but also of other immunoglobulin isotypes. While a majority of this immunoglobulin is a result of polyclonal B-cell activation, up to 5% or more represent species as well as stage-specific antibodies, reacting with a wide variety of parasite antigens. The level of total antimalarial antibodies increases with age, and is usually taken as a measure of the length and intensity of exposure, and sometimes may indicate protection against malaria. Meanwhile, the efficiency of antibody-mediated inhibition is usually insufficient to confer complete protection. Some protective mechanisms do not involve antibodies, for example, protection against sporozoite infection of liver cells is primarily mediated by T-cells\textsuperscript{37}. Antibodies against merozoite surface-associated proteins may block RBC invasion\textsuperscript{38}, or may block merozoite release from schizonts, either by binding to surface exposed antigens or by entering the infected RBC through leaky membrane at the time of rupture\textsuperscript{39}. In conjunction with effector cells, parasite antigen specific antibodies act via a mechanism named antibody-dependent cellular inhibition (ADCI), whereby binding of antibodies to monocytes through Fc receptors leads to inhibition of parasite growth\textsuperscript{40,41}.

Antibodies produced against malaria parasites are of different isotypes and subclasses, with different functional capabilities regarding being protective or otherwise. In most cases, the IgG isotype has been shown to be protective against malaria, as well its subclasses IgG1 and IgG3, and mainly these antibodies are found to prevail in most
malaria infected individuals from endemic areas, supporting the functional role of these antibody subclasses $^{42-44}$. The other subclasses, IgG2 and IgG4, have been speculated to be non-protective, although in some malaria endemic areas, elevated levels of IgG2 have been related to decreased risk of infection, $^{45}$. Elevated levels of IgE appear to be associated with pathogenesis, as indicated in patients with severe and cerebral malaria $^{46,47}$. Regarding IgM, elevated levels as compared to controls have been observed in several studies performed in relation to malaria specific antigens, but its functional role till date has not been properly elucidated.

**Cell mediated responses**

As human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by CD8+ T cells has no role in defence against blood-stage parasites. However, during the preerythrocytic liver stage, effector functions of the CD8+ T cells are important $^{48}$, and contribute to protection against severe malaria $^{49,50}$. In the case of asexual blood-stage parasites, CD4+ T cells are pivotal in the development and regulation of both humoral and cell mediated immune responses. Depending on the cytokine produced, both murine and human T helper cells can be divided into Th1 and Th2 cells $^{51}$. The Th1 are thought to be involved in the initial resolution of acute parasitaemia through cell mediated effector mechanisms, including clearance of parasitized red cells by phagocytes and production of Th1 cytokines $^{52-54}$. The Th2 cells are believed to contribute to eventual clearance of parasites via T-B cell co-operation and the subsequent antibody response $^{52,53}$. It was however shown that CD4+ T cells from malaria exposed individuals naturally exposed to malaria, respond to blood stage antigens of *P. falciparum* by proliferation, production of IFN$\gamma$ and / or IL-4 secretion *in vitro*. Such IL-4 production was neither associated with proliferation nor with IFN$\gamma$ production, but was well correlated to serum antibodies to the peptides used to activate the T cells $^{55}$. $\gamma\delta$ T cells, whose activation is initiated by IL-2, IL-4 and IL-15, have been shown to expand both in mice and humans during malaria infection $^{56}$. They also recognize schizont derived–phosphorylated molecules $^{57}$ and produce proinflammatory cytokines. The T regulatory cells of the immune system which are thought to be involved in immunosuppression, are still under investigation regarding immunity to malaria infection $^{58}$. 
Vaccine development

The development of vaccines as a tool to protect man against infectious diseases was initiated more than 200 years ago, when Edward Jenner used cow pox to achieve immunity against fatal small pox \(^{59}\). Since then, vaccines have been the mainstay of fight against pathogenic organisms, and success has been achieved with diseases such as polio, diphtheria and tetanus. Achievements have also been seen concerning smallpox, and 39% decrease in deaths caused by measles. New effective vaccines against *Haemophilus influenza* type B, Hepatitis B virus and *Neisseria meningitis* have been introduced, and are now being incorporated in vaccine programmes in developing countries. The majority of effective vaccines today are based on inactivated or live attenuated virulent organisms, which are being referred to as first generation vaccines. Despite the successful records for these types of vaccines, there are still disadvantages encountered with them. For inactivated vaccines, it is cumbersome to prepare sufficient amounts of material from organisms which can not be cultured efficiently *in vitro*, and efficient killing of the organisms must be guaranteed without loss of their immunogenic properties. Setbacks associated with attenuated vaccines include the presence of contaminations derived from the cultures of the organisms and the hazard of reversion to virulence. The second generation vaccines (subunit vaccines), which originate from defined antigens from the pathogens, may be composed of recombinant proteins or synthetic peptides, and these types of vaccines have no risk of pathogenicity, since they can not replicate in the host. Moreover, there is a recent approach to vaccine development, which is based on nucleic acids encoding defined antigens from the pathogen.
**Immune response to vaccines**

The aim of vaccination is to generate an immune response with capacity to protect the recipient against the natural infection. Furthermore, it must be able to induce a long-lived immunological memory through the adaptive immune system, which is initiated by antigen uptake by an antigen presenting cell (APC), proteolytic processing, and presentation on the surface of the APC in association with either major histocompatibility complex (MHC I or MHC II). For an endogenous antigen, the peptide derived antigen will be processed through the cytosolic pathway and presented through MHC I, thereby CD8+ T cells are activated. However, for an exogenous antigen, processing will be via the endocytic pathway, followed by activation and presentation on APCs (dendritic cells, B cells, macrophages) associated with MHC II, and in this case the CD4+ T cell subset will be activated. Since the different APCs differ in their mechanism of antigen uptake, expression levels of MHC and co-stimulatory molecules, a vaccine should be delivered and presented to the immune system in a selective way, which facilitates the association with uptake by the appropriate APCs.

After antigen encounter, the CD4+ T cells develop into T helper (Th) 1 or Th 2 effector cells depending on the cytokine milieu. The Th1 effector cells produce proinflammatory cytokines, such as IFN-γ and TNF-α, and these lead to further activation of the CD8+ T cells and their cytotoxic effector function. The Th2 cells, on the other hand, produce interleukins, such as IL-4, 5, 10 and 13, which further stimulate B cells to undergo activation into effector B cell: (plasma cells), which secrete antibodies (IgD, IgM, IgE, IgA or IgG). The immune response induced by vaccination should be of long duration and should have the capacity to be effectively reactivated by a natural infection. Memory cells, which are only about 5-10 % of the lymphocyte population, are phenotypically different from the naïve cells, and they proliferate and produce cytokines faster. Whether the persistence of antigen on follicular dendritic cells is needed or not for maintenance of immunological memory, is not clear. Several studies have indicated that memory B and T cells can survive without antigen, while others have suggested otherwise 60. However, cytokines such as IL-15 is crucial for the longevity of memory T cells.
Recombinant Vaccines

The advent of recombinant DNA technology and protein engineering enabled the expression of immunogenic proteins in bacterial, yeast or mammalian cells, after being cloned in a suitable expression vector, and such expressed antigens are used for vaccine development 61-64. The first such recombinant protein vaccine approved for human use, is the hepatitis B vaccine, which was developed by cloning the gene for the major surface antigen of hepatitis B virus (HbsAg) and expressing it in yeast cells 65. The basics of this technology is to transfer a gene encoding an antigen, responsible for inducing good humoral responses sufficient for protection, to a non-pathogenic expression vehicles 64, thereby making the production of the antigen safer and generally more efficient. There are several limitations with recombinant proteins; they are generally poor immunogens when administered alone and are unable to induce effector T–cell responses, such a CD8+ CTLs, that are necessary for elimination of the intracellular pathogens.

Malaria vaccine development

Considering the fact that traditional methods of controlling malaria have neither been practical nor cost effective in most malaria endemic areas, there is the dire need for an affordable and effective malaria vaccine. Attempts to develop a malaria vaccine began in the early twentieth century 66, and in spite of advances in biomedical technology and periodic bouts of unsubstantiated optimism in the field, no effective vaccine is available for widespread use till date. Plasmodium species have evolved multiple mechanisms of immune evasion at the individual and population levels, including stage specific antigen expression, allelic diversity, variability within T cell epitope sequences and antigenic variation. During the course of its complex life cycle, the Plasmodium parasite expresses different, complex mixtures of antigens. Therefore, a vaccine against a single stage in the parasite life cycle may need to be 100% effective, because parasites which progress to the next stage may express a new set of antigens, that may be unaffected by the vaccine induced response.
The rationale behind the development of a malaria vaccine is supported by previous studies, which have shown that antisporeozoite vaccines based on irradiated sporozoites elicited sterile immunity in humans\textsuperscript{67}, and also that passive transfer of IgG from semi-immune individuals can provide some protection against malaria\textsuperscript{68}. Most malaria antigens are stage-specific and therefore there are distinct immune mechanisms operating against the different stages of the complex life cycle.

**Pre-erythrocytic vaccines**

Most malaria vaccine studies have been focusing on the pre-erythrocytic stages of the life cycle. This is the clinically silent stage of the parasite’s life cycle. A vaccine targeted at these stages would need to elicit sustained high antibody responses as well as an efficient CD8\(^+\) T cell memory. It will possibly prevent sporozoite invasion of hepatocytes, or the development of the exo-erythrocytic stages within the hepatocytes. Such a vaccine would eliminate disease manifestation and further transmission, but most importantly, it would benefit individuals who have previously not been exposed to the parasite, but might later be at risk of high morbidity and mortality. For the pre-erythrocytic stages, several vaccine candidates have been studied and have been evaluated in clinical trials. They have been based on repeat sequences from the circumsporozoite protein (CSP), which have been tested in various adjuvant formulations, but the immunogenicity of these was low in trials carried out in humans\textsuperscript{69}.

Another vaccine candidate from sporozoites\textsuperscript{70} which has progressed to field studies, is thrombospondin related anonymous protein (TRAP). This antigen is not crucial for sporozoite formation, but it ensures sporozoite motility and thereby successful invasion of the mosquito salivary glands and human hepatocytes\textsuperscript{71}. Antibodies against TRAP inhibit the invasion of hepatocytes by sporozoites, and this was shown to correlate with control of parasite densities \textit{in vivo}\textsuperscript{72, 73}. TRAP attached to a multi-epitope string, consisting of CD8\(^+\), CD4\(^+\) and B cells epitopes derived from six other pre-erythrocytic antigens, provided in various heterologous prime boost regimes, entailing DNA or pox
viral particles, has been tested in a series of phase IIa and phase IIb clinical trials \(^{74, 75}\). In a recent clinical trial, it was indicated that the hepatic burden of the parasites was reduced by 92%, and circulatory memory T cells elicited sterile immunity for as long as 20 months in some volunteers \(^{76, 77}\). Recently, another phase IIb efficacy trial has been initiated in Kenya, in order to assess protection against febrile malaria in children \(^{75}\).

The presently most advanced vaccine candidate is RTS,S/AS02A comprising of a fusion between half of the CS protein and the hepatitis B surface antigen (RTS,S), which is expressed in yeast cells and used with the oil-in-water adjuvant AS02 \(^{78}\). Recently, two field trials were carried out in The Gambia and Mozambique. A vaccine efficacy trial was conducted in Gambian semi immune adults during a period of low transmission season, and followed up on occurrence of new infections during 16 weeks of active malaria transmission, \(^{79}\). While efficacy during the first 9 weeks of follow up was 71%, it was zero at later time points. The RTS,S vaccine was also evaluated in children aged 1-4 in Mozambique, this vaccine imparted 30% reduction in clinical malaria incidence, a delayed time to first infection by 45%, and reduced incidence of severe malaria by 58% at a 6 month follow up \(^{80}\).

**Asexual blood-stage vaccines**

A vaccine targeted against antigens expressed on the asexual blood stages of the parasites’ life cycle is aimed at preventing the complications of the disease, such as cerebral malaria or anemia. Since these are the stages responsible for pathology caused by the disease, antibodies to the target antigens should be able to inhibit parasite sequestration, to induce neutralizing antibodies against parasite derived materials, or to eliminate/reduce the parasite load, which might further reduce mortality. Despite encouraging progress, the lack of immune correlates of protection, and insufficient predictive animal models, as well as polymorphism and strain variability of most asexual blood-stage antigens, constitute major challenges to the development effort of asexual stage vaccines. Inhibition of parasite invasion, as often measured in *in vitro* assays, is not always predictive of immune status in endemic areas.
The first asexual blood-stage malaria vaccine submitted to clinical trial was SPf66, a synthetic, multi-stage peptide vaccine, mixed with alum as an adjuvant. The vaccine was tested in several Phase III field trials involving thousands of volunteers, but these trials showed mixed results. Thus, it was difficult to justify for further trials, although one may suspect that the vaccine would have fared better with more potent adjuvants. The most advanced asexual blood-stage vaccines at this time are based on the use of different merozoite associated antigens, such as merozoite surface protein 1 (MSP-1), MSP-2 and MSP-3, the apical membrane antigen 1 (AMA-1) and the glutamate-rich protein (GLURP). Antibodies to MSP-1 have been shown to block parasite invasion of RBCs \textit{in vitro}. AMA-1 is a natural target of protective responses \textit{in vivo}. Both AMA-1 and MSP-1 have their 3D conformation stabilized by intramolecular disulphide bonds, which are critical for optimal immunogenicity of the molecule. MSP-1 contains two cysteine-rich epidermal growth factor (EGF)-like, domains that generate protective antibody responses, and are conserved across all species of Plasmodium.

The MSP/RESA vaccine (combination B) is one of the most advanced in asexual vaccine development. It is a mixture of three recombinant asexual blood stage antigens: block 3 and 4 of MSP-1, the 3D7 form of the polymorphic MSP-2, and the last 70% of RESA in a Montanide adjuvant formulation. In a Phase I/IIb trial in Papua New Guinea, there was 62% reduction in parasite density in vaccinees. However, the vaccine contained the 3D7 allelic form of MSP-2, which made the vaccine ineffective against the FC27 allele genotype of parasites. A new formulation is being developed using both variants of MSP-2 in order to target both genotypes.

Much work has concentrated on the entire MSP-1 molecule, its 42 kDa C-terminal moiety, or a further processed 19 kDa fragment. These were expressed either as such or as parts of fusion molecules, using baculovirus, Escherichia coli, or yeast (Saccharomyces or Pichia). Experimental vaccinations with recombinant MSP-1 42 kDa and 19 kDa fragments have been shown to protect both mice and Aotus monkeys against lethal parasite challenge, but a Phase I trial of the 19 kDa fragment, carried out at
Baylor University (USA), demonstrated that it was poorly immunogenic and had unacceptable side-effects. The MSP-1 42 kDa fragment formulated in AS02 adjuvant was found to be safe and very immunogenic in human volunteers in the USA, Kenya and Mali. The vaccine is currently being tested in pediatric efficacy trials in Kenya. Another vaccine studied is based on the AMA-1 protein formulated in the AS02 adjuvant. Like MSP-1, the vaccine potential of AMA-1 is supported by the observation that antibodies to the protein inhibit invasion of RBCs in vitro. The high polymorphism of the AMA-1 antigen is however a matter of concern. A MSP-1/AMA-1 fusion antigen, made of the C terminal region of AMA-1 and the 19 kDa fragment of MSP-1, was produced using Pichia pastoris. The vaccine (PfCP-2.9), which was formulated with Montanide ISA 720 as an adjuvant, showed good immunogenicity in rabbits and non-human primates. The vaccine was found to be safe and immunogenic, and plans are underway for further clinical development. The combination of the PfCP-2.9 vaccine with a fragment of the PfEBA-175 antigen, expressed in P. pastoris yeast, elicited in both rabbits and monkeys, antibodies that inhibited parasite growth in vitro.

The merozoite surface protein 3 (MSP-3), has been proposed to be a target of protective antibodies from immune adults, and a vaccine has been developed as a long synthetic peptide. The vaccine construct contains B and T cell epitopes, that were selected based on their targeting by cytophilic antibodies, which interact with monocytes in the antibody-dependent cellular inhibition (ADCI) assay. A Phase I study of the vaccine was carried out in Burkina Faso, and the vaccine was shown to be safe and to induce long-lasting antibodies that display ADCI activity in vitro, as well as in vivo in a new mouse model of P. falciparum malaria. Another long synthetic peptide vaccine, the rationale of which is based on the induction of antibodies with ADCI activity, is based on the glutamate-rich protein (GLURP), also suggested to be a target of protective antibodies from immune adults. The GLURP vaccine was formulated either in alum or Montanide ISA 720 and tested in a Phase I clinical trial. Yet another candidate being developed in Asian–African collaboration is based on the serine repeat antigen (SERA), also known as P126 antigen. The antigen accumulates in the parasitophorous vacuole of trophozoites and schizonts and is processed into three fragments (18 kDa, 47 kDa, 50
kDa) \(^{93}\). Cross-sectional studies, conducted in the Solomon Islands, Brazil and Uganda \(^{94}\) have shown a significant association between responses to SERA and lower parasitemia \(^{84}\). Various SERA-based constructs have been tested and shown to induce \textit{in vitro} parasite killing activity in a dose dependent manner through both complement-mediated inhibition and ADCI \(^{84}\). Furthermore, protective efficacy of a subunit SERA vaccine against challenge in \textit{Aotus} and squirrel monkeys has also been demonstrated \(^{84}\). A candidate SERA-based vaccine currently is in Phase I studies in Japan.

In addition to the above mentioned malaria vaccines, transmission-blocking vaccines against the sexual stage antigens, would interfere with parasite development within the mosquito. They are intended to protect communities from infection, the leading vaccine candidate in this field contain \textit{P. falciparum} ookinetes surface antigens Pfs25 and Pfs28 \(^{95}\). However, to ensure perfect protection from malaria disease, a malaria vaccine should consist of antigens from different stages of the parasite’s life cycle. Importantly, induction of an efficient transmission blocking by such a vaccine would prevent the transmission of parasites which may have mutated due to the immune pressure elicited by the vaccine against the asexual blood stages.
THE PRESENT STUDY

Background

The *P. falciparum* antigen 332 (Pf332) is a megadalton protein of schizont-infected red blood cells, and is a member of a family of glutamic acid rich proteins, also including antigen Pf11.1, a megadalton protein of gametocytes, and Pf155-RESA, a 155-kDa protein of ring-infected red blood cells. These antigens have been reported to share related amino acid repeat sequences, which are antigenically cross-reactive ⁹⁶. Pf332, identified by Mattei *et al* ⁹⁷, consists of 5508 amino acids, with an overrepresentation of glutamic acids (30%) and valine (13%) ⁹⁸. It is expressed during the trophozoite stage, and transported from the parasitophorous membrane to the outer erythrocyte membrane during schizogony ⁹⁹. The Pf332 gene is located in the subtelomeric region on chromosome 11. Genes located in this region are prone to frequent breakage and healing. Although the Pf332 gene appears to be subject to some degree of breakage induced variation ¹⁰⁰, irreversible loss has never been detected in any of the parasite strains analysed so far ¹⁰¹, ¹⁰². In a colocalization study, it was shown that Pf332 is being transported in vesicles together with RIFIN and PfEMP-1 ¹⁰³.

The initial rationale to consider Pf332 as a blood stage malaria vaccine candidate, was based on that a Pf332-reactive human monoclonal antibody (mAb 33G2) inhibits parasite growth and cytoadherence *in vitro* ¹⁰⁴. Furthermore, various rabbit polyclonal antibodies specific for Pf332 ¹⁰⁵, ¹⁰⁶, and human polyclonal IgG antibodies, affinity-purified on Pf332 repeats, also display similar parasite growth inhibitory capacity ¹⁰⁷. In addition, increased titers of Pf332-reactive IgG antibodies in humans are associated with decreased number of malaria incidents ¹⁰⁸. Experimental animal vaccinations with Pf332 have mainly been conducted with a central fragment of the antigen, denoted EB200 ¹⁰⁹. This fragment has been identified as a target of opsonizing antibodies in hyperimmune sera from *P. falciparum*-exposed squirrel monkeys ¹¹⁰. In conducting further studies, it was observed that the presence of these antibodies correlated with protection against disease ¹¹¹.
Objectives

Despite efforts made towards vaccine development, malaria remains uncontrolled due to the little understanding of the natural protective immunity against this disease. The earlier studies carried out regarding Pf332, mainly focused on the central part of the antigen, denoted EB200. This fragment is quite overrepresented with glutamic acid repeats and there have been difficulties in defining the actual target for parasite growth inhibitory antibodies due to its antigenic cross reactivity with other malaria antigens. In order to address this problem, it was proposed that a less repetitive fragment of the antigen Pf332-C231 should be studied. The C231 fragment is located in the C-terminal region of the antigen Pf332 and consists of 231 amino acids. It is well represented with various amino acids and most importantly has a cysteine, which might be involved in the folding of the protein. The specific aims of the research work carried out in this thesis were:

- To study the immunological capabilities of the fragment Pf332-C231 in an experimental model (Paper I)

- To study the profile of the antibody response to C231 in naturally exposed individuals (Paper II)

RESULTS AND DISCUSSION

The majority of all functional studies on malarial proteins has been initiated by introducing genes downstream of promoters ported by plasmids, and transfection into *E. coli* for recombinant expression. There are however other expression systems such as baculovirus, yeast, mammalian and insect cells, but the approach which we have used in our study (paper I), poses some advantages such as; simplicity and low cost, availability of compatible molecular tools, absent of post translational modifications, higher yield per unit biomass. In our study (Paper I), we were able to clone our recombinant protein, using the plasmid vector and expression was carried out in *E. coli*. In molecular biology,
expression in *E. coli* has been debarred by the formation of insoluble aggregates of the recombinant protein being expressed, and to circumvent this problem, we enhanced the purification of our protein, using a solubility enhancing fusion tag. The 6xHis tag is a small, poorly immunogenic tag, which does not interfere with the structure or function of the purified protein. Regarding immunogenicity of this protein, high antibody levels were obtained in rabbits, and antibodies raised against C231 could recognize the native protein expressed in infected erythrocytes. Malaria vaccine candidates of the asexual blood stages of *P. falciparum* are evaluated on the basis of their ability to induce antibodies with anti-parasite activity. Such antibodies may have different effector functions e.g., inhibition of invasion or inhibition of parasite growth/development, depending on the target antigen. In *in vitro* experiments carried out with anti-C231 antibodies (Paper I), we observed inhibition of merozoite invasion, as reflected by inhibition of parasite development with infected RBCs.

Antibodies are thought to be the primary immune effectors in the defense against erythrocytic stage *P. falciparum*. The level of total antimalarial antibodies increases with age and is usually taken as a measure of the length and intensity of exposure, and sometimes may indicate protection against malaria. We performed analysis with recombinant C231 as antigen in comparison to crude malaria extract, and looked at the profile of isotypes and IgG subclasses of antibodies in malaria exposed individuals from Senegal. In general, we detected anti-C231 response in all sera tested regarding all isotypes (IgG, IgM, IgE) investigated, and the levels increased significantly with age.

The polarization of antibody responses towards IgG1 and IgG3 subclasses, which bind to Fc\(\gamma\) receptors on the surface of monocytes, macrophages, and neutrophils, is believed to play a key role in immunity to blood-stage *P. falciparum* infection. These cytophilic antibodies (IgG1 and IgG3) mediate parasite-killing responses, such as opsonization and phagocytosis of extracellular parasites or parasitized red blood cells, and antibody-dependent cellular inhibition of intracellular parasites. However, there are studies suggesting a protective role of non-cytophilic IgG2 *in vivo* \(^{112, 113}\), which may be explained by a 131 R/H polymorphism in the Fc\(\gamma\) receptor IIa. As a result, IgG2 is
cytophilic in individuals carrying the H131 allele positive FcγIIa gene. When analyzing the subclasses of C231 specific IgG antibodies in sera from individuals naturally primed to *P. falciparum* (Paper II), we observed a bias towards IgG2 and IgG3 relative to IgG1. We conclude that the IgG subclass distribution of naturally acquired antibodies to Pf332-C231 in malaria exposed individuals in Senegal may be epitope driven. Since polarization towards IgG2 was more evident for anti-C231, this may be affected by cumulative or current exposure to malaria by the subject's age and FcγRIIa genotype. These findings have clear implications for the rational design and evaluation of antimalarial vaccines that induce antibody-mediated protection. However, further studies and analysis should be carried out for possible correlation of IgG2 and H131 allele of FcγIIa.
Concluding remarks and future perspectives

The development of an effective malaria vaccine encompasses a whole array of setbacks. The complexity of the parasite cycle offers various candidates there have doubts whether these candidates will induce the right type of immunological response, even if it does in experimental models and some clinical trials, it may not be able to induce the long term immunological memory essential for long term protection. Antigenic diversity as reflected by allelic polymorphisms and antigenic variation are also factors that limit the efficacy of a malaria vaccine development, particularly in the asexual blood stage, an area in which this study is based on. Although, there are suggestions of combining antigens from various developmental stages of the parasite, but this might lead to selective pressure.

The natural immunity to malaria consists of a complex mixture of diverse immune responses, some non-protective and some protective or inhibitory. However a subunit vaccine which is aimed for in our study, is needed to evoke additive responses that are more beneficial and advantageous than those generated due to natural exposure. In concluding the studies presented in this thesis, we have shown that the C-terminal fragment of antigen Pf332 is immunogenic and anti-C231 antibodies have parasite inhibitory capacities. Also it was well recognized by sera from individuals in various age groups from various malaria endemic areas. Anti-C231 antibodies may also be associated to protection from clinical malaria, which further confirms previous studies carried out with EB200, a fragment of Pf332 \(^{108}\). This study further emphasizes the inclusion of antigen Pf332 as a subunit vaccine against *P. falciparum* malaria.

Amidst all findings mentioned above, further studies will be carried out addressing the following;

Antibodies induced by protein are commonly directed against surface exposed regions of the protein and certain epitopes may be immunodominant. One strategy for the selection of antigenic sequences to be included in a subunit vaccine against *P. falciparum* malaria,
is to define epitopes seen by antibodies which have the ability to interfere with parasite development. As it was earlier revealed, mAb 33G2, which was shown to inhibit merozoite invasion of RBCs, harbours an epitope of five amino acids long sequence, VTEEI \(^96\). In line with this, the elucidation of specific B-cell epitopes by analyzing the ability of peptides to induce antibodies may reveal different epitope specificities regarding Pf332-C231.

As mentioned earlier, malaria vaccine candidates directed to blood stages of infection are evaluated based on their ability to induce antibodies with anti-parasite activity. We intend to carry out more studies regarding the parasite inhibitory capacity of affinity purified human anti-C231 antibodies from Liberians, as in our earlier study (Paper I) we only used total rabbit antibodies. The parasite neutralizing capacity of these human antibodies will also be tested with human monocytes as this mimics the immunological situation \textit{in vivo}.
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