CHARACTERIZATION OF NOVEL SURFACE PROTEINS OF *PLASMODIUM FALCIPARUM* AND THEIR ASSESSMENT AS MOLECULAR TARGETS FOR A MALARIA SUBUNIT VACCINE

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"All truths are easy to understand once they are discovered; the point is to discover them."

Galileo Galilei

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SUMMARY

Each year there are more than 250 million cases of malaria, claiming nearly one million deaths of which most are among children below the age of five from sub-Saharan Africa. An effective malaria vaccine could prove to be the most cost-effective and efficacious means of preventing severe disease and death from malaria. To date, no approved malaria vaccine is available and only a few candidate vaccines were able to induce some protective efficacy. Limited success in the development of a malaria vaccine may partly be due to the reliance on a hand full of antigens discovered more than 20 years ago. Since the fully annotated *Plasmodium falciparum* genome has become available in 2002, reverse vaccinology presents a new opportunity to identify novel malaria vaccine candidate antigens.

In this thesis, we anticipated the rational selection of novel malaria subunit vaccine candidates. Proteins on the surface of extracellular stages of the malaria parasite are accessible to immune surveillance by antibodies. Thus, our strategy is based on the selection of hypothetical surface proteins of extracellular parasite stages and subsequent functional characterization using specific monoclonal antibodies.

Functional *in vitro* and *in vivo* assays require antibodies capable of recognizing the endogenous antigen in its native context. We developed an entirely cell-based approach that bypasses the problematic step of protein purification. By presenting the antigen on the surface of mammalian cells in its native conformation for immunisation and hybridoma selection, this procedure promotes the generation of monoclonal antibodies capable of binding to the native endogenous target proteins. This was exemplified by three hypothetical surface proteins of *P. falciparum,* whereof two contain complex folds comprising numerous disulfide bonds. The developed new strategy for the generation of monoclonal antibodies may be applied for a wide range of cell-surface proteins.

The generated monoclonal antibodies were used to study the selected hypothetical *P. falciparum* surface proteins. PF14_0325 was found to be expressed in late asexual

blood stages and PFF0620c, a member of the 6-cysteine protein family implicated in cell-cell interactions, in gametocytes and sporozoites. Both proteins await further investigation in assay systems assessing sporozoite and sexual blood stage inhibition. A third candidate, designated cysteine-rich protective antigen (CyRPA), is expressed in merozoites and localizes to its apical pole. CyRPA-specific antibodies inhibited parasite growth *in vitro* as well as *in vivo*. The inhibitory mechanism was found to be independent of immune cells or complement, although complement augmented the effect *in vivo*. Antibodies blocked invasion of merozoites into erythrocytes, indicating that CyRPA has a function in merozoite invasion. The protein was found to be highly conserved. Together, this renders CyRPA a candidate component for a malaria blood stage vaccine.

The *in vivo* growth inhibitory effect of antibodies specific for malaria *P. falciparum* blood stage antigens was assessed by passive immunisation experiments in *P. falciparum* infected NOD/*scid-IL2Rγ*^{null} mice engrafted with human erythrocytes. In contrast to previously described passive immunisation studies in other SCID mouse lines, this model evinced a dose-response relationship. Thus we propose this model for comparison of the relative *in vivo* inhibitory potency of malaria specific antibodies.

Applying the principle of reverse vaccinology, we identified and characterized surface proteins of extracellular malaria stages. Thereby we identified CyRPA as target of merozoite invasion-inhibitory antibodies. We expect that characterization of further hypothetical parasite proteins with this strategy will identify additional vaccine candidate antigens from the extracellular stages of *P. falciparum*. This will increase the choice of vaccine antigens for incorporation into an effective multivalent, multi-stage malaria subunit vaccine.

INTRODUCTION

Even though we only realize it once we lost it, good health is one of the most important dimensions of our quality of life. Besides, health is a major resource for social, economical and personal development [1]. In the Universal Declaration of Human Rights from 1948, the General Assembly of the United Nations declared health as a human right [2].

Global burden and epidemiology of malaria

Aspiring effective health promotion calls for the assessment of the global burden of diseases. Globally, malaria is placed on rank 12 of the leading causes of burden of disease. It is responsible for 2.2% of total DALYs (disability-adjusted life years), a measure that combines years of life lost due to premature mortality and years of life lost due to time lived in states of less than full health or disability into a single metric [3]. Restricted to sub-Saharan Africa, malaria claims 8.2 % of total DALYs, being number four after HIV/AIDS, lower respiratory infections and diarrhoeal diseases [4]. Malaria mainly affects children and is responsible for 16% of the total mortality in African children below the age of five [5].

According to the WHO, malaria occurs in 108 countries, mainly in sub-Saharan Africa, but also in Asia, Oceania, Central and South America, and the Caribbean. Approximately 40% of the world population (3.3 billion people) are at risk [6]. Each year malaria causes 250 million cases, claiming nearly one million deaths of which most are children below the age of 5 or pregnant women from sub-Saharan Africa [6]. In some heavy-burden countries, malaria accounts for 30-50% of all hospital admissions and is responsible for up to 40% of public health expenditures [7]. Malaria particularly affects poor people who cannot afford treatment and have limited access to health care [8,9]. Hence, malaria brings about a downward spiral of poverty. Consequently, reduction of malaria morbidity also disburdens the health system and increases the standards of living of affected countries [10].

Malaria is transmitted exclusively through the bite of *Anopheles* mosquitoes. Thus, transmission depends on climatic conditions like rainfall patterns, temperature and humidity, which affects the abundance and survival of mosquitoes. Consequently, in some areas malaria transmission is seasonal, with the peak infection rate during and just after the rainy season, whereas in other areas malaria occurs all year round.

Malaria is caused by protozoal endoparasites of the genus *Plasmodium*. Human malaria can be caused by infection of five different malaria species; *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale and P. knowlesi*. *P. falciparum* is the most deadly, accounting for 92 % of all cases [11].

P. falciparum life cycle

The life cycle of *P. falciparum* involves an alternation of asexual and sexual generations and obligatory heteroecism between *Homo sapiens* and *Anopheles*. Parasites are transmitted to the human host by the bite of infected mosquitoes. During the blood meal, sporozoites, the fusiform infectious stage, are inoculated into the human skin and reach the liver via the blood circulation, where they infect hepatocytes. Within 6 days the parasites develop into multinucleated schizonts, from which up to 30’000 1.5 µm long ovoid cells, called merozoites, are formed. The rupture of the infected hepatocyte releases merozoites into the bloodstream. Within 1-2 min of release, merozoites attach to specific receptors on the surface of erythrocytes, which initiates active invasion into the erythrocyte. Within a parasitophorous vacuole, the parasite undergoes maturation into ring-stages, trophozoite-stages and upon mitotic division into schizont-stages. 8-32 merozoites are formed from each schizont, which, upon rupture of the erythrocyte, are released into the blood stream, where each one starts a new asexual cycle by infecting another erythrocyte. In the case of *P. falciparum* the asexual cycle takes 48h. After repeated asexual cycles, some parasites develop into sexual stages. Male and female gametocytes persist in the blood for up to 22 days. When taken up by a feeding mosquito, the male gametocytes exflagellate into 8 single-nucleated microgametes, which fertilize female macrogametes. Gamete fusion induces formation of a motile zygote, the so-called ookinete. The ookinete penetrates the mosquito gut wall and

develops between the epithelium and the basal lamina into an oocyst. The oocyst undergoes schizogony leading to the formation of thousands of sporozoites. Eventually oocysts rupture and freed sporozoites travel via the hemolymph to the salivary glands. During another blood meal of the mosquito, sporozoites are transmitted to a new human host. Development within the mosquito takes 8-14 days depending on the ambient temperature.

Pathology, treatment and intervention strategies

Clinical symptoms are solely caused by the asexual stages of the parasite. Therefore, clinical symptoms develop not until 7 days after the infectious mosquito bite. Severity of the disease depends largely on the infecting species and the immunological status of the affected person. Particularly susceptible are non-immune humans from malaria non-endemic areas as well as children of the age of 6 months to 3 years living in endemic countries. Initial symptoms are fever, nausea, headache, muscular pain and chills and describe uncomplicated malaria. *P. falciparum* malaria can progress to severe malaria with symptoms like severe anaemia, hypoglycaemia, respiratory distress, cerebral malaria (a rapidly developing encephalopathy) or cardiovascular collapse and shock. Malaria during pregnancy can cause miscarriage, low birth weight and premature delivery [12].

Malaria is treated by oral drugs including chloroquin, mefloquin, and artemisinin. However, resistance to chloroquin and sulfacoxine-pyrimethamine has spread to large geographical areas [13]. This leaves artemisinin-based combination therapy as the best available treatment option [14]. Prophylactic chemotherapy is applied for non-immune travellers to endemic areas. Exposition prophylaxis is achieved by bed nets, insect repellents, appropriate clothing, or vector control by insecticide-treated bed nets and indoor residual spraying of insecticides. Vector control was shown to effectively reduce malaria transmission, prevalence of severe malaria and malaria-associated mortality [9,15].

Immunity to malaria

In areas of low endemicity, malaria causes morbidity and mortality in patients of all ages. In contrast, in highly endemic areas, the incidence of severe malaria largely depends on the age of the host [16]. In newborns, the prevalence of parasitemia increases sharply at about 20 weeks of age but the infants remain more or less resistant to high parasitemia, fever and severe disease until about 6 months of age. For the next one to two years, risk of severe malaria increases rapidly, followed by a gradual decrease till the age of 5. Although largely protected from severe disease, children remain susceptible to uncomplicated malaria episodes till they reach adolescence. Adults living in high endemic countries rarely experience malaria episodes but may remain infected. Parasite density gradually decreases with age [17,18]. Protection against disease is lost if a person moves to a non-endemic area [19].

Protection of adults is mediated by natural immunity. This immunity protects against symptomatic disease, death and high-density parasitemia, but is not effective in offering sterile immunity. The rate at which immunity is acquired correlates upon the degree of exposure [20]. In addition, the acquired protection is short lived and its maintenance is dependent on sustained exposure [21]. Protection in infants till the age of 6 moths is possibly associated with fetal hemoglobin and the presence of maternal immune immunoglobulin acquired from the immune mother *in utero* or from breast milk [22,23].

Naturally acquired immunity predominantly targets the asexual blood stages [21]. No protection against pre-erythrocytic stages is acquired. This is evident by the fact that adults living in malaria-endemic areas cleared from parasites are readily reinfected and reinfection rates were shown to be identical for adults and infants [24].

Different effector arms of the immune system contribute to protective immunity. Innate, as well as adaptive cellular and humoral immune responses were shown to be effective. Their specific roles are briefly described below:

The role of the innate immune system in malaria infections is not well understood. However, studies in humans and mice showed that shortly after infection cytokines, including IFN-γ, are released [25,26]. IFN-γ was shown to be essential for the control of parasitemia, but at the same time it is implicated in malaria associated pathoglogy [27,28].

Immunization with high doses of irradiated sporozoites delivered by the bites of infected mosquitoes has been shown to induce long lived sterile protective immunity against sporozoite challenge [29–31]. By rodent studies this immunity was shown to be mediated in part by sporozoite specific antibodies but mainly by $CD8^+$ T cells that efficiently eliminate liverstage parasites [32]. CSP was identified as the immunodominant protein involved in T-cell mediated protection induced by immunization with sporozoites [33]. Furthermore it was shown that IFN-γ is a critical effector molecule, that IL-4 secreting CD4+ T cells are required for induction of the CD8+ T cell responses, that Th1 CD4+ T cells provide help for optimal CD8+ T cell effector activity and that components of the innate immune system, including γδ T cells, natural killer cells and natural killer T cells, also play a role [34]. However, it is important to note that exposure by natural transmission does not induce protective immunity to infection. Naturally exposed individuals harbour few numbers of circulating $CD8^+$ T cells specific for parasite-derived epitopes [35,36]. But it is not clear if these $CD8^+$ T cells have any anti-parasite activity and if they contribute to the immunity acquired with age in individuals of malaria endemic areas.

Antibody-independent cell-mediated immunity was also found to have a role in blood stage immunity. It was shown that mice lacking B cells and antibodies are able to control blood stage infection [37,38]. However, the relative importance of cellular or humoral immunity was found to vary among different rodent malaria models [37,38]. Studies further demonstrated that $CD4^+$ T-cell lines and clones could adoptively transfer protection against malaria [39–41]. Along with $CD4^+$ T cells, γδ T cells and $CD8^+$ T cells play a role in malaria immunity [39,42,43]. T cells are thought to confer protection by production of inflammatory molecules downstream of $CD4^+$ T-cell activation like IL-12, IFN-γ and TNF-α [44]. The presence of IFNγ and TNFα promotes synthesis of reactive oxygen intermediates and nitric oxide, which have

been shown to mediate parasite killing [45,46]. Also in humans T cells were shown to be able to control malaria parasite growth independently of antibodies. Protective immunity induced by repeated ultra-low doses of *P. falciparum* infected erythrocytes and drug cure induced protective immunity characterized by the presence of a proliferative T-cell response, involving $CD4^+$ and $CD8^+$ T cells, IFNy response and induction of high concentrations of nitric oxide synthase activity in peripheral blood mononuclear cells [47]. Less is known about the contribution of T-cells to naturally acquired protection in humans. Cell mediated immunity is not readily induced by natural infection in humans [48]. However, IFNy-secretion and high proliferative responses to blood stage antigens were shown to be associated with clinical protection [48–50].

An important role for antibodies in malaria immunity has been demonstrated by passive immunisation studies in humans [51,52]. Serum antibodies of immune adults transferred to non-immune individuals suffering from severe clinical malaria conferred a rapid drop in parasitemia and cease of symptoms [51,52]. Antibodies induced by *P. falciparum* infection are mainly directed against asexual blood stage antigens and are specific for proteins expressed on the membrane of infected erythrocytes, the surface of extracellular merozoites, or for proteins released from secretory organelles upon invasion. Various mechanisms have been described by which antibodies can interfere with parasite growth. They largely depend on the antibody specificity. Antibodies directed against surface proteins of merozoites were shown to agglutinate merozoites and thereby inhibit merozoite dispersal [53–55]. Other antibodies were shown to prevent antigen processing or binding to surface ligands required for the merozoite invasion process [56–58]. Furthermore, antibodies can mediate clearance of free merozoites by opsonization, leading to enhanced phagocytosis of merozoites or to complement-mediated damage [59–61]. Cytophilic antibodies bound to merozoite surface proteins were shown to induce destruction of merozoites by neutrophilic respiratory burst [62] or inhibition of division of intraerythrocytic parasites mediated by a soluble factor released by monocytes (a mechanisms called antibody-dependent cellular inhibition) [63–65]. On the other hand, antibodies recognizing parasite proteins on the surface of infected

erythrocytes were demonstrated to prevent cytoadherence and thus their sequestration, presumably allowing clearance of infected cells by the spleen [66–68]. Furthermore, such antibodies can induce phagocytosis of infected red blood cells or their destruction by complement or antibody-dependent cell-mediated cytotoxicity [69–73]. Antibodies can also neutralize malaria endotoxins that contribute to malaria pathology [74,75]. Although all of these mechanisms have been described, their relative contribution to naturally acquired protection is not fully understood. Important to note is that not all malaria-specific antibodies are protective. Depending on the specificity, antibodies may even enhance invasion, prevent the binding of inhibitory antibodies, or have no effect at all [76,77].

Variant-specific immunity

A special role for acquired immunity has been ascribed to antibody responses to PfEMP1 and other variant surface antigens (VSA) expressed at knob-structures on the membrane of infected erythrocytes [78]. PfEMP1 mediates the adhesion of infected erythrocytes to a range of receptors in the host vasculature and thereby prevents clearance of infected cells in the spleen [79,80]. The *P. falciparum* genome contains approximately 60 *var* genes, each encoding a distinct PfEMP1 variant with particular antigenic and adhesive properties [81]. Individual infected erythrocytes only express a single variant at a time, but are capable of switching the expressed variant [82–84]. Antibody-mediated agglutination experiments could show that parasites causing clinical disease express VSA to which the patient has no pre-existing antibodies [85–88]. In response to disease, a VSA-specific antibody response is triggered, but offers no protection to other variants [85–88]. Hence, sequential parasite waves observed in malaria patients are understood as changes in the expression of variant antigens. Variant-specific antibodies are induced and control the infection but expression then switches to a different variant not recognized by the pre-existing antibodies [89]. Different VSA mediate adhesion to different host receptors with different efficiency. VSAs mediating sequestration to certain tissues have been associated with specific clinical presentations, like cerebral malaria and pregnancy-associated malaria [90–92].

The fact that the surface proteins are highly polymorphic, that malaria parasites can undergo surface antigen variation, and that triggered antibody responses are more or less variant specific may explain the low onset of protective immunity. Protection requires repeated exposure to a vast range of variants leading to the attainment of a broadly protective antibody repertoire. Naturally acquired immunity among adults is thus assumed to be the cumulative product of exposure to multiple parasite infections over time. However, data from transmigrant studies showed that adults acquire immunity more rapidly than children [93,94]. Within a two-year period migrants acquired protection comparable to age-matched life-long residents. This may indicate that age as an intrinsic factor independent from exposure, may determine development of protective immunity [95].

Malaria vaccine development

An ideal malaria vaccine should be safe, easy to administer, cheap, easy to manufacture, stable, and ought to induce life-long protection. Despite good progress in malaria research, no malaria vaccine has been licensed for use. To date, only three candidate vaccines were able to confer partial protection [96–98]. The best strategy to pursue for the development of an efficacious malaria vaccine is still up for debate. Which parasite stage should be aimed at? Which antigens should be targeted? What kind of immune response should be triggered? What are the long-term effects of vaccine introduction on malaria epidemiology?

Individuals exposed to malaria develop protective immunity, but naturally acquired immunity is non-sterilizing, takes very long to develop, and is short-lived. This implies two intrinsically different approaches for the develop of a malaria vaccine; i) either a vaccine needs to induce the same kind of immune responses responsible for naturally acquired protection but just more rapidly and more long-lasting, or ii) a vaccine needs to evoke a type of immune response that would not normally be induced upon natural infection but is potent in preventing infection or disease. For the first strategy, major obstacles present sequence polymorphisms and antigenic variation of immunodominant antigens. Approaches to solve these problems include the formulation of multivalent vaccines covering various variants and combinations

with potent adjuvants in order to induce stronger and broader responses resulting in heterologous protection [99]. Approaches for the second strategy include the targeting of protective antigens or epitopes that are normally little immunogenic and consequently are more conserved.

Pre-erythrocytic vaccines

As described above, protective immunity to the pre-erythrocytic stages is not acquired by natural exposure [24]. However, sterile immunity to the pre-erythrocytic stage can be induced experimentally. Repeated infection with attenuated sporozoites renders humans completely resistant to challenge infections [100]. The protection was associated with effector memory T cell responses [101]. Additionally, although naturally acquired antibodies specific for sporozoites do not confer protection from re-infection [102], antibodies specific for sporozoite surface proteins have been described to block invasion of hepatocytes *in vitro* [103,104]. Thus, a vaccine would have to target the pre-erythorcytic stages either by inducing antibody responses that block invasion of sporozoites into hepatocytes or effector $CD4^+$ and $CD8^+$ T cells that interfere with development of liver stages. A vaccine that efficiently targets pre-erythrocytic stages may be capable of inducing sterile immunity and would consequently prevent morbidity and mortality associated with blood stage infection as well as transmission.

The most prominent pre-erythrocytic malaria vaccine antigen is CSP, the predominant surface protein of sporozoites [105]. The most advanced malaria vaccine, RTS,S, consists of the hepatitis B surface antigen fused to the central repeat and thrombospondin domain of CSP formulated in different AS adjuvants [106]. Approximately 30-50% of children and infants immunized with RTS,S in clinical phase II trials conducted in endemic countries were protected from clinical malaria [97,107–110]. This vaccine is currently undergoing a phase III trial at 11 sites in seven countries in Africa, involving over 15’000 children (www.clinicaltrials.gov (NCT00866619)). Another pre-erythrocytic vaccine candidate antigen is TRAP (thrombospondin related adhesive protein) found in the micronemes and on the surface of sporozoites. It has been implicated in gliding motility and hepatocyte invasion [111,112]. A TRAP-based vaccine was partially protective in naïve adults

through TRAP-specific IFN-γ producing T cells, but failed to induce protection in children in Africa [113,114]. Also, utilization of whole sporozoites as a vaccine is currently being reconsidered. Production of irradiation-attenuated sporozoite compliant with regulatory requirements for clinical use was achieved [115], but clinical trials using sporozoites given intradermally or subcutaneously showed only limited protection.

Asexual blood stage vaccines

Vaccines targeting malaria blood stages aim for elimination or at least reduction of parasite load in the blood and consequently associated morbidity and mortality. One rationale behind blood-stage vaccines is the fact that naturally acquired immunity is mainly blood stage specific. Blood stage vaccine candidate antigens comprise proteins expressed on the surface of merozoites, like merozoite surface protein 1 (MSP1) [116], MSP2 [117], MSP3 [118–120] and glutamate-rich protein (GLURP) [121,122], or proteins released from secretory organelles upon invasion, like membrane antigen 1 (AMA1) [123] and erythrocyte-binding antigen-175 (EBA-175) [124]. Naturally acquired immune responses to all these proteins have been variably associated with protection from symptomatic disease [125]. Furthermore, for all antigens vaccine-induced antibody responses were shown to inhibit parasite growth *in vitro*. Inhibition by MSP1, AMA1 and EBA-175 antibodies is attributed to blockage of merozoite invasion [126–130]. In contrast, MSP3 and GLURP specific inhibition is dependent on monocytes and affects parasite replication [118,121,131]. Vaccines based on all these antigens, except MSP2, were reported to induce protection from subsequent challenge in monkeys [132–136]. Parasite proteins transported to the surface of infected erythrocytes, like PfEMP1, are well-described targets of protective immune responses [137]. But due to their high degree of antigenic diversity and their capacity for clonal antigenic variation, development of PfEMP1 as vaccine is a challenging task. The identification of conserved epitopes capable of inducing antibodies preventing sequestration of a wide range of variants may pave the way for a PfEMP1-based vaccine [138,139]. Furthermore, certain PfEMP1 variants are associated with specific clinical presentations [90–92]. For example, conserved epitopes of PfEMP1 encoded by *var2csa* are currently developed as

pregnancy associated malaria vaccine [140]. Var2csa is expressed by *P. falciparum*-infected erythrocytes and mediates adhesion to the placental lining during pregnancy and is responsible for pregnancy-associated malaria that can result in the mother's death, low birth weight of the infant, or death of the fetus or newborn [12,91].

Only a handful of blood-stage vaccines have been tested in phase II efficacy trials in humans. "Combination B" vaccine, based on recombinant MSP1, MSP2 and RESA (ring-infected erythrocyte surface antigen) with montanide ISA720, offered no protection to blood stage challenge in malaria naïve adults [141]. However, this vaccine reduced parasite density and reduced infection rates with MSP2 allelic variant included in the vaccine but not with the alternate allelic variant [96]. MSP1-42 in AS02A elicited high antigen-specific antibody concentrations but no protection in young children in Western Kenya [142]. A vaccine based on recombinant AMA1 formulated with Alhydrogel failed to induce protective efficacy in young children in Mali [143]. The same vaccine formulated with alhydrogel and CPG7907 also showed no clinically relevant vaccine effect in blood stage challenge of malaria naïve adults, but a significant correlation between parasite multiplication rate and both vaccine-induced growth-inhibitory activity and AMA1 antibody titres in the vaccine group was observed [144]. The vaccine FMP2.1, based on recombinant AMA1 administered in adjuvant system AS01B or AS02A, showed no protective effect in sporozoite challenge experiments in naïve adults, but data suggest reduced parasite growth rates [145]. Recently, it was reported that virosome formulated malaria peptidomimetics derived from AMA-1 and CSP reduced the rate of clinical malaria episodes in children vaccinees by 50% [98]. However, the relative contribution of AMA1 to the protective effect remains unclear.

Anti-toxin vaccine

A completely different approach is the development of vaccines not directed against the parasite, but aiming for the neutralization of parasite-derived molecules that are responsible for disease. One target of this category is glycosylphosphatidylinositol (GPI), a component of the parasite membrane, which is reported to have toxin-like effects and to induce pro-inflammatory responses and clinical symptoms [146].

Immunization of mice with synthetic GPI glycan reduced clinical symptoms but had no effect on parasite replication [147].

Transmission blocking vaccines

The principal of transmission-blocking vaccines is the induction of antibodies that, if ingested as part of the blood meal, would target antigens on gametes, zygotes or ookinetes and prevent parasite development in the mosquito midgut [148]. Such a vaccine would confer no protection to the vaccinated individual unless used in the entire population and thereby reducing transmission rates. Transmission-blocking vaccines are regarded as an important tool for the recently revived ambition for malaria elimination [149]. The basic concept of transmission-blocking vaccines derives from experimental data showing that naturally acquired as well as vaccine induced antibodies specific for surface proteins of sexual stages block development of mosquito stages [150–152]. These antibodies block parasite maturation either by interfering with fertilization or by inducing antibody-dependent complement lysis [152,153]. Sexual stage antigens considered for transmission-blocking vaccines include Pfs25, Pfs48/45, and Pfs230. Antisera of mice immunized with all these antigens conferred high reduction in the average oocyst numbers per feeding mosquito [154–157].

Transmission-blocking as well as pre-erythrocytic vaccines are in dispute as they reduce exposure to blood stage parasites and consequently naturally acquired clinical immunity might be lost or its onset might be delayed in time. Depending on the prevailing transmission intensity, the introduction of a vaccine that does not provide complete protection could potentially worsen the overall burden of disease. A reduction in attack rates reduces malaria-associated morbidity and mortality [158–160], but if exposure drops below the threshold required to acquire sustained clinical immunity, the vaccine may actually be of detrimental overall outcome [161,162].

Objective

To date, only a handful of proteins are being tested as malaria vaccine candidates. Current candidates represent less than 0.5% of the entire *P. falciparum* genome and their initial identification dates back more than 25 years. Up till now, only 7 distinct antigens have been tested in efficacy trials and only three candidate vaccines were able to induce some protective efficacy so far. Since the fully annotated *P. falciparum* genome has become available in 2002, systematic screening of the more than 5000 hypothetical proteins represents a new opportunity to identify novel malaria vaccine candidates. The use of rational selection criteria and comparative analysis of vaccine candidates will aid in the development of a highly effective malaria vaccine.

In this thesis, we anticipate the rational discovery of novel malaria subunit vaccine candidates. Our strategy is based on the selection of hypothetical parasite proteins that are accessible to the host immune surveillance and their subsequent functional characterization using specific monoclonal antibodies. This includes three main elements:

1. Identification of candidate hypothetical proteins based on the predicted protein localization, expression pattern, homologies and domain predictions.

2. Assessment of expression, localization, processing and protein function of selected candidates and determination of their potential as molecular target for a malaria subunit vaccine by functional assays with specific monoclonal antibodies.

3. Generation of antibodies capable of recognizing the endogenous antigen in its native context is considered crucial for their use in functional *in vitro* and *in vivo* assays; therefore we aimed at developing a novel protein expression and immunisation strategy favouring generation of mAbs binding to the native folded proteins.

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RESULTS PART 1

An efficient system to generate monoclonal antibodies against membrane-associated proteins by immunisation with antigen-expressing mammalian cells

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Abstract

Background

The generation of monoclonal antibodies specific for protein antigens usually depends on purified recombinant protein for both immunisation and hybridoma screening. Purification of recombinant protein in sufficient yield and purity is a tedious undertaking and can be demanding especially in the case of membrane proteins. Furthermore, antibodies generated against a purified recombinant protein are frequently incapable of binding to the endogenous protein in its native context.

Results

We describe a strategy to generate monoclonal antibodies against membrane or membrane-associated proteins that completely bypasses any need for purified recombinant antigen. This approach utilises stably transfected mammalian cells expressing recombinant antigens on their cell surface for immunisation of mice. The transfected cells are also used for measuring seroconversion, hybridoma selection and antibody characterisation. By presenting the antigen in its native conformation for immunisation and hybridoma selection, this procedure promotes the generation of antibodies capable of binding to the endogenous protein. In the present study, we applied this approach successfully for three predicted GPI-anchored proteins of the malaria parasite Plasmodium falciparum.

Conclusions

The described entirely cell-based technology is a fast and efficient approach for obtaining antibodies reactive with endogenous cell-surface proteins in their native conformation.

Background

Since the development of the B-cell hybridoma technology for the generation of monoclonal antibodies (mAbs) in 1975 by Kohler and Milstein [1], mAbs have become molecular tools of great value. Due to their high specificity, mAbs are used throughout biology for the characterisation of protein function and distribution. Besides their usage in research, mAbs are also widely utilised as diagnostic and therapeutic agents [2][3]. Due to this wide range of applications the generation of mAbs became a standard procedure. However the generation of mAbs against protein antigens can still be problematic, since for studies in physiological settings, it is important that the mAbs recognise the target protein in its native conformation.

Frequently, mAbs are raised against synthetic peptides derived from the predicted sequence of the target protein. Unfortunately, these Abs, though strongly reactive with peptide, frequently fail to recognise the native protein [4]. Another standard procedure to generate mAbs uses purified recombinantly expressed proteins. Prokaryotic expression systems are the most widely used expression hosts. But when studying mammalian surface proteins it is often necessary to use mammalian expression systems, as they are more likely to produce functional proteins with the appropriate disulfide-bonds and posttranslational modifications [5][6]. Although introduction of affinity tags simplifies purification, it often remains difficult to obtain recombinant protein in native conformation and in sufficient yield and purity. This applies most notably to membrane and membrane-associated proteins, as they are likely to lose their native structure during the purification processes [7].

When attempting to generate mAbs capable of recognising the native protein, it is also critical to use the target protein in its native conformation not only in the immunisation step but also for the screening procedure. Many standard hybridoma-screening protocols make use of recombinant proteins immobilized on solid supports, which may significantly alter protein conformation [8].

With the objective of generating mAbs specifically recognising membrane-associated proteins in their native conformation, we applied a methodology that bypasses any

need for purified recombinant protein. This strategy utilises antigens expressed on the surface of stably transfected mammalian cells both for immunisation of mice and for immunoassays, such as testing seroconversion, hybridoma selection and mAb characterisation.

In the present study, we applied this approach for three predicted GPI-anchored proteins of *Plasmodium falciparum*. *P. falciparum* is the causative agent of malaria tropica, which claims 300-600 million clinical cases and more than 2 million deaths each year [9]. Malaria is transmitted to humans by the bite of an infected female *Anopheles* mosquito. The inoculated sporozoites infect hepatocytes where the parasites undergo schizogony resulting in the rupture of the infected liver cell and release of free merozoites, which infect erythrocytes. Upon intra-erythrocytic schizogony red blood cells rupture and release more merozoites. Some of these differentiate into gametocytes, which, when taken up by a feeding mosquito bring about the sexual cycle, resulting in the development of sporozoites located in the salivary gland of the mosquito. Highly specific cell-cell interactions between the invasive forms of the parasite and the corresponding host cells are pivotal steps in the complex life cycle of *P. falciparum,* which depend on specific molecular interactions of cell surface molecules. Being exposed to potentially parasite inhibitory antibodies makes parasite proteins involved in cell-cell interactions of particular interest with respect to vaccine development. Most proteins that coat the surface of the extracellular forms of *P. falciparum* are known or presumed to be GPI anchored [10]. We anticipated the generation of mAbs against hypothetical proteins of *P. falciparum* predicted to be a GPI-anchored membrane protein in order to get a tool to characterize their properties and potential as vaccine candidate antigens. Based on being predicted to be expressed in invasive stages and to contain a GPI-anchor motif, three hypothetical proteins were selected: *PFF0620c, PFD1130w* and *PF14_0325.* For *PFD1130w* and *PF14_0325* published transcriptional analysis data showed elevated expression levels in late stages of the asexual blood stage cycle [11][12]. For both predicted proteins no function can currently be assigned as no significant homology to functional domains of other characterized gene products could be identified. *PFF0620c* contains two S48-45 sexual stage antigen-like domains,

which are characterized by the presence of 6 cysteines. Other members of this protein family have been shown to be located on the surface of the parasite and some of them are known to play a role in cell-cell interactions [13]. Mass spectrometry data indicate expression of *PFF0620c* at the sporozoite stage [14].

To achieve surface localisation of the recombinant *P. falciparum* proteins on mammalian cells despite potential differences of the secretory machinery between *Plasmodium* and host cells, the *P. falciparum* coding sequences were altered in several ways. The endogenous secretion signal sequences and GPI-attachment sites were replaced with the signal peptide of bee venom melittin and the transmembrane domain of mouse glycophorine A, respectively. To allow assessment of surface localization, antibody-tags were introduced on both sides of the transmembrane domain.

We expect that this entirely cell-based system, capable of generating mAbs against membrane-associated antigens, is applicable for a wide range of cell-surface proteins.

Results

Expression of predicted GPI-anchored *P. falciparum* proteins on the cell surface of mammalian cells

The three predicted open reading frames (ORFs) PFF0620c, PFD1130w and PF14_0325 of *P. falciparum* were expressed on the cell surface of HEK cells using the expression plasmids pcDNA3.1_BVM_PFF0620c_FLAG_GLP_His, pcDNA3.1_BVM_PFD1130W_FLAG_GLP_His and pcDNA3.1_BVM_PF14_0325_FLAG_GLP_His, respectively (Figure 1). To ensure expression on the cell surface, the predicted ORFs were modified in several ways: i. the endogenous *P. falciparum* sequences were codon-optimised for expression in mammalian cells; ii. the endogenous secretion signal sequences were replaced by the secretion signal sequence of bee-venom melittin; iii. for membrane anchoring the transmembrane domain encoding sequence of mouse glycophorin was used instead of the predicted GPI-attachment signal sequences; iv. to allow expression analysis, a FLAG tag was inserted N-terminally of the transmembrane domain and a hexa-His tag was placed at the C-terminus. The two tags were positioned just before and after the transmembrane domain to facilitate verification of the extracellular localisation of the recombinantly expressed malaria antigens (Figure 2 and additional files 1 and 2).

HEK-derived cell lines expressing *P. falciparum* PFF0620c, PFD1130W and PF14_0325 were established by stable transfection. Expression of recombinant proteins was confirmed by Western blot analysis using anti-His tag and anti-FLAG tag antibodies. While protein bands appeared when lysates of the three stably transfected cell lines were analysed, no staining was obtained with non-transfected HEK cells (data not shown). Also in immune fluorescence analysis (IFA) transfectants showed strongly increased staining both with anti-His tag and with anti-FLAG tag antibodies (data not shown).

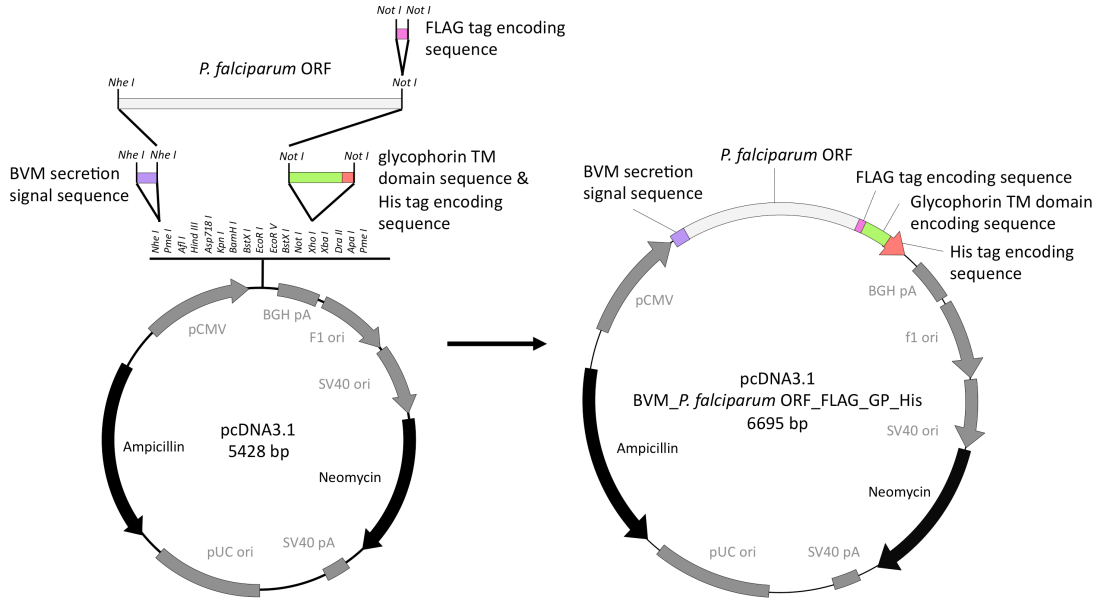

Figure 1. Construction of expression plasmids. Schematic diagram of pcDNA3.1 BVM_PFF0620C_FLAG_GP_His, pcDNA3.1 BVM_PFD1130W_FLAG_GP_His and pcDNA3.1 BVM_PF14_0325_FLAG_GP_His (pcDNA3.1 BVM_*P. falciparum* ORF_FLAG_GP_His). The commercially available pcDNA3.1 expression plasmid (Invitrogen) was modified to contain chimeric genes consisting of the secretion signal sequence of bee venom melittin, the codon-optimised sequence encoding the *P. falciparum* proteins PFF0620C, PFD1130W or PF14_0325 without secretion signal sequence and GPI-attachment signal sequence, a FLAG tag encoding sequence, a sequence encoding the transmembrane domain of mouse glycophorin-A and a His tag encoding sequence.

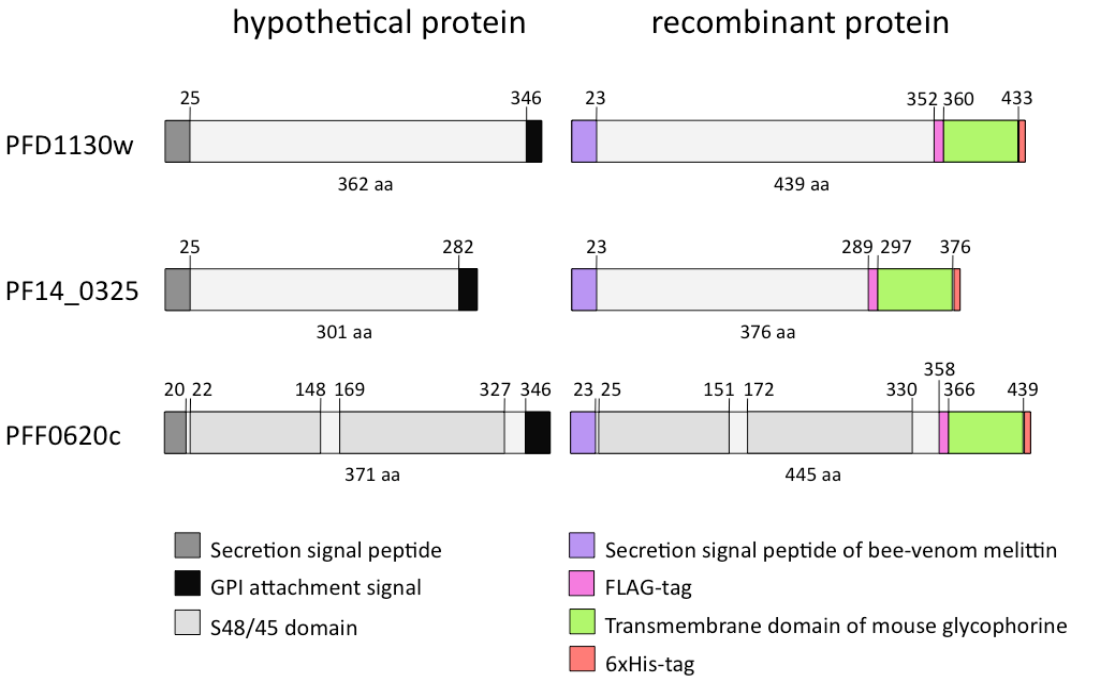

Figure 2. Schematic representation of the modified hypothetical *P. falciparum* proteins PFF0620C, PFD1130W or PF14_0325. The hypothetical *P. falciparum* proteins PFF0620C, PFD1130W or PF14_0325 (left), and their modification for ectopic expression (right) are schematically depicted. The diagram shows the location of the secretion signal peptide, the GPI-attachment signal sequence, PFAM predicted domains, FLAG tag, hexa-His tag and transmembrane domains. Respective amino acid positions of domains are indicated

To obtain highly expressing cell lines, transfectants were separated into high-expressing cell-pools by fluorescent-activated-cell-sorting after surface staining with anti-FLAG tag antibodies. After several days in culture the mean fluorescence intensities of the high-expressing cell pools of the transfectants PFF0620c-HEK, PFD1130W-HEK and PF14_0325-HEK, were ten, seven and three times higher compared to non-transfected HEK cells (Figure 3B).

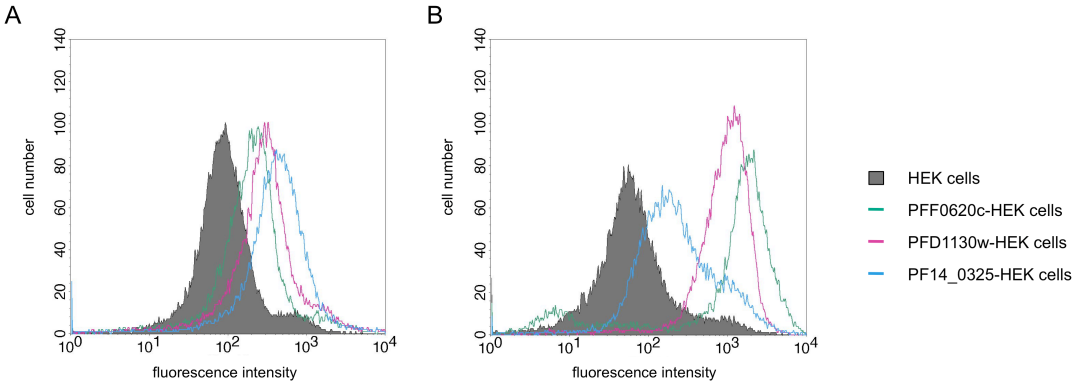

Figure 3. Detection of the *P. falciparum* proteins PFF0620C, PFD1130W or PF14_0325 displayed on stably transfected HEK cells. Histogram plots show flow-cytometric analysis of non-transfected and high-expressing cell pools of transfected HEK cells (PFF0620C-HEK, PFD1130W-HEK, PF14_0325-HEK) stained with anti-His tag antibody (A) or anti-FLAG tag antibody (B).

Live-cell staining of PFF0620c-HEK, PFD1130W-HEK or PF14_0325-HEK cells with anti-FLAG tag antibody yielded strong signals. In contrast, staining with anti-His tag antibody gave strong signals on methanol fixed cells but not on living cells (Figure 4). From these results we deduced that PFF0620c, PFD1130W and PF14_0325 were expressed and anchored in the cell membrane with the FLAG tag located extracellularly and the His tag intracellularly.

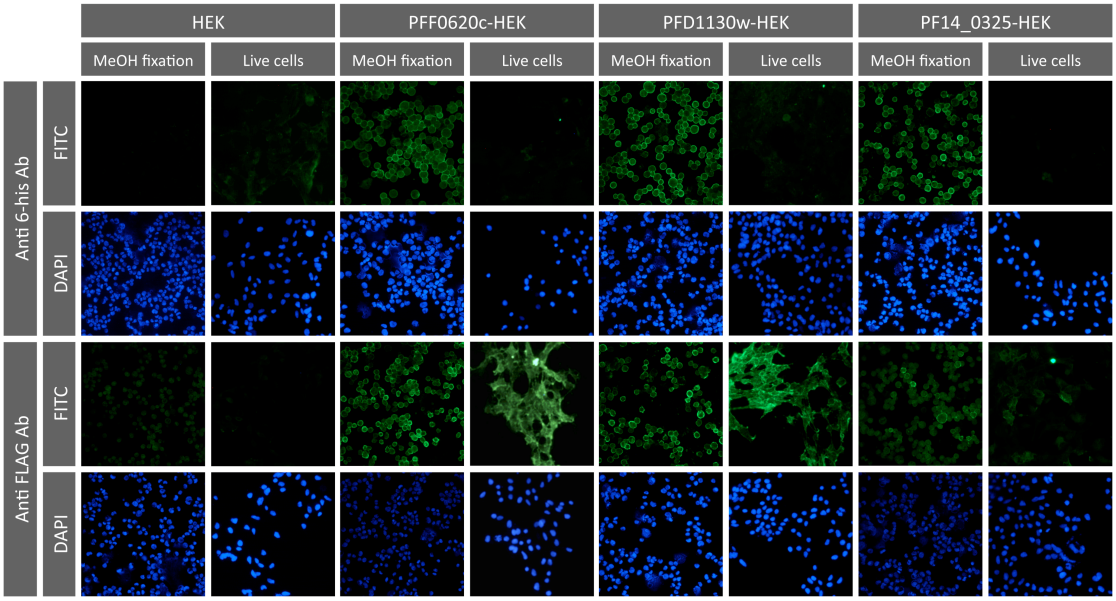

Figure 4. Cell-surface expression of PFF0620C, PFD1130W or PF14_0325 on stably transfected HEK cells. Fluorescence staining of living or methanol fixed untransfected HEK cells, PFF0620C-HEK cells, PFD1130w-HEK cells and PF14_0325 HEK cells after staining with anti-His tag or anti-FLAG tag antibodies and FITC-labelled anti-mouse IgG antibodies. Nuclei were stained with DAPI.
With the anti-FLAG tag antibody both living and methanol-fixed transfectants were stained, whereas the anti-His tag antibody only stained methanol-fixed transfectants, indicating intracellular localisation of the His tag and extracellular localisation of the FLAG tag together with the P. falciparum derived protein domain.

Development of malaria antigen specific antibodies in mice immunised with transfected HEK cells

The high-expressing cell pools PFF0620c-HEK, PFD1130W-HEK and PF14_0325-HEK were used to immunise NMRI mice. Mice received intravenous injections of 10^6 cells on three consecutive days and another suite of three daily injections two weeks later. Development of serum antibody titres was analysed by flow cytometry comparing immune-staining of the corresponding transfectants with that of non-transfected HEK cells (Figure 5). The fluorescence intensity observed with transfectants was two- to four-fold higher than that of non-transfected control HEK cells. This indicated that the mice had mounted an antibody response against the recombinant malaria antigens expressed on the surface of the transfected HEK cells.

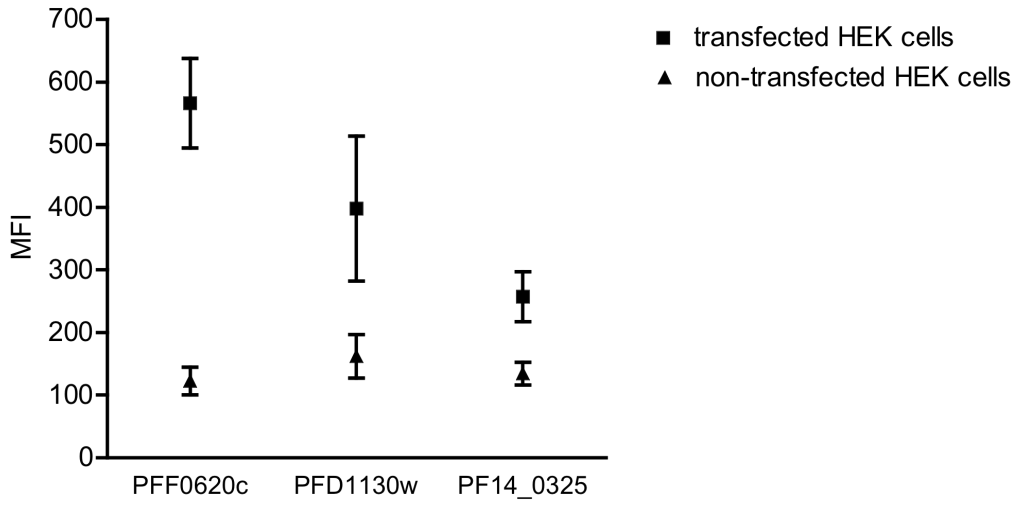

Figure 5. Staining of transfected HEK cells by serum antibodies of immunised mice. The graph shows mean fluorescence intensities (MFI) obtained by flow-cytometric analysis of transfected and non-transfected HEK cells after staining with serum of mice (dilution 1:600) immunised with the corresponding cell line (PFF0620C-HEK, PFD1130W-HEK & PF14_0325-HEK). Values are means of two mouse sera and error-bars indicate the range.

Spleen cells of mice immunised with the transfected HEK cells were fused with PAI myeloma cells to generate B cell hybridomas. Fused cells were distributed in 1152 microtitre culture plate wells. To identify hybridoma cells that produce malaria antigen specific antibodies a two-step screening procedure was used that completely obviated the requirement for purified recombinant proteins. First all culture wells were tested for IgG production by ELISA. Between 18 and 29% of the tested wells were IgG positive (Table 1). In a second step all wells positive for IgG production were screened by IFA for antibodies binding to transfected cells. Transfected and non-transfected HEK cells spotted onto multiwell glass slides were stained with individual hybridoma supernatants and analysed by fluorescence microscopy (Figure 6). Non-transfected HEK cells served as a negative control for each sample. In all three fusions numerous culture supernatants positive on the transfected cells were also positive on non-transfected cells. However, each fusion yielded also numerous wells containing antibodies strongly reactive with transfectants but not reactive with untransfected HEK cells. Among these, eight wells were identified that contained antibodies reactive with an epitope present on all three transfectants but not on untransfected HEK cells. Most likely, these antibodies are specific for the FLAG tag or

the membrane anchor domain. All other culture supernatants reactive with transfectants, but not with untransfected HEK cells were specific for the transfected cells used for immunisation and did not stain the two other transfectants (Table 1). From wells of this category, 17, nine and two hybridoma clones (Table 2) were derived by recloning from the PFF0620c, PFD1130W and PF14_0325-fusion, respectively.

Table 1. Specificity of hybridoma culture supernatants

Antigen	IgG ELISA screen[a]			IFA screen on transfected and non-transfected HEK cells[b]									
	total[c]	IgG positive[d]		total[e]		negative[f]		Pf protein specific[g]		HEK cell specific[h]		anchor specific[i]	
PFF0620c	1152	335	29%	142	42%	86	60%	25	18%	30	21%	1	0.7%
PFD1130w	1152	294	26%	294	100%	200	68%	10	3%	83	28%	1	0.3%
PF14_0325	1152	202	18%	202	100%	125	62%	2	1%	68	34%	7	3.0%

[a] For each fusion hybridoma supernatants of 12 96-well-plates were analysed for the presence of mouse IgG by sandwich-ELISA
[b] Wells screened positive by IgG ELISA were tested for binding to the corresponding transfected and the non-transfected HEK cells by IFA
[c] Number of wells analysed by ELISA
[d] Number and percentage of all tested wells that were IgG positive
[e] Number and percentage of IgG positive wells that were analysed by IFA
[f] Number and percentage of all wells tested by IFA that showed no reactivity in IFA
[g] Number and percentage of all wells tested by IFA that only stained the transfectant cell line used for the immunisation of mice
[h] Number and percentage of all wells tested by IFA that stained both transfected and non- transfected HEK cells by IFA
[i] Number and percentage of all wells tested by IFA that stained all three transfectants, but were negative for non-transfected HEK cells

The specificity of the monoclonal antibodies was further confirmed by Western blot analysis (Figure 7). With the exception of one anti-PFF0620c mAb, all 28 mAbs stained the corresponding recombinant protein in the lysate of the transfectant used for immunisation, but not in lysates of the other transfectants or of untransfected HEK cells (Table 2). Interestingly, in Western blot analysis anti-PFF0620c mAbs only bound to the non-reduced form of the recombinant protein. In contrast, anti-PFD1130w and anti-PF14_0325 mAbs bound both to the reduced and non-reduced recombinant proteins (data not shown).

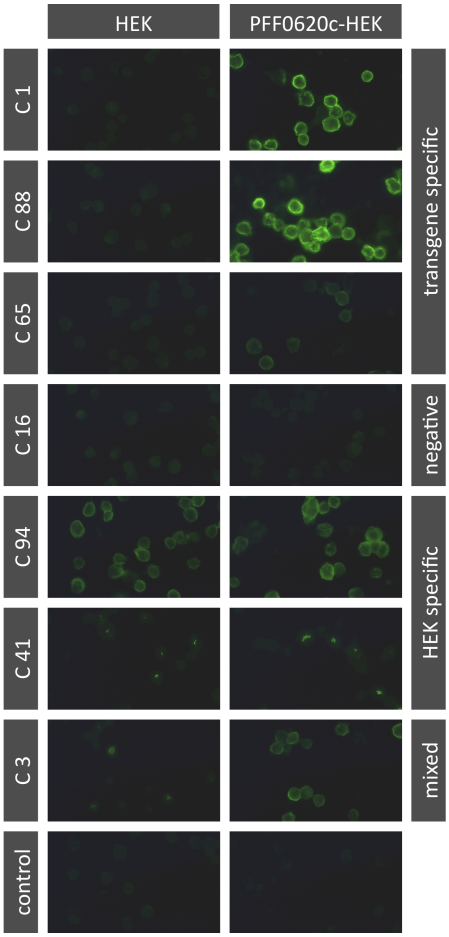

Figure 6. Immunofluorescence microscopic screening of hybridoma cell culture supernatants for antibodies binding to cell-surface expressed PFF0620C. PFF0620C-HEK cells (right column) and untransfected HEK cells (left column) were fixed with methanol and stained with hybridoma supernatants of individual cell culture wells taken 15 days after the fusion. A FITC-labelled anti-mouse IgG antibody served as secondary antibody. Representative fluorescence micrographs from the PFF0620C-fusion are shown, demonstrating typical results for hybidoma lines producing PFF0620C-specific (line 1, 2 & 3), non-binding (line 4), or HEK cell antigen specific (line 5 & 6) antibodies. For some wells mixed specificity was seen, indicating presence of two ore more cell clones with one producing Abs specific for the transgene and the other producing Abs specific for a HEK-cell protein (line 7).

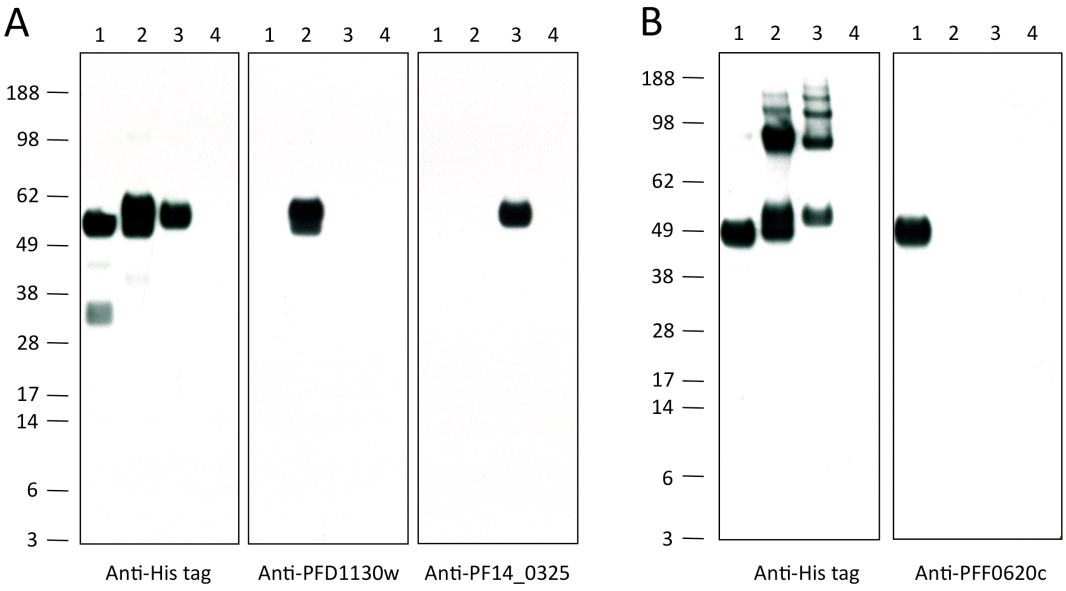

Figure 7. Western blot analysis of generated monoclonal antibodies with the recombinant *P. falciparum* proteins. Specificity of representative monoclonal antibodies for the corresponding recombinant proteins is demonstrated by Western-blot analysis. Reduced (A) and non-reduced (B) protein lysates of PFF0620c- (line 1), PFD1130w- (line 2), PF14_0325-expressing HEK cells (line 3) and non-transfected HEK cells (line 4) were probed with anti-His tag mAb, anti-PFD1130w 02 mAb, anti-PF14_0325 04 mAb and anti-PFF0620c 07 mAb, respectively.

Reactivity of the generated monoclonal antibodies with the endogenous target antigens.

To determine whether the generated mAbs selected against ectopically expressed PFF0620c, PFD1130W or PF14_0325 would bind to the endogenous proteins of P. falciparum parasites, we performed IFA and Western-blot analysis using cultivated blood-stage parasites and mosquito salivary gland-derived sporozoites (Figure 8 & Table 2). All nine generated anti-PFD1130W mAbs recognised a discrete band of about 40 kDa, corresponding to the predicted molecular weight of the PFD1130W protein. Furthermore all nine mAbs stained blood-stage parasites in IFA, resulting in a distinctive staining pattern (Figure 8 & Table 2). Both anti-PF14_0325 mAbs were Western-blot positive on blood stage parasite lysate and stained specifically late blood stage parasites in IFA (Figure 8 & Table 2). Thirteen of the seventeen mAbs specific for PFF0620c reacted in IFA with mosquito salivary gland sporozoites, but not with blood stage parasites in IFA (Figure 8 & Table 2). No reactivity was seen in Western blot with sporozoite lysate.

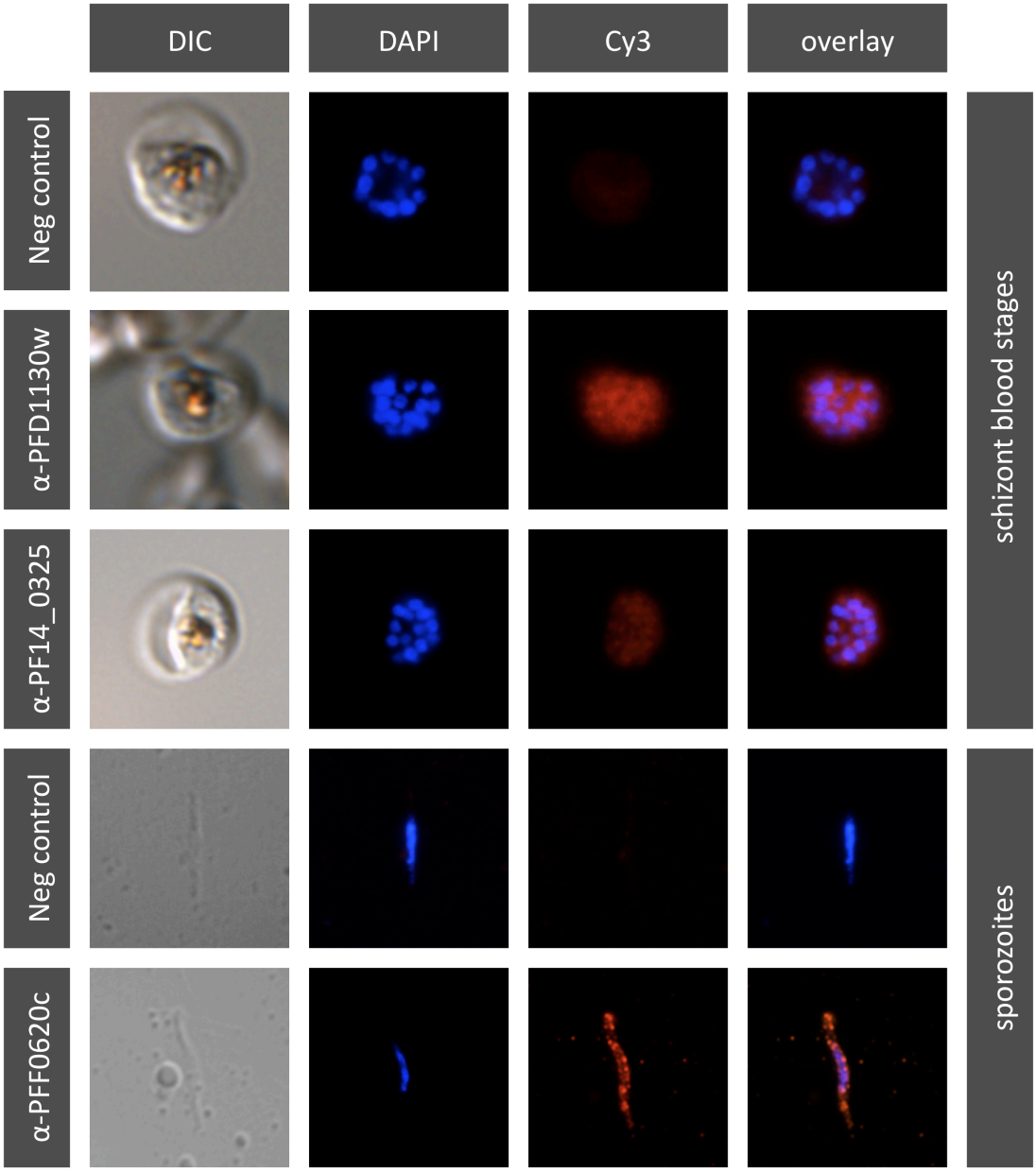

Figure 8. Reactivity of generated monoclonal antibodies with the endogenous *P. falciparum* proteins. Fluorescence staining (column 2, 3 & 4) and DIC micrographs (column 1) of *P. falciparum* parasites. While anti-PFF0620C mAb specifically stained salivary gland sporozoites (line 5), anti-PF14_0325 mAb (line 3) and anti-PFD1130W mAb (line 2) specifically reacted with schizont blood stage parasites. Staining with only the Cy3-labelled rabbit anti-mouse IgG secondary antibody served as negative control (line 1 & 4). Parasite nuclei were stained with DAPI.

Table 2. Reactivity patterns of all generated mAbs with HEK cells and *P. falciparum* parasites

Hybridoma Clone	Isotype	Western blotting analysis				IFA			
		HEK	PFF0620c HEK	blood stages	sporozoites	HEK	PFF0620c HEK	blood stages	sporozoites
PFF0620c 01	IgG2a	−	+	−	−	−	+	−	+
PFF0620c 02	IgG2a	−	+	−	−	−	+	−	−
PFF0620c 06	IgG1	−	+	−	−	−	+	−	+
PFF0620c 07	IgG2a	−	+	−	−	−	+	−	+
PFF0620c 08	IgG1	−	+	−	−	−	+	−	−
PFF0620c 09	IgG1	−	+	−	−	−	+	−	+
PFF0620c 10	IgG1	−	+	−	−	−	+	−	+
PFF0620c 11	IgG1	−	+	−	−	−	+	−	+
PFF0620c 13	IgG2a	−	+	−	−	−	+	−	+
PFF0620c 17	IgG2b	−	+	−	−	−	+	−	+
PFF0620c 19	IgG1	−	+	−	−	−	+	−	−
PFF0620c 21	IgG2b	−	+	−	−	−	+	−	+
PFF0620c 22	IgG1	−	+	−	−	−	+	−	+
PFF0620c 25	IgG1	−	+	−	−	−	+	−	+
PFF0620c 27	IgG2b	−	+	−	−	−	+	−	−
PFF0620c 29	IgG1	−	+	−	−	−	+	−	+
PFF0620c 32	IgG1	−	−	−	−	−	+	−	+
		0/17	16/17	0/17	0/17	0/17	17/17	0/17	13/17

Hybridoma Clone	Isotype	HEK	PFD1130w HEK	blood stages	sporozoites	HEK	PFD1130w HEK	blood stages	sporozoites
PFD1130w 02	IgG2b	−	+	+	−	−	+	+	−
PFD1130w 04	IgG3	−	+	+	−	−	+	+	−
PFD1130w 05	IgG2a	−	+	+	−	−	+	+	−
PFD1130w 06	IgG2a	−	+	+	−	−	+	+	−
PFD1130w 08	IgG1	−	+	+	−	−	+	+	−
PFD1130w 09	IgG3	−	+	+	−	−	+	+	−
PFD1130w 10	IgG1	−	+	+	−	−	+	+	−
PFD1130w 12	IgG2a	−	+	+	−	−	+	+	−
PFD1130w 13	IgG1/ IgG2b	−	+	+	−	−	+	+	−
		0/9	9/9	9/9	0/9	0/9	9/9	9/9	0/9

Hybridoma Clone	Isotype	HEK	PF14_0325 HEK	blood stages	sporozoites	HEK	PF14_0325 HEK	blood stages	sporozoites
PF14_0325 02	IgG1	−	+	+	nd	−	+	+	nd
PF14_0325 04	IgG1	−	+	+	nd	−	+	+	nd
		0/2	2/2	2/2		0/2	2/2	2/2	

nd = no data

Discussion

Membrane proteins, including membrane associated proteins, are the gatekeepers of the cell and selectively mediate the flow of information and nutrients between the outside and inside of the cell. Functions of membrane proteins include: transport of substances across membranes, enzymatic activity, signal transduction, intercellular joining, cell-cell recognition, attachment to the cytoskeleton and extracellular matrix. Even though only 25% of all proteins are membrane proteins, about 60% of today's approved drug targets are membrane proteins [15]. Also for subunit vaccine design cell surface associated proteins, which can be accessed by antibodies, are of prime interest. Nonetheless the basic knowledge about these proteins lags far behind that of soluble proteins. This is due to difficulties in ectopic expression, purification and protein stability. Therefore investigation of membrane proteins is a demanding undertaking and generation of mAbs against membrane proteins represents a difficult task.

Here we described a procedure to generate mAbs specific for cell-surface associated proteins, which completely bypasses any need for purified antigen. This is highly desirable as it supersedes the laborious task of antigen purification and ensures presentation of target proteins in their native conformation. In a study using antigen expressing transfectant cells to generate single chain antibodies it was shown that cell-based selection was far more efficient in generating Abs cross-reactive with the native endogenous protein than selection with purified recombinant protein [16].

The principal steps of the applied procedure are the following: (I) generation of stably transfected mammalian cells, expressing high levels of the target antigen on their surface in a native conformation; (II) immunisation of mice with transfected cells; (III) hybridoma cell generation; (IV) selection of hybridoma cells by a two step screening procedure involving an anti-IgG ELISA and an IFA with transfected and non-transfected cells. Only one single transfected cell line is required for the whole process including immunisation, serum analysis, screening of hybridoma cell lines

and antibody characterisation. Furthermore the very same cell line may also be utilised for the study of receptor-ligand interactions.

In contrast to other entirely cell-based strategies to generate monoclonal antibodies [17][18][16] our procedure used a dual tagging strategy to be able to assess surface expression of the recombinant protein. In the case of all three transfectants, the His tag was only accessible upon fixation with methanol in IFA, whereas the FLAG tag was accessible on live cells. From these results we deduced that all three cell lines presented recombinant protein on their cell surface. Flow cytometric analysis showed that the staining intensity with the anti-FLAG tag and anti-His tag Ab was varying between the three different transfectants. The finding that transfectants showing the lowest staining with the anti-FLAG tag Ab showed the strongest staining with the anti-His tag Ab could indicate that the percentage of correctly orientated protein molecules is varying between the different transfectants. However, differences in accessibility of the two tags in the context of the different core proteins could also be the reason for these differences. Especially accessibility of the FLAG tag, which is positioned between the transmembrane domain and the parasite-derived part of the protein, could depend on the structural features of the parasite protein sequences.

In order to generate recombinant proteins with natural conformation, it is most favourable to use expression hosts of the same biological kingdom, since they likely produce the same post-translational modifications and recognise the same signals for synthesis, processing, and secretion as the organism, which the sequence was originally derived from. As we were dealing with Plasmodium proteins, this objective could not be fulfilled, because currently no Apicomplexa-based expression system is described. Anticipating surface expression and natural conformation, the protein encoding sequence was genetically modified in several ways. Firstly codon-usage was optimised for mammalian cells, as the plasmodium genome is extremely A+T-rich. The overall (A + T) composition is 80.6%, and rises to 90% in introns and intergenic regions [19]. Furthermore the endogenous GPI-attachment signal sequence was replaced by a transmembrane alpha helix of mouse glycophorin-A to ensure C-terminal membrane anchoring despite potential phylogenetic differences

in the GPI-attachment machinery [20][21]. Whether recombinantly expressed proteins will contain the same posttranslational modifications as the endogenous protein was uncertain. Plasmodium species have undergone a process of gene loss that has removed much of their capacity to produce glycosylated proteins other than GPI-anchored proteins [22]. Therefore recombinant expression of P. falciparum proteins in mammalian cells takes a risk of generating glycosylated forms of the protein, which are not found in the parasite. Generated mAbs might therefore not be capable of recognising the unglycosylated endogenous protein. However, in the study presented here 24 out of 28 generated mAbs were capable of recognising the endogenous protein in IFA. Therefore potential differences in the glycosylation status of recombinant and endogenous protein were inconsequential for antigen-antibody interactions. Beside protein glycosylation, disulfide bonds play an important role in the folding and stability of proteins, especially for proteins secreted to the extracellular medium. PFF0620c is predicted to contain two s48/45 domains. These domains are known to contain six position-conserved cysteine residues, resulting in a conserved set of disulfide bonds [23][24]. Generated anti-PFF0620c mAbs only reacted with recombinant PFF0620c when analysed under non-reducing conditions, indicating that PFF0620c expressed in HEK cells contained disulfide bridges. As 13 out of 17 mAbs recognised endogenous PFF0620c in IFA, disulfide bridge formation in HEK cells is likely to have resulted in endogenous protein-like structures.

To our knowledge, the present study shows for the first time that antigen-expressing tranfectants of xenogenic origin can well be used for immunisation of mice to efficiently generate antigen-specific mAbs. In the described procedure we used a human derived cell line (HEK) for immunisation of mice. Usage of HEK cells has the advantage that they are easy to grow, transfect very readily and are capable of expressing large amounts of recombinant proteins [25]. In contrast, our attempts to express the three Plasmodium proteins in NIH/3T3 cells using a pcDNA3.1 or a pEF6 expression vector were not successful (data not shown). One might expect that mice immunised with cells of xenogenic origin generate an excess of antibodies against HEK cell derived antigen, thereby repressing antibody generation specific for the

parasite proteins of interest. Indeed we saw strong antibody reactivity against HEK cells. However, this procedure generated also mAbs against the target antigens at high frequencies (Table 1). On the other hand one could speculate that immunisation with xenogenic transfectants possibly results in a stronger activation of the immune system than immunisation with allogenic cells. Interestingly, the percentage of transgene-specific clones generated in the three individual fusions correlated with the malaria antigen expression level of the corresponding transfected cells used for the immunisation of mice. This indicates that high expression levels of the target antigen are needed. It remains to be elucidated in more detail how expression strength and cell line origin affect the efficiency of mAb generation by this procedure. Anyhow, our results indicate that any kind of cell line, which expresses the antigen in decent amounts, can be used for the generation of mAbs by the described procedure.

The two-step screening procedure consisting of an IgG-ELISA followed by IFA of transfected cells was quick, and the readout straightforward. In other studies using antigen-expressing cells for immunisation and hybridoma selection, either hybridoma supernatants have been analysed by flow cytometry or a reporter cell line has been used [16][26]. These screening strategies represent alternatives to the two-step screening procedure described here. This would have the advantage that hybridoma supernatants are screened for reactivity to living transfectants and not against cells that had undergone a fixation processes. However, both for flow cytometric and for reporter cell analysis, antigen-expressing cells need to be available in appropriate quality and number over the extended and not entirely predictable time period when hybridomas are ready for primary and secondary screening. In contrast, the described IFA-screen can be performed with slides prepared prior to the fusion and stored at -80°C.

Conclusions

These results demonstrate that our cell-based system of immunisation and hybridoma selection offers a rapid and efficient mean of obtaining mAbs reactive with the native form of membrane-associated proteins. The strategy may easily be extended to a wide range of cell-surface proteins.

Methods

Bacterial strains and media

E. coli strain Top10 (TOP10 Chemically Competent E. coli Cells, Invitrogen) was used for the amplification of plasmids. Bacteria were grown in LB medium containing 100 µg/ml ampicillin.

Construction of plasmids and transformation

A double-stranded oligonucleotide encoding the secretion signal sequence of bee-venom melittin was ligated to NheI digested pcDNA3.1 (Invitrogen) resulting in plasmid pcDNA3.1_BVM. Mouse glycophorin-A mRNA was obtained from mouse bone marrow cells and cDNA generated by two-step reverse-transcription PCR using primers designed to amplify specifically the cDNA of the transmembrane encoding region of glycophorin-A. This sequence was then ligated to NotI digested pcDNA3.1_BVM resulting in plasmid pcDNA3.1_BVM_GP_His. The plasmid was constructed such that unique NheI and NotI sites were preserved in the multiple cloning site. Then plasmid pUC57 containing codon optimised synthetic genes encoding P. falciparum genes PF14_0325, PFD1130W or PFF0620C respectively that lack sequences for the secretion signal peptide and for the GPI-attachment signal peptide (Genscript), were ligated into NotI/NheI digested pcDNA3.1_BVM_GP_His. Thereafter a double-stranded oligonucleotide encoding a FLAG tag was ligated into the NotI site resulting in expression plasmids pcDNA3.1_BVM_PF14_0325-_FLAG_GP_His, pcDNA3.1_BVM_PFD1130W_FLAG_GP_His and pcDNA3.1_BVM_-PFF0620C_FLAG_GP_His.

Culture of eukaryotic cells

The human embryonic kidney cell line 293 HEK was obtained from the American Type Culture Collection (CRL-1573, ATCC). 293 HEK cells were cultured in DMEM supplemented with 10% foetal calf serum, glutamine and penicillin/streptomycin at 37°C in a humidified incubator.

Establishment of HEK 293 cell lines stably expressing PF14_0325, PFD1130W or PFF0620C

293 HEK cells were transfected with pcDNA3.1_BVM_PF14_0325_FLAG_GP_His, pcDNA3.1_BVM_PFD1130W_FLAG_GP_His and pcDNA3.1_BVM_PFF0620C-_FLAG_GP_His respectively using JetPEITM (PolyPlus) transfection reagent. One day prior to transfection, a total of 5 x 105 293 HEK cells were seeded in 35-mm dishes. Transfection was performed following the manufacturer's protocol. 3 µg of expression plasmid and 6 µl transfection solution was used. Antibiotic selection was started 48 h after transfection. The selection medium containing 500 ug/ml of Geneticin (Gibco) was exchanged every 3-4 days.

Generation of anti-FLAG tag and anti-His tag mAb

The mAbs His-6/9 and FLAG-27 were raised in Naval Medical Research Institute (NMRI) mice injected intraperitoneally with 20 µg of the respective peptides CGGHHHHH and CGGDYKDDDDL conjugated to KLH (Imject® Maleimide Activated mcKLH, Pierce) and emulsified in aluminum hydroxide gel (Alhydrogel-2%, Brenntag Biosector) containing CPG-OGN according to Davis et al 1998 [27]. The animals received up to four booster injections each at 3-week intervals with the same antigen preparation. As soon as the animals showed a specific immune response to the immunogen, the best responders were boosted and after 3 days, the spleens were removed and the isolated cells fused to PAI myeloma cells, a variant of the P3-x63-AG8 myeloma [1][28].

Immunofluorescence staining of methanol-fixed HEK cells

HEK cells were collected using enzyme free dissociation buffer (Cell dissociation buffer enzyme-free Hanks'-based, Gibco), washed with PBS and spotted on multiwall glass slides (multitest slide, 12-well, 7mm, ICN Biomedicals Inc.). When air-dried, cells were fixed in methanol for 10 min. Immunostaining was performed by incubating the wells with 30 µl of an appropriate mAb diluted in PBS or hybridoma culture supernatant for 20 min in a humid chamber at 37°C. After rinsing twice and washing for 15 min in PBS, 30 µl of 125 µg/ml FITC-conjugated goat anti-mouse IgG antibodies (RAM/IgG(H+L)/FITC, Nordic Immunological Laboratories) diluted in PBS

were added to the wells and incubated for 20 min in a humid chamber at 37°C. Finally, slides were rinsed twice and washed for 15 min in PBS, mounted with mounting solution (50% PBS 50% glycerol) and covered with a coverslip. Stainings were assessed by fluorescence microscopy on a Leica CTR500 fluorescence microscope and a Leica DFC300 FX digital camera.

Immunofluorescence staining of living HEK cells

For immunofluorescence staining of live HEK cells chamber slides (4-well chamber-slide, Lab-Tek™, NuncTM) were used. Wells were coated with 100mg/l poly-D-lysine in H2O in a humid box at room temperature over night. After washing the wells three times with sterile H2O, 40'000 cells were seeded per well. Three days later the immunostaining was performed by incubating the wells with 500 µl of an appropriate mAb diluted in serum-free culture medium for 30 min on ice. After washing two times with serum-free culture medium 500 µl of 100 µg/ml FITC-conjugated goat anti-mouse IgG antibodies (RAM/IgG(H+L)/FITC, Nordic Immunological Laboratories) diluted in serum-free culture medium were added to the wells and incubated for 30 min on ice. Finally, the wells were rinsed twice with serum-free culture medium and once with D'PBS (Dulbecco's Phosphate-Buffered Saline containing calcium, Gibco). The slides were mounted with mounting solution containing DAPI (ProLong® Gold antifade reagent with DAPI, Invitrogen) and covered with a coverslip. Stainings were assessed as described above.

Western Blotting analysis

HEK cells were collected using enzyme free dissociation buffer (Cell dissociation buffer enzyme-free Hanks'-based, Gibco), and washed two times with PBS. To prepare cell lysate, 106 cells were lysed with 0.1ml of lysis buffer (1% NP40, 10% glycerol, 2mM EDTA, 137mM NaCl, 20mM TrisHCl, pH8, Protease Inhibitors) for 10 min on ice. The lysate was cleared by centrifugation at 20'000 g for 5 min.

Blood stage parasite lysates were prepared essentially as described previously by saponin lysis of P. falciparum 3D7-infected erythrocytes [29]. In brief, cultured parasites were washed once with PBS. Pelleted infected red blood cells were lysed by mixing with 20 volumes of 0.015% (w/v) saponin in PBS and incubated on ice for

20 min. Finally, the pelleted parasites were resuspended in 3 volumes of PBS and stored at −80 °C until further use.

For SDS-PAGE cell- or parasite lysate was resolved on precast 4-12% gradient gels (NuPAGE® Novex 4-12% Bis-Tris Gel, Invitrogen) with MES running buffer according to the manufacturer's directions. The proteins were electrophoretically transferred to nitrocellulose membrane using a dry-blotting system (iBlot, Invitrogen). After blocking the membrane over night in blocking buffer (5% Milk in PBS) at 4°C, specific proteins were detected with appropriate dilutions of mAbs in blocking buffer for 1 h at room temperature. The membrane was then washed four times for 5 minutes in blocking buffer and incubated with horseradish peroxidase conjugated anti-mouse IgG mAb (GAM/IgG (γ-chain)/HRP) diluted 1:10'000 in blocking buffer at room temperature for 1 h. After washing again, blots were developed using ECL Western blotting detection reagents (ECL Western Blotting Substrate, Pierce) to visualise bands.

FACS and Flow cytometric analysis of HEK cells

For sorting stably transfected cells into high-expressing cell pools, cells were dissociated with enzyme-free dissociation buffer (Cell dissociation buffer enzyme-free Hanks'-based, Gibco), washed with blocking buffer (PBS containing 3% BSA). The cells were then incubated with 200 µl of 100 µg/ml FLAG-27 mAb diluted in blocking buffer for 15 min on ice. The cells were then washed with blocking buffer and incubated with 200 µl of 100 µg/ml FITC-conjugated goat anti-mouse IgG antibodies (RAM/IgG(H+L)/FITC, Nordic Immunological Laboratories) diluted in blocking buffer for 15 min on ice. After a final wash the labelled cells were analysed and sorted using a Becton Dickinson FACSAria running Diva software. All analyses were performed using appropriate scatter gates to exclude cellular debris and aggregates. Gating settings were set to collect highly labelled cells. Post-sorting, the cells were collected in culture medium with 20% FCS and plated in 35 mm wells

For monitoring surface expression of PF14_0325, PFD1130W or PFF0620C on transfected cell lines, cells were stained as described above. FACS analysis was performed on a FACScan (Becton Dickinson) using CellQuest software (Becton

Dickinson). 20'000 thousand events were collected for each sample. Untransfected cells served as negative control. For analysing seroconversion of immunised mice, transfected cell lines were stained using a 1:600 serum dilution in blocking buffer. Cells were staining and analysed as described above.

Immunisation of mice

All procedures involving living animals were performed in accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzverordnung) of the Swiss Bundesamt für Veterinärwesen. Naval Medical Research Institute (NMRI) mice were immunised by intravenous injections of 106 stably transfected HEK cells. Cells were thawed, washed and resuspended in 0.9 % NaCl. Injections were accomplished on three consecutive days and after two weeks again on three consecutive days. Two to three weeks after the boost, blood was collected and the serum was tested for the presence of anti-PF14_0325, anti-PFD1130W or anti-PFF0620C antibodies, respectively by IFA and flow cytometry using stably transfected 293 HEK cells. Mice immunised with PF14_0325-HEK cells were boosted a second time after 4 weeks.

Fusion and cell-based selection

Animals with serum strongly reactive with expressing cells were selected for fusion. These received a final injection of 106 cells two and one day before the fusion. Mice were sacrificed and the spleen was removed. Spleen cells were harvested by trituration under sterile conditions and fused with the myeloma cell partner (PAI mouse myeloma cells, derived from P3-x63-AG8) using polyethylene glycol 1500 (Roche Diagnostics). The fusion mix was plated into multiwell plates and hybridomas were selected by growing in HAT medium supplemented with culture supernatant of mouse macrophages P388. Wells were screened for specific IgG production between 2-3 weeks post-fusion by ELISA and IFA as described below. Cells from wells positive in initial screens were cloned by limiting dilution to obtain monoclonal populations.

IgG ELISA screen

MaxisorpTM plates (Nunc) were coated overnight at 4°C in a humid box with 100 µl of 5 µg/ml goat anti-mouse IgG (γ-chain specific) mAb (Sigma) diluted in PBS. After two washings with PBS containing 0.05% Tween-20, wells were blocked with

blocking buffer (50 mM Tris, 140mM NaCl, 5mM EDTA, 0.05% NONidet P40, 0.25% gelatine, 1% BSA) for 1 h at 37°C and afterwards washed two times. 50 µl hybridoma supernatants were added to the wells and incubated for 1 h at 37°C. After washing 4 times, plates were incubated with 50 µl horseradish peroxidase-conjugated goat anti-mouse IgG (γ-chain specific) (Sigma) diluted 1:1000 in blocking buffer for 1 h at room-temperature in a humid box in the dark. After washing 4 times, TMB peroxidase substrate solution was added and the colour change monitored.

Antibody production and characterisation

Identification of antibody subclasses was performed using a Mouse Monoclonal Antibody Isotyping Kit (ISO2, Sigma). For large-scale mAb production hybridoma cell lines were cultured in 500 ml roller-bottles (Corning). MAbs were purified by affinity chromatography using protein A or protein G Sepharose.

P. falciparum blood stage culture

P. falciparum strain 3d7 was cultured essentially as described previously [29]. The culture medium was supplemented with 0.5% AlbuMAX (Gibco) as a substitute for human serum [30]. Cultures were synchronised by sorbitol treatment [31]. Erythrocytes for passages were obtained from the Swiss Red Cross (Switzerland).

Immunofluorescence staining of *P. falciparum*

Erythrocytes from *in vitro* cultures of *P. falciparum* strain 3d7 were fixed in paraformaldehyde-glutaraldehyde as described previously [3]. Cells were blocked by incubation in 3% BSA in PBS for 1h. Immunostaining was performed by incubation with an appropriate mAb dilution in blocking solution for 1 h. After three washes with PBS, 5 µg/ml cyanine dye (Cy3)-conjugated affinity-pure F(ab')2 fragment goat anti-mouse IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories) diluted in blocking solution was added and incubated for 1 h at RT. Finally cells were washed three times with PBS, mounted with mounting medium containing DAPI (ProLong® Gold antifade reagent with DAPI, Invitrogen) and covered with a coverslip. Antibody stainings were assessed as described above.

For immunfluorescence staining of sporozoites, air-dried unfixed P. falciparum (strain NF54) salivary gland sporozoites attached to microscope glass slides were

incubated with mAbs and detected with cyanine dye (Cy3)-conjugated affinity-pure F(ab')2 fragment goat anti-mouse IgG (Fc-specific) antibodies (Jackson Immuno Research Laboratories) as previously described [32].

List of abbreviations

Ab: antibody; Abs: antibodies; BSA: bovine serum albumin; DAPI: 4'-6-Diamidino-2-phenylindole; DIC: differential interference contrast; ELISA: Enzyme-linked Immunosorbent Assay; FACS: Fluorescence activated cell sorting; FCS: foetal calf serum; GPI: Glycosylphosphatidylinositol; HAT: Hypoxanthine Aminopterin Thymidine; HEK: Human Embryonic kidney cells; IFA: Immuno-fluorescence Assay; IgG: Immunoglobulin G; kDa: kilo Dalton; mAb: monoclonal antibody; mAbs: monoclonal antibodies; MFI: mean fluorescence intensity; PBS: phosphate-buffered saline; RT: room temperature

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AD was responsible for experimental design, performed the experiments and data analysis described in this study and drafted the manuscript. **JB** participated in the study design and designed the expression vectors. **HM** contributed to the conception of the study, participated in its design and assisted in data interpretation. **GP** contributed to the conception of the study, participated in the study design, coordinated the collaborations that made this study possible and revised the manuscript. All authors have read and approved the final manuscript.

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Supporting Information

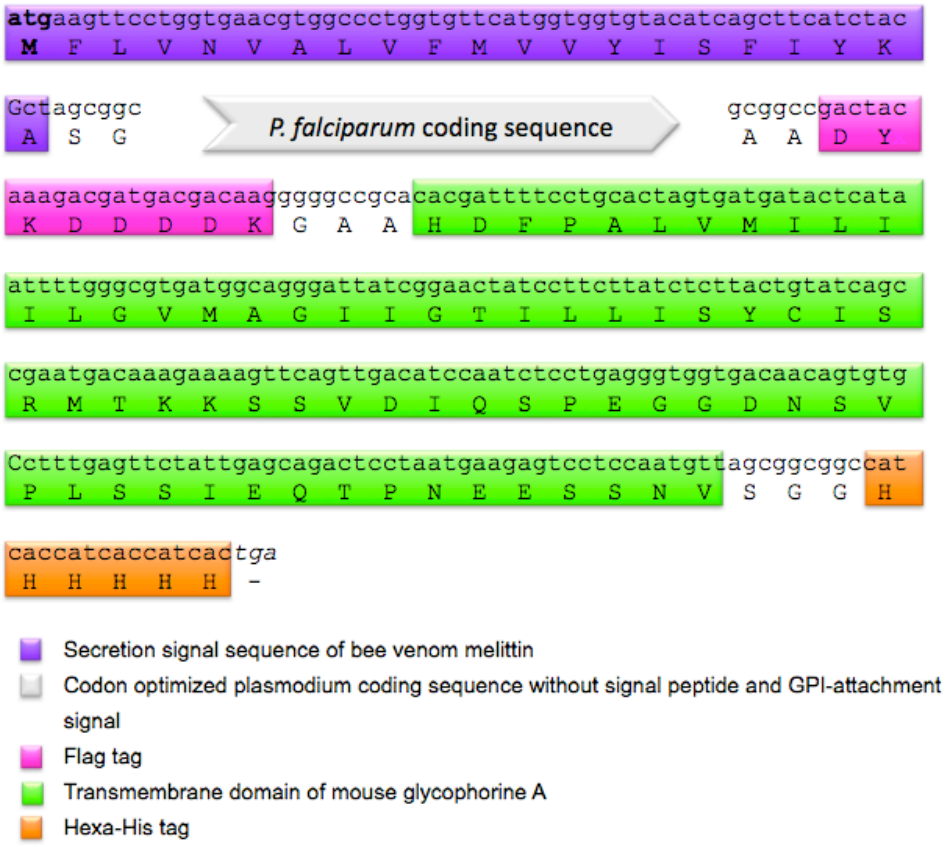

Figure S1. DNA and amino acid sequence of the antigen modifications. Protein-coding DNA sequence and amino acid sequence of the modifications applied to the recombinant proteins, which allow for surface expression and detection of the recombinant protein.

PFD1130w

ATGAAGTTCCTGGTGAACGTGGCCCTGGTGTTCATGGTGGTGTACATCAGCTTCATCTACGCTAGCGGCATAAATTGTGACAGCCG
GCATGTGTTCATTAGAACCGAGTTGTCCTTCATAAAGAATAATGTGCCCTGCATTCGCGACATGTTCTTCATTTACAAGCGCGAGT
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GAGTACTTCCTCATCTGCGGGGTGAGCCCATACAAGTTTAAGGACGATAATAAGAAGGATGATATACTGTGTATGGCCTCCCACGA
CAAGGGAGAGACGTGGGGAACTAAGATAGTGATCAAGTATGATAATTACAAGCTCGGCGTGCAGTACTTCTTCCTCAGGCCTTACA
TCAGTAAGAACGACCTGTCCTTTCACTTTTATGTTGGAGACAACATCAATAATGTGAAAAACGTGAACTTTATAGAATGTACACAT
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AGAAGTATGGAAACACGACCGCCGGCTGTTACGGGGGAACCTTCGTGAAGATCGACGAAAATAGGACCCTGTTCATCTACTCTTCA
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TTGACATCCAATCTCCTGAGGGTGGTGACAACAGTGTGCCTTTGAGTTCTATTGAGCAGACTCCTAATGAAGAGTCCTCCAATGTT
AGCGGCGGCCATCACCATCACCATCACTGA

PF14_0325

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CGCGGCCGACTACAAAGACGATGACGACAAGGGGGCCGCACACGATTTTCCTGCACTAGTGATGATACTCATAATTTTGGGCGTGA
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GAGGGTGGTGACAACAGTGTGCCTTTGAGTTCTATTGAGCAGACTCCTAATGAAGAGTCCTCCAATGTTAGCGGCGGCCATCACCA
TCACCATCACTGA

PFF0620c

ATGAAGTTCCTGGTGAACGTGGCCCTGGTGTTCATGGTGGTGTACATCAGCTTCATCTACGCTAGCGGCTATAAAATCAACGGCGT
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AACCAGGAAACCAGCTACGCCACAAAGAACAAGAATGCGGCCGACTACAAAGACGATGACGACAAGGGGGCCGCACACGATTTTCC
TGCACTAGTGATGATACTCATAATTTTGGGCGTGATGGCAGGGATTATCGGAACTATCCTTCTTATCTCTTACTGTATCAGCCGAA
TGACAAAGAAAAGTTCAGTTGACATCCAATCTCCTGAGGGTGGTGACAACAGTGTGCCTTTGAGTTCTATTGAGCAGACTCCTAAT
GAAGAGTCCTCCAATGTTAGCGGCGGCCATCACCATCACCATCACTGA

Secretion signal peptide of bee venom melittin

Codon-optimized protein-coding sequence of *P. falciparum* gene without signal peptide and GPI-attachment signal sequence

Flag-tag

Transmembrane part of mouse glycophorine A

Hexa-His tag

Start codon

Stop codon

Figure S2. Protein-coding DNA sequence of the expression vectors. Protein-coding DNA sequence of the expression vectors used to express the three different recombinant proteins on the surface of HEK cells.

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RESULTS PART 2

Passive immunoprotection of *Plasmodium falciparum* infected mice designates the Cysteine-Rich Protective Antigen as candidate malaria vaccine antigen.

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Abstract

Screening of predicted *P. falciparum* open reading frames for proteins that elicit parasite-inhibitory antibodies has led to the identification of the Cysteine-Rich Protective Antigen (CyRPA) as promising blood-stage candidate protein for inclusion in a malaria subunit vaccine. CyRPA is a cysteine-rich protein, harbouring a predicted signal sequence. The stage-specific expression of CyRPA in late schizonts resembles that of proteins known to be involved in merozoite invasion. Immunofluorescence staining localised CyRPA at the apex of merozoites. The entire protein is highly conserved as shown by sequencing of the CyRPA encoding gene from a diverse range of *P. falciparum* isolates. CyRPA-specific monoclonal antibodies substantially inhibited parasite growth *in vitro* as well as in a *P. falciparum* animal model based on NOD-*scid IL2Rγ*^{null} mice engrafted with human erythrocytes. In contrast to other *P. falciparum* mouse models this system generated very consistent results and therefore represents an unprecedented *in vivo* model for quantitative comparison of the functional potencies of malaria specific antibodies. Our data suggest a role for CyRPA in erythrocyte invasion by the merozoite. Inhibition of merozoite invasion by CyRPA-specific monoclonal antibodies *in vitro* and *in vivo* renders this protein a promising malaria asexual blood stage vaccine candidate antigen.

Introduction

Albeit being preventable and curable, malaria was estimated to have claimed 781'000 deaths in 2009 [1]. Besides other control measures, implementation of an effective vaccine against malaria is regarded as a cost-effective measure to reduce mortality and morbidity. Three different malaria vaccine strategies can be distinguished: infection-blocking vaccines targeting pre-erythrocytic stages, anti-morbidity vaccines targeting the erythrocytic stages and transmission-blocking vaccines targeting the sexual stages. It is assumed that a highly effective malaria vaccine needs to be targeting multiple stages of the parasite life cycle [2,3].

Importance of a parasite blood-stage component in a malaria subunit vaccine is evident, as clinical symptoms of malaria are mainly attributed to asexual blood stages. In exposed humans protection from symptomatic disease is acquired after repeated exposure over years [4]. Naturally acquired immunity is attributed at least in part to antibody responses targeting blood-stage antigens. This was demonstrated by passive immunotherapy studies, in which transfer of immunoglobulins from immune individuals to malaria patients led to very substantial reductions of parasitaemia and clinical symptoms [5–7].

Feasibility of an asexual blood-stage vaccine is supported by studies in humans and animal models [8–13]. So far research in this field focused on a few protein candidates including merozoite surface protein 1 (MSP-1) [14], MSP-2 [8], MSP-3 [15–18], apical membrane antigen 1 (AMA-1) [19], erythrocyte binding antigen 175 (EBA175) [20], glutamate-rich protein (GLURP) [15,21] and serine repeat antigen 5 (SERA5) [22]. These antigens are all merozoite proteins, either located on the merozoite surface or contained within apical invasion organelles. In contrast to intra-erythrocytic stages, which are largely hidden within the red blood cells, free merozoites are directly accessible to antibodies. Antibodies are thought to interfere with the invasion of erythrocytes by binding to merozoite surface proteins or proteins released from apical organelles. Merozoite invasion involves a complex series of orchestrated molecular interactions but nevertheless takes less than a minute to be completed [23,24]. After low-affinity attachment of a freshly released

merozoite to an erythrocyte, the merozoite reorients to its apical pole. This interaction triggers the release of microneme and rhoptry neck resident proteins, which mediate high affinity attachment by the establishment of a tight junction. This in turn initiates the release of rhoptry bulb resident proteins, which are involved in the formation of a membrane to surround the parasitophorous vacuole. Mediated by an actomyosin motor complex the tight junction is then moved towards the anterior pole, terminating with the sealing of the parasitophorous vacuole membrane at the posterior pole of the merozoite (for a review see reference [25]). Most proteins currently regarded as key malaria blood stage vaccine candidate antigen were shown or are thought to be involved in the invasion process. These antigens are targets of invasion inhibitory antibodies, which interfere with different steps of the invasion process. Parasite growth inhibitory antibodies specific for MSP-1, the most abundant protein on the surface of merozoites assumed to be involved in the initial attachment, were for example shown to inhibit merozoite invasion either by preventing MSP-1 processing or by agglutination preventing the dispersal of released merozoites [26,27]. By contrast, inhibitory antibodies specific for AMA-1, a micronemal protein and constituent of the tight junction, were shown to sterically interfere with the assembly of the protein complex forming the tight junction [28]. Not for all invasion inhibitory antibodies the mode of action is identified, but suggested mechanisms also include opsonisation and destruction of merozoites by phagocytic cells [29,30], complement activation [31] or neutrophil respiratory bursts [32],

Thus far, relatively few blood-stage antigens are in clinical development as vaccine components [33]. Unfortunately, the most advanced blood-stage vaccine antigens, AMA-1 and MSP-1, did not demonstrate efficacy in African children so far [34–36]. However, a multistage vaccine comprising an AMA-1 component showed a 50% reduced incidence rate of clinical malaria episodes in children vaccinees compared to control children [37]. But whether protection is associated with AMA-1-specific responses remains to be shown. The extensive polymorphisms of current candidate antigens is deemed to be a major hurdle for blood-stage vaccine development [38–42]. Hence, although a couple of blood stage antigens are under vaccine

development, it is eligible to search for more vaccine candidates. The comparison of a large range of protein antigens in preclinical assays would allow a more rational prioritization of candidates for inclusion into a vaccine. Since the availability of the *P. falciparum* genome in 2002, reverse vaccinology is considered an opportunity to identify novel vaccine candidates in a more rational way [43–51]. Based on the genome-wide transcriptomic and proteomic information generated since, we selected the *P. falciparum* open reading frame (ORF) PFD1130w for further characterisation. PFD1130w was selected because the available information suggested that the protein is a merozoite protein involved in erythrocyte invasion: i) transcription has been shown to be up-regulated in late asexual blood stages [47,48] ii) the encoded protein is predicted to contain a N-terminal secretion signal peptide [52] ii) a genome-wide *in silico* study based on gene co-expression, sequence homology, domain-domain and yeast two-hybrid interaction data showed that PFD1130w clustered into an interaction network implicated in merozoite invasion [49] and iii) the *pfd1130w* gene lies in close proximity to genes encoding proteins known to be involved in red blood cell invasion, such as reticulocyte-binding protein homolog proteins RH4 and RH5, SURFIN4.2 and GAPM2 (Figure 1) [53–56]. Based on these data we selected PFD1130w for generation of mouse monoclonal antibodies [57], which allowed us to further characterize the protein and to perform functional assays.

In this study we carried out an in-depth characterisation of PFD1130w and demonstrate its potential as malaria asexual blood stage vaccine candidate. PFD1130w is a highly conserved cysteine-rich protein that we designated Cysteine-rich Protective Antigen (CyRPA). Our data on localisation, stage-specific expression pattern and functional assays suggest a role of CyRPA in erythrocyte invasion by the merozoite. Importantly, CyRPA elicits antibodies that inhibit merozoite invasion *in vitro* and *in vivo*. Furthermore, our passive immunisation studies in *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice with anti-CyRPA mAbs identified this animal model as unprecedented system to quantitatively evaluate functional potencies of malaria specific antibodies.

Results

Sequence analysis of *P. falciaprum* CyRPA

Pfd1130w/cyrpa is 1188 bp long with a 99 bp intron and is localized in the subtelomeric region of chromosome 4 in close proximity to genes encoding proteins known to be involved in red blood cell (RBC) invasion, such as reticulocyte-binding protein homolog proteins RH4 and RH5, SURFIN4.2 and glideosome-associated protein with multiple-membrane span 2 (GAPM2) (Figure 1) [53–56]. The hypothetical protein encoded by *pfd1130w* is predicted to contain a N-terminal secretion signal peptide. Orthologs of CyRPA are only present in the genomes of *P. knowlesi* (PKH_052740) and *P. vivax* (PVX_090240) and are absent in *Plasmodium* species infecting rodents. Comparison of these protein sequences revealed conservation with 36% and 38% identity of *P. falciparum* CyRPA with the *P. knowlesi* and the *P.vivax* orthologs, respectively. Furthermore, cysteine residues in the orthologs are positionally conserved (Figure 2). BLAST search identified the mosquito-stage protein PSOP12 (PFE0680) as paralog of CyRPA [58]. This protein shares nine positionally conserved cysteine residues in the homologous N-terminal part, but has an additional 6-cysteine-protein domain at the C-terminus [59].

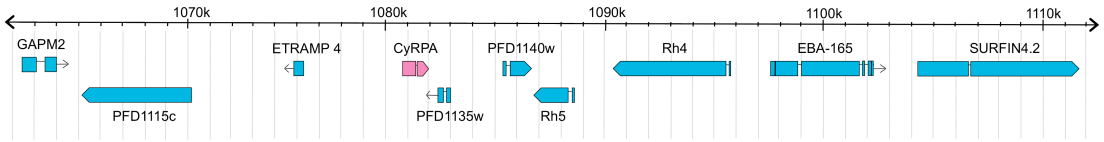

Figure 1. The CyRPA encoding gene is flanked by several invasion-associated genes. The *CyRPA* encoding gene (pink) is positioned in the subtelomeric region of chromosome 4. ORFs in close proximity include genes encoding glideosome-associated protein with multiple-membrane spans 2 (GAPM2), reticulocyte-binding homologs 4 and 5 (RH4 and RH5), and SURFIN4.2. These proteins are known or presumed to be involved in merozoite invasion [53-56]. Positional data were obtained from PlasmoDB (www.plasmoDB.org).

Figure 2. Amino acid sequence alignment of *P. falciparum* CyRPA with orthologs in *P. knowlesi* and *P. vivax*. Full-length putative orthologs of *P. falciparum* CyRPA are only found in the genomes of *P. vivax* (PVX_090240) and *P. knowlesi* (PKH_052740). Sequence identities are 38% and 36%, respectively. "*": identical, ":": conservation between groups of strongly similar properties, ".": conservation between groups of weakly similar properties substitution. Ten out of twelve cysteine residues are positionally conserved (shaded in grey). Amino acid residues of the predicted secretion signal sequences are colored in blue. DNA-sequencing of the *cyrpa* gene of a set of *P. falciparum* strains revealed one nonsynonimous SNP at base pair position 1116. Hence, at amino acid residue 339 (red) the strains 3D7 (airport malaria), MAD20 (PNG), FC27 (PNG), RFCR3 (The Gambia), W2met (Indochina), Hb3 (Honduras), Ro-33 (Ghana), 7G8 (Brazil), IFA4 (Tanzania), IFA6 (Tanzania), IFA10 (Tanzania), IFA12 (Tanzania), IFA18 (Tanzania), IFA19 (Tanzania) encode an arginine residue, whereas strains K1 (Thailand), FCR3 (The Gambia), ITG2F6 (Brazil) and FVO (Vietnam) encode a serine residue.

CyRPA is highly conserved

In order to assess potential sequence diversity of the CyRPA protein, *cyrpa* genes of twelve *P. falciparum* standard strains from different geographical origin (3D7, K1, MAD20, FC27, FCR3, RFCR3, W2met, Hb3, Ro-33, 7G8, ITG2F6 and FVO) and six field isolates from Tanzania (IFA4, IFA6, IFA10, IFA12, IFA18 and IFA19) were amplified by PCR and sequenced. Apart from one non-synonymous single nucleotide polymorphism at base pair position 1116 in *P. falciparum* strains K1, FCR3, ITG2F6 and FVO, all sequences obtained were identical to the *P. falciparum* 3D7 reference sequence. In the protein sequence this single nucleotide polymorphism results in an arginine/serine dimorphism at amino-acid position 339 (Figure 2).

Stage specific expression of CyRPA in schizonts and free merozoites

The *PFD1130w* gene encodes a 362 amino acid long protein with a predicted molecular mass of 42.8 kDa. Using highly synchronized asexual blood stage parasite cultures, we assessed the expression profile of CyRPA in *P. falciparum* across the intra-erythrocytic developmental cycle (IDC) at the protein level by Western Blot analysis with CyRPA-specific mAbs at 8h intervals. A discrete band of about 36 kDa was detected in schizont stages, free merozoites and very early ring stages but not in late ring and early trophozoite stages (Figure 3A). Stage specific expression of CyRPA in schizont stages and free merozoites was affirmed by indirect immunofluorescence staining of synchronized blood stage parasites with anti-CyRPA mAbs (Figure 3B). These results are in agreement with transcriptional data for *PFD1130w*, showing elevated transcript levels in late stages of the asexual blood cycle of *P. falciparum* with maximal expression measured at 40 to 48 hours post-invasion [47,48,60]. Additionally, mass spectrometry based evidence for expression of *PFD1130w* was reported in schizont stages [61].

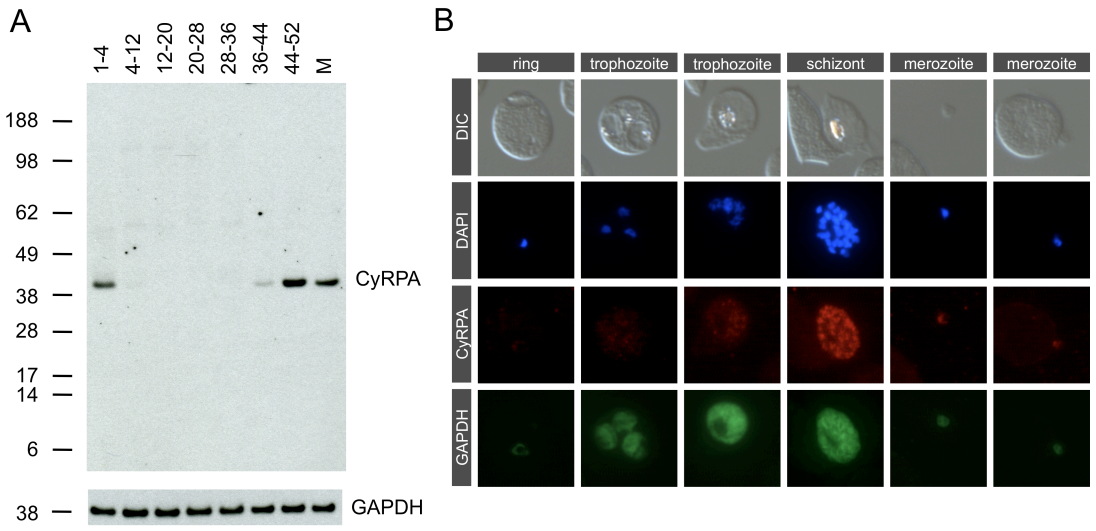

Figure 3. Stage-specific expression of CyRPA in late asexual blood stage parasites. (A) Western blot analysis with lysates of tightly synchronized *P. falciparum* 3D7 blood stage parasites with anti-CyRPA mAb c12 (upper panel). The blot was probed for equal loading with an anti-GAPDH mAb (lower panel). 1-4, 4-12, 12-20, 20-28, 28-36, 36-44, 44-52: hours post-invasion; M: free merozoites. (B) Indirect immunofluorescence stainings of asexual blood stage parasites confirmed stage specific expression in schizont stages and free merozoites. Methanol/acetone fixed *P. falciparum* 3D7 parasites were probed with anti-CyRPA mAb c06 (red) and anti-GAPDH mAb (green). Exposure times were identical for all pictures of the same channel.

CyRPA is localized at the merozoite apex

CyRPA has a putative N-terminal secretion signal sequence and is cysteine-rich (Figure 2). No transmembrane domains are predicted and no significant homology to well characterized functional domains could be identified. To determine the localisation of CyRPA in schizont stages and free merozoites more accurately, we performed co-localisation studies using antibodies specific for the cytosol (glyceraldehyd-3-phosphat-dehydrogenase, GAPDH), the micronemes (apical membrane antigen 1, AMA-1) [62], the rhoptry bulb (rhoptry-associated protein 1, RAP-1) [63], and the merozoite surface resident proteins MSP-1 [64] and MSP-5 [65] (Figure 4). The pattern of CyRPA staining in free merozoites included a dot towards the merozoite apical end and a weaker staining dispersed over the anterior pole of the merozoite. The apical dot did not co-localize with micronemes or rhoptries. But stainings of CyRPA resembled MSP-5-specific stainings. Apical dots of MSP-5 and CyRPA largely overlayed, but the degree of additional faint staining of the merozoite body differed, being spread over the anterior part for CyRPA and just at the apical pole for MSP-5. Accordingly, schizonts were stained by CyRPA-specific mAbs in a

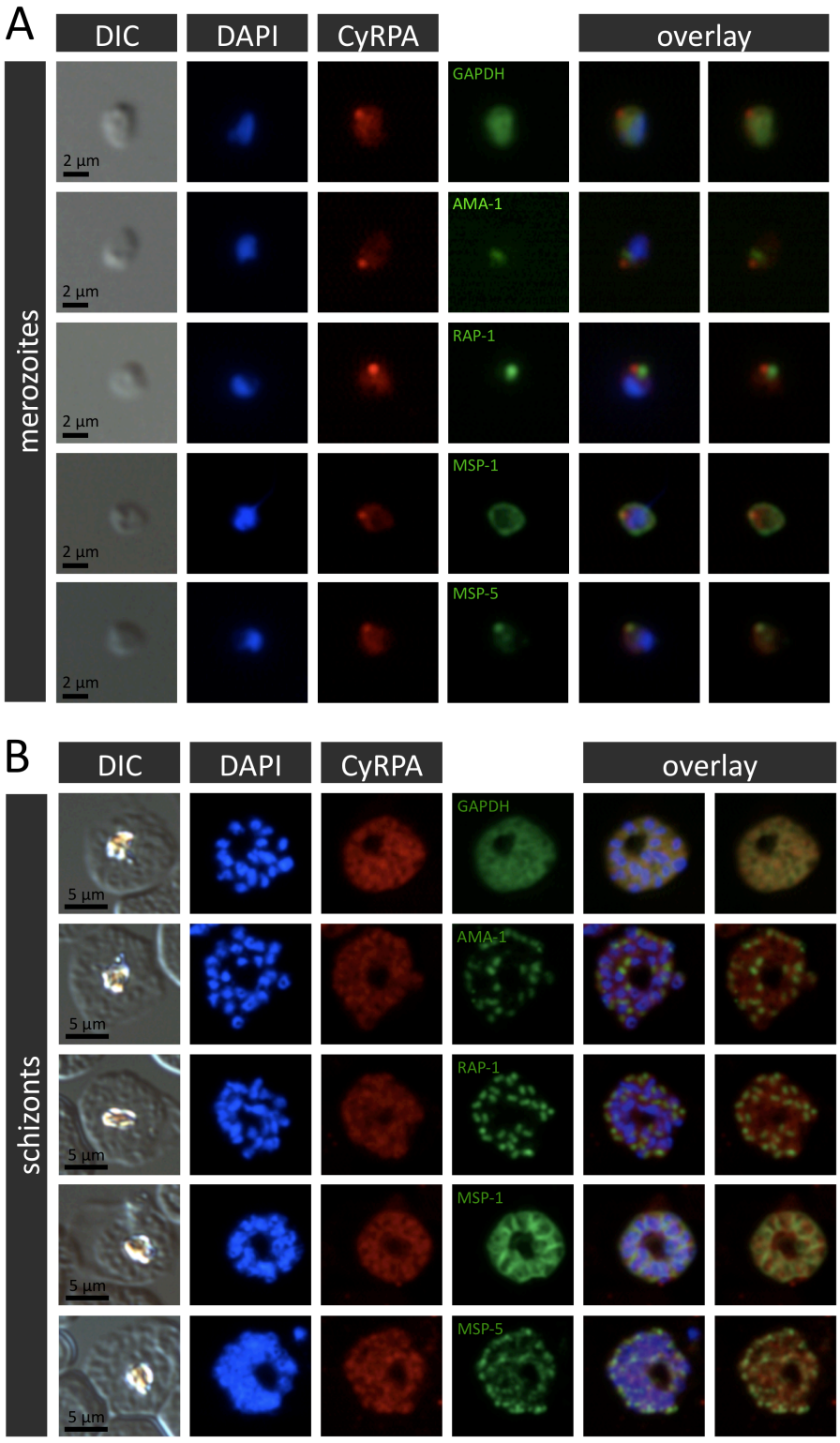

Figure 4. Localisation of CyRPA to the merozoite apex by immunofluorescence staining. *P. falciparum* 3D7 merozoites (A) or schizont stages (B) were co-immunostained with anti-CyRPA mAb c06 (red) and anti-GAPDH antibodies (marker for cytosol), AMA-1 (marker for micronemes), RAP-1 (marker for rhoptries), MSP-1 (marker for merozoite surface) or MSP-5. Nuclei were stained with DAPI.

patchy to dotty manner, not co-localizing with rhoptries or micronemes. CyRPA- and MSP-5-specific stainings showed a relatively similar pattern. Hence, CyRPA localizes to an apical structure distinct from rhoptries and micronemes.

Anti-CyRPA mAbs inhibit merozoite invasion *in vitro*.

Considering the apical localisation of CyRPA, and since CyRPA was predicted to be implicated in merozoite invasion [49], we assessed anti-CyRPA mAbs for *in vitro* parasite growth inhibitory activity. Growth inhibition assays were conducted for two cycles of merozoite invasion. Of the anti-CyRPA mAbs tested, seven out of nine consistently showed growth inhibitory activity in a concentration dependent manner (Figure S1). In contrast, two anti-CyRPA mAb (c05 and c13) as well as control mAb (6xHis-tag specific) had no effect on parasite growth (Figure 5A and Figure S1). All mAbs tested were produced and purified in parallel in the same manner and results were reproducible with independent mAb production batches (data not shown). At a concentration of 500 µg/ml the anti-CyRPA mAb c12 inhibited parasite growth by 65.5 ± 1.7%. The growth inhibitory effect was statistically significant for mAb concentrations of 500 ($p=0.0006$), 250 ($p<0.0001$) and 125 µg/ml ($p=0.0030$), as judged by a two-sided t test.

In order to identify the step at which the anti-CyRPA mAbs exert their effect, parasite growth was compared in the presence or absence of inhibitory mAbs during one blood stage cycle of highly synchronized parasites. 250 µg/ml of the anti-CyRPA mAb c12 was added to early ring stages and parasitemia was monitored after 13, 27 and 31 hours by flow cytometry. No significant difference in the development of trophozoites from ring stages was observed at any time point compared to the PBS control (Figure 5B). When the anti-CyRPA mAb c12 was added to a synchronized parasite culture at the schizont stage and parasitemia was monitored during development into new ring and subsequently into trophozoite stages, a significant reduction in parasitemia compared to the PBS control was measured (Figure 5C). This reduction emerged as soon as the parasitized erythrocytes had ruptured and the released merozoites had infected new erythrocytes ($p=0.0283$ for the difference in parasitemia of PBS vs. anti-CyRPA mAb samples at time point 12 +15h; two-sided t-test). In contrast, the subsequent development of the intra-erythrocytic parasites

from ring stages into trophozoites was not affected by the mAbs. These results verify that the parasite growth inhibitory activity of anti-CyRPA mAbs is not due to inhibition of intracellular parasite development, but due to specific inhibition of merozoite invasion.

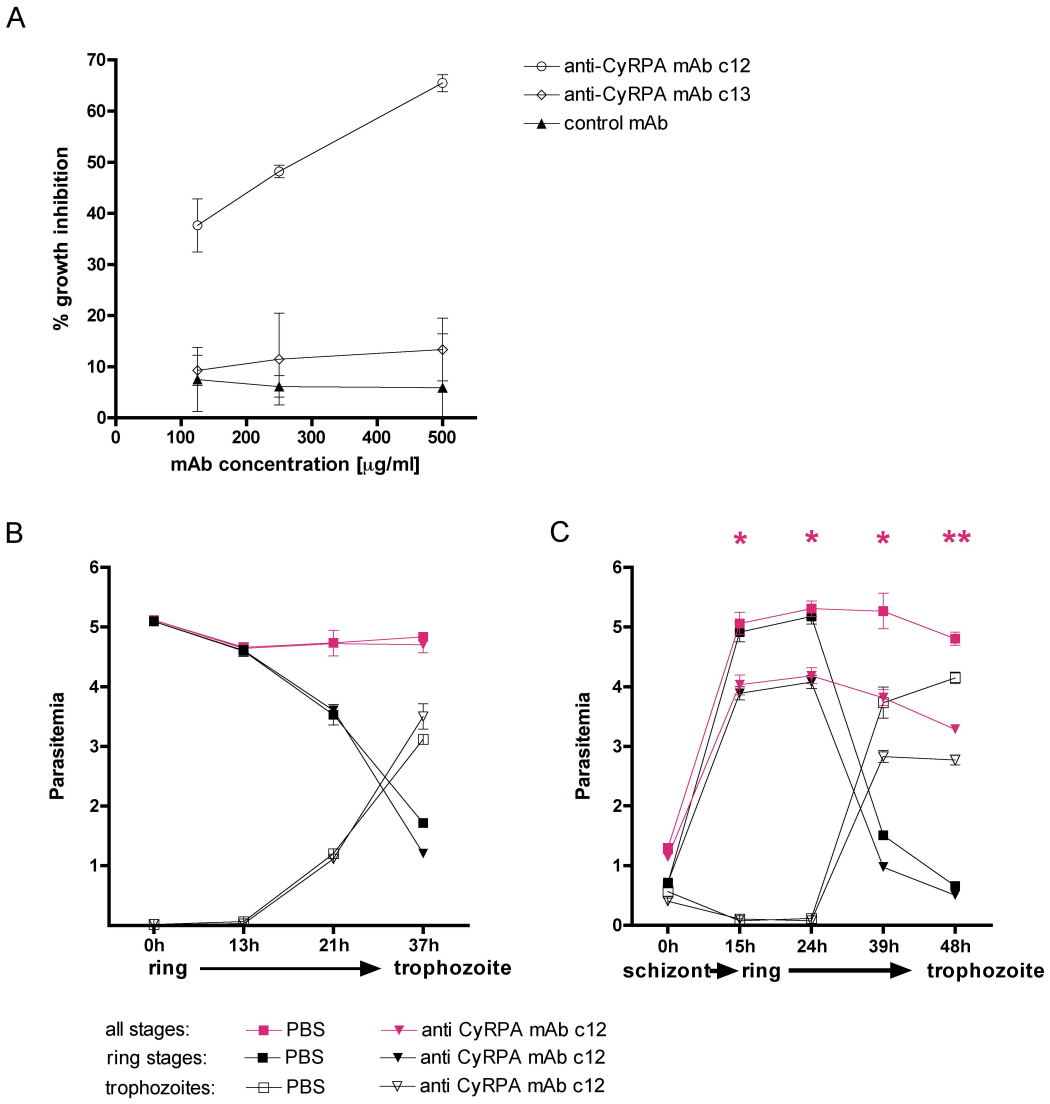

Figure 5. CyRPA specific mAbs inhibit parasite growth *in vitro*. (A) Synchronized *P. flaciparum* 3D7 blood stage parasites were cultured for two cycles in the presence of different concentrations of purified anti-CyRPA mAbs c12 and c13. Percent parasite growth inhibition was calculated against the parasitemia of PBS control wells. Anti-6xHis-tag mAb was used as negative control mAb. Each symbol represents the mean of a triplicate experiment, and error bars indicate the standard deviation. (B) Anti-CyRPA mAb c12 was added to highly synchronous early ring stages and parasitemia was monitored during development into trophozoite stages (squares) and compared to PBS controls (triangles). Parasitemia was measured after 0, 13, 21 and 37 hours. (C) Anti CyRPA mAb c12 was added to highly synchronous schizont stages and parasitemia was monitored during the infection of new erythrocytes and the development into ring and trophozoite stages (triangles) and compared to the PBS control (squares). Parasitemia was measured after 0, 15, 24, 39 and 48 hours. Each symbol represents the mean of a duplicate experiment, and error bars indicate the standard deviation. Total parasitemia is shown in pink, ring stages are shown as closed black symbols and trophozoite stages are shown as open black symbols. Asterisks indicate significant differences in total parasitemia between Ab-treated cultures and control cultures (unpaired t-test).

Anti-CyRPA mAbs inhibit parasite growth *in vivo*

CyRPA has no orthologs in the rodent malarial species and therefore cannot by studied with conventional mouse models with rodent parasites. Therefore we evaluated the *in vivo* parasite inhibitory activity of anti-CyRPA mAbs in a *P. falciparum* SCID mouse model. This model uses non-myelodepleted NOD-*scid IL2Rγ*^{null} mice engrafted with human erythrocytes in order to allow the growth of *P. falciparum* [66]. Groups of three mice with a parasitemia of 0.87 ± 0.12 % were injected once with 0.5 mg, 2.5 mg or 5 mg anti-CyRPA mAb c12, respectively. The control group received 2.5 mg subclass-matched control mAb (6xHis-tag specific). Parasitemia of all mice was monitored for the next five days (Figure 6A). In mice that had received the control mAb, a sigmoidal increase in parasitemia was measured, reaching 21.0 ± 3.6 % on day 6. Parasitemia in mice having received 2.5 mg anti-CyRPA mAb 13, which has shown no *in vitro* growth inhibitory effect, increased similarly. In contrast, parasitemia of mice having received 2.5 mg or 5 mg anti-CyRPA mAb c12 increased only marginally, reaching 3.5 ± 1.2 % and 3.7 ± 1.1 % on day 6. The difference in parasitemia on day 6 in mice receiving 2.5 mg of mAb c12 compared to the negative control group was highly significant (two-sided t-test; p=0.0013). Also, a five times lower dose of mAb c12 (0.5 mg) still reduced parasite growth (15.0 ± 1.2 % parasitemia on day 6). Titration of mAbs in the circulation of the passively protected mice by ELISA showed that antibody levels remained high over the entire study period, ranging from 57-117 % on day 6 compared to day 2, and immunofluorescence staining of persisting parasites showed that they still expressed CyRPA (data not shown).

On day 6 blood cells of mice having received 2.5 mg anti-CyRPA mAb c12 were used in a follow-up experiment to infect three naïve groups of NOD-*scid IL2Rγ*^{null} mice engrafted with human erythrocytes (Figure 6B). A rapid and continuous increase in parasitemia indicated good viability of the transferred parasites (14.7 ± 1.3 % parasitemia on day 6 in the untreated group). To assess if parasites growing in anti-CyRPA mAb c12 treated mice (Figure 6A) have developed resistance to antibody inhibition, recipient mice were treated with a single i.v. injection of 2.5 mg anti-CyRPA mAb c12 (Figure 6B). Again, parasitemia was significantly reduced in

comparison to mice treated with an isotype-mached control mAb (two-sided t-test; p=0.0001).

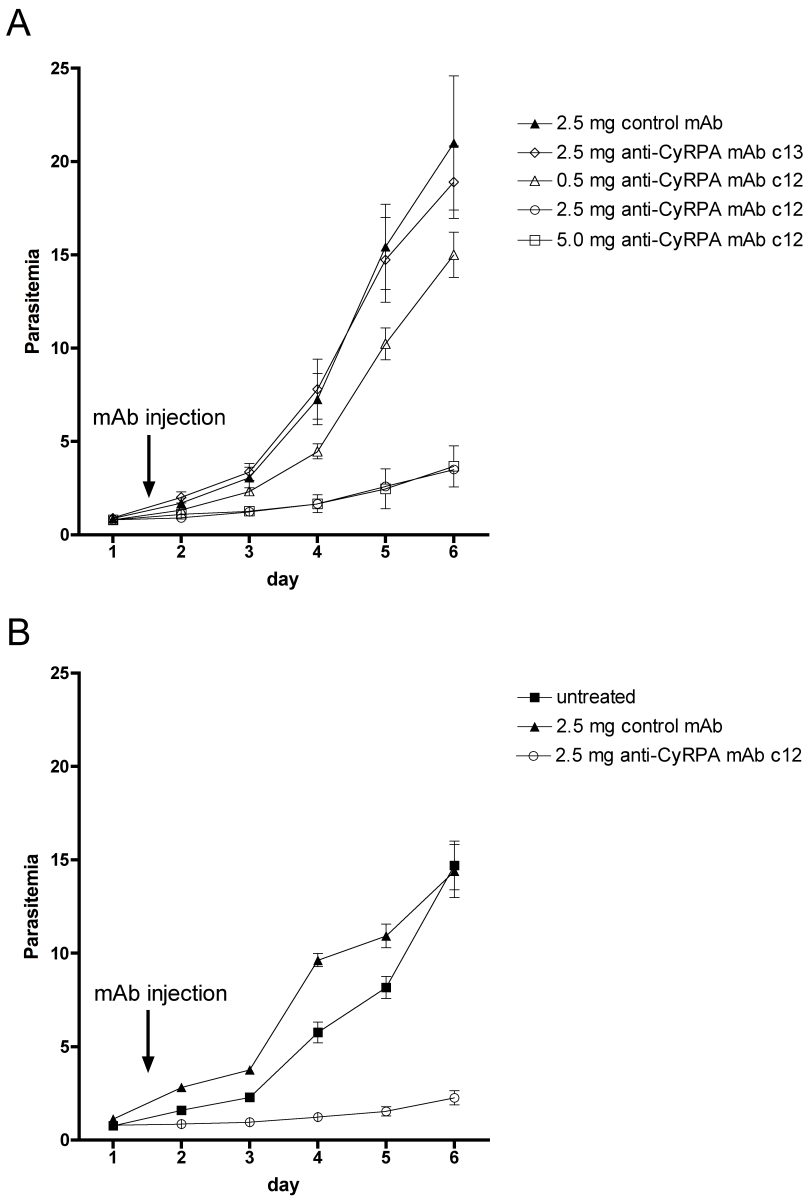

Figure 6. CyRPA specific mAbs inhibit parasite growth in the NOD-*scid IL2Rγ*^{null} mouse model. (A) *P. falciparum*-infected NOD-*scid IL2Rγ*^{null} mice received purified anti-CyRPA mAbs or isotype/subclass-matched control mAbs by i.v. injection. The arrow indicates the day of mAb injection and values are the mean parasitemia in peripheral blood of three mice per group. Data are means ± SD. (B) On day six, blood cells of mice having received 2.5mg anti-CyRPA mAb c12 were used to infect fresh NOD-*scid IL2Rγ*^{null} mice. Once parasitemia exceeded 0.5%, mice received a single dose of 2.5 mg anti-CyRPA mAb c12 or control mAb by i.v. injection. Parasitemia was monitored over the next five days. Data are mean parasitemia of three mice per group ± SD.

Fine specificity of anti-CyRPA mAbs

Of nine CyRPA specific mAbs analysed, two (c05 & c13) showed no growth inhibitory effect and seven (c02, c04, c06, c08, c09, c10, c12) showed inhibitory activity. To explain these differences in biological activity, fine specificities of the different mAbs were analysed. Firstly, antibody-antibody competition experiments were performed by ELISA using plates coated with recombinant CyRPA protein expressed in *E.coli* (Table 1). mAbs c02, c04, c06, c08, c09 and c12 competed against each other. These mAbs did not compete with and were not competed by mAbs c05, c10 and c13. While mAbs c05 and c13 competed with each other, mAb c10 did not compete with and was not competed by any of the other anti-CyRPA mAbs. According to these results the nine mAbs were grouped into three epitope groups (I, II, III), with the two non-inhibitory mAbs forming epitope group III.

Secondly, the anti-CyRPA mAbs were tested for their reactivity with overlapping protein fragments of CyRPA. For this purpose, HEK cells transiently transfected with expression plasmids encoding fragments of CyRPA comprising amino acid 26-352, 26-251, 26-181, 127-352, 236-352, 74-251, 74-181, 26-142 and 127-251 were analyzed by Western blot analysis and life cell staining. In Western blot analysis CyRPA-specific mAbs bound to fragment 26-181, 26-251 and 26-352 but not to the two overlapping subfragments 26-141 and 74-181. This indicates that all nine mAbs recognize conformational epitopes that is present in fragment 26-181, but not formed in subfragments 26-141 and 74-181 (Figure S2 and data not shown). Life cell staining of HEK cells expressing the protein fragments on their cell surface with different anti-CyRPA mAbs revealed five distinctive reactivity-patterns (A, B, C, D, E) (Table 1). When combining results from antibody-antibody competition experiments and epitope mapping, the seven growth inhibitory anti-CyRPA mAbs classified into three fine specificity groups. These were distinctive from the fine specificities of the two non-inhibitory mAbs c05 and c13, which were the only mAbs reacting with the N-terminally truncated fragment comprising aa 74-251 (Table 1).

The identified dimorphism at amino-acid position 339 does not lei within the sequence stretch aa 26-181 relevant for binding of the analyzed anti-CyRPA mAbs

(Table 1). Accordingly, anti-CyRPA mAb 12 inhibited growth of *P. falciparum* strains expressing either variants (3D7 vs. K1) (data not shown).

Natural immunogenicity of CyRPA

In order to examine if natural exposure to *P. falciparum* leads to the development of anti-CyRPA antibodies, human sera were analyzed by ELISA for their reactivity with purified recombinant CyRPA expressed in *E. coli* (Figure 7). Sera were collected from healthy adults living in a *P. falciparum* malaria endemic area of West-Africa. Sera from healthy Swiss adults without any history of malaria served as negative control. Whereas sera from controls showed no reactivity with CyRPA (median OD 0.2090), sera from malaria exposed adults showed significantly higher reactivity (median OD 0.4070; Mann-Whitney test $p<0.0001$). However, only 6 out of the 24 African sera tested showed strong reactivity.

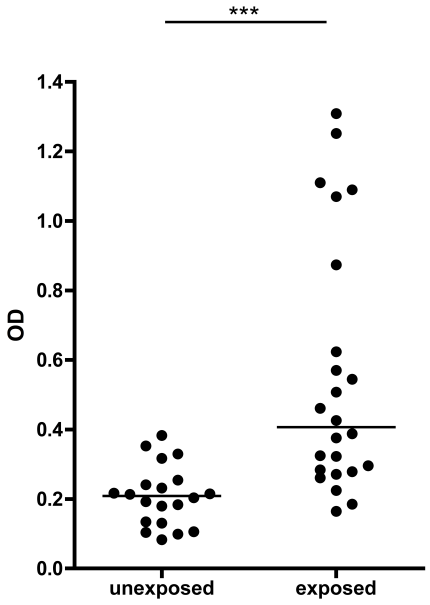

Figure 7. Serum of some individuals from a malaria endemic area contain CyRPA specific IgG. Reactivity of human sera from Europeans (unexposed) or from West Afrikans (malaria exposed) with recombinant CyRPA assessed by ELISA. Shown are IgG levels expressed as optical density (OD) at 405 nm of serum samples diluted 1:400. Horizontal lines designate medians of anti-CyRPA responses and asterisks indicate statistical difference between the two groups (Mann-Whitney test).

Table 1. Fine specificity of anti-CyRPA mAbs.

Anti-CyRPA mAb	c10	c02	c06	c08	c09	c12	c04	c05	c13
Parasite growth inhibition	yes	yes	yes	yes	yes	yes	yes	no	no

A Antibody-antibody competition ELISA

	Detecting anti-CyRPA mAb								
Competing anti-CyRPA mAb	c10	c02	c06	c08	c09	c12	c04	c05	c13
c10	x	–	–	–	–	–	–	–	–
c02	–	x	x	x	x	x	x	–	–
c06	–	x	x	x	x	x	x	–	–
c08	–	x	x	x	x	x	x	–	–
c09	–	x	x	x	x	x	x	–	–
c12	–	x	x	x	x	x	x	–	–
c04	–	x	x	x	x	x	x	–	–
c05	–	–	–	–	–	–	–	x	x
c13	–	–	–	–	–	–	–	x	x
epitope group	I	II	II	II	II	II	II	III	III

B Reactivity of anti-CyRRPA mAbs with CyRPA fragments

	Anti-CyRPA mAb								
CyRPA fragment	C10	c02	c06	c08	c09	c12	c04	c05	c13
	x	x	x	x	x	x	x	x	x
	–	x	x	x	x	x	x	x	x
	–	x	x	x	x	x	x	x	x
	–	–	–	–	–	–	x*	–	–
	–	–	–	–	–	–	x*	–	–
	–	–	–	–	–	–	–	x*	x*
	–	–	–	–	–	–	–	–	x*
	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–
epitope group	A	B	B	B	B	B	C	D	E

(A) Antibody-antibody competition ELISA results of nine anti-CyRPA mAbs. x; antibody competition, -; no antibody competition. According to their competition pattern, mAbs were assigned to epitope groups I, II or III. (B) Capability of nine anti-CyRPA mAbs to bind to fragments of CyRPA expressed on the cell surface of HEK cells assessed by live-cell immunofluorescence staining. x; staining, -; no staining. According to their reactivity pattern, mAbs were assigned to epitope groups A, B, C, D or E. *; no reactivity in Western blot analysis of HEK cell lysates (data not shown).

Discussion

With the release of the fully annotated genome of *P. falciparum* in 2002, genome-wide searches for potential vaccine candidate antigens have become possible [45,46]. Based on published transcriptome and proteome data, suggesting expression in extracellular stages and surface localisation, we have selected sets of uncharacterized ORFs for evaluation of their potential as vaccine candidate antigens [47,49,52,57,67] (and unpublished results). Among the proteins characterized, CyRPA exhibited outstanding properties, demonstrating the utility of systematic genome-wide approaches for vaccine antigen selection. However, for each individual antigen detailed evaluations, including functional assays, remain indispensable to evaluate their potential as vaccine candidate.

One major problem in the development of a malaria asexual blood-stage vaccine is the lack of functional assays with proven predictive potential. Established assays only assess certain potential antibody-mediated effector functions against blood stage parasites [18,68–70], i.e. growth inhibition by antibodies alone (GIA) or antibody-dependent cellular inhibition (ADCI). Still, these assays can provide valuable information on the protein function. Antibodies against CyRPA showed a strong parasite growth inhibitory activity in GIA. This activity targeted the process of merozoite invasion into RBCs, but not the development of intra-erythrocytic stages. These results point towards a complement- and leukocyte-independent mode of action, such as blocking of molecular interactions involved in merozoite invasion [26,28].

Plasmodium *in vitro* cultures are very sensitive to physiological changes. Thus, the *in vitro* growth inhibition assay is criticized for being very sensitive to impurities of antibody preparations tested. We addressed this by testing more than one independently produced batch of the individual mAbs and obtained consistent results. Importantly, the mouse *in vivo* model offers the advantage of a constant physiological environment given that factors like nutrient supply, electrolyte balance and pH is very well controlled by the animal [71]. Since *P. falciparum* cannot infect rodent erythrocytes and rodent malaria parasites lack many antigens of *P.*

falciparum, including CyRPA, non-human primates or SCID-mice engrafted with human erythrocytes are the only *in vivo* models available to study growth-inhibitory effects of CyRPA specific antibodies. It was previously shown that the *P. falciparum* model in NOD-SCID mice genetically deficient in IL-2 receptor γ chain (NOD-*scid IL2Rγ*^{null}) engrafted with human erythrocytes is a reliable test system for drug evaluation *in vivo* [66][72]. Also in our hands, parasite growth in infected NOD-*scid IL2Rγ*^{null} mice was very consistent and reproducible. However, this model has never been used previously for passive immunisation studies. Anti-CyRPA mAbs administered at the beginning of the exponential growth phase exerted a strong, dose-dependent parasite growth inhibitory effect. To the best of our knowledge, our study describes the first immunoglobulin transfer assay using a *P. falciparum* murine model in which a dose-response relationship has been reported. In contrast to the previously described model [73] this system therefore permits comparison of the relative inhibitory potency of malaria specific antibodies *in vivo*. Thus, we propose this model as a reliable *in vivo* model to test protective efficacy of vaccine antigens against *P. falciparum* blood stages.

Growth inhibitory capacities of different anti-CyRPA mAbs assessed in the *in vivo* model correlate with the results obtained in the *in vitro* assays, strengthening credibility of both systems. The inhibitory effect of CyRPA-specific Abs seen *in vivo* can well be ascribed to the same effector mechanisms as *in vitro*, namely blocking of invasion–relevant processes. Yet, we cannot exclude involvement of additional immune effector mechanisms. NOD-*scid IL2Rγ*^{null} mice are incapable of mounting adaptive immune responses and additionally lack a functional common IL-2 receptor γ-chain which is required for high affinity binding of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 to their receptor [74]. As a consequence, NOD-*scid IL2Rγ*^{null} mice have no NK cells and show additional defects in innate immunity. Involvement of complement or cellular effector mechanisms in the observed protection is nevertheless possible, as in these mice the percentage of macrophages/monocytes and granulocytes is normal or even increased [75].

Anti-CyRPA mAbs inhibited, but did not completely block, parasite growth. The multiplication rate *in vivo* was much reduced but remained above one, leading to a

very slow rise in parasitemia. Increasing the applied dose from 2.5 to 5 mg per mouse did not reduce multiplication further, indicating a saturation of the inhibitory mechanism. From our functional data we deduce that anti-CyRPA mAbs reduce parasite growth by partially inhibiting some processes crucial for invasion of erythrocytes by merozoites, be it a specific protein interaction or processing of invasion relevant proteins. *P. falciparum* merozoites are described to use alternative invasion pathways to evade antibody mediated immunity [76]. Use of a CyRPA-independent invasion pathway of limited efficiency could explain persistence of infection associated with a reduced multiplication rate. Since parasites that survived treatment with anti-CyRPA mAb showed normal multiplication rates when transferred into naive mice, and remained sensitive to re-exposure to anti-CyRPA mAbs, no rigid switch to an alternative invasion pathway seems to occur. The fact that, at least over a short time period, no CyRPA-Ab resistant parasites were selected is of importance when considering the inclusion of CyRPA into a malaria subunit vaccine.

Interestingly, among a set of nine mAbs specific for CyRPA, seven mAbs showed a parasite growth inhibiting effect whereas two did not. No confined CyRPA sequence stretch could be defined as epitope, since all mAbs seem to recognize conformational epitopes not present in short CyRPA sequence stretches. However, the fine specificity of inhibitory anti-CyRPA mAbs was distinct from the fine specificity of non-inhibitory anti-CyRPA mAbs. These results indicate that protection by anti-CyRPA mAbs is likely due to blockage of a molecular process required for invasion and not simply due to opsonization. CyRPA thus may be involved in distinct interactions with erythrocyte structures and/or parasite proteins involved in erythrocyte invasion. The fact that only anti-CyRPA antibodies of a certain fine-specificity showed invasion inhibitory activity demonstrates once more the shortcoming of assessing immunity or vaccine-potential of antigens by solely measuring antibody titres by ELISA. Besides CyRPA, a range of other blood stage antigens, including AMA-1 and MSP-1, where shown to induce inhibitory as well as non-inhibitory or even inhibition-blocking antibodies [77,78]. ELISA cannot differentiate between functional and non-functional antibodies. For the same reason

it is favourable to perform passive immunoprotection experiments with mAbs in animal models to identify novel vaccine targets rather than using active immunisation. MAbs represent highly defined agents with a distinct fine-specificity and affinity, whereas immunisation induces antibodies with a wide range of binding properties. Hence, functional assays with polyclonal antibodies poses the risk of generating misleading results due to interference or masking of functional by non-functional antibodies.

Most merozoite antigens associated with protection identified so far are located on the merozoite surface or within apical organelles. From a gene interaction network associated with invasion constructed from gene co-expression, sequence homology, domain-domain and yeast two-hybrid data, CyRPA was selected as one of the genes to be expressed as GFP-fusion protein in order to validate functional annotations [49]. In that study, expression of CyRPA as GFP-fusion protein in transgenic parasites was shown to result in a predominantly apical distribution. Consistent with this finding we showed by indirect immunofluorescence staining with CyRPA-specific mAbs that CyRPA localises at the merozoite apex. Additionally we could show that CyRPA does not localize to micronemes or rhoptries. The CyRPA-specific staining showed similarity to the staining of MSP-5, possibly indicating that CyRPA shares the same subcellular localisation as MSP5, a protein considered as blood stage vaccine candidate [79,80]. Although MSP-5 is described as a merozoite surface protein, its exact localisation is sparsely characterized and immunofluorescence staining of schizonts with MSP-5 specific antibodies was shown previously to result in a rather dotty staining pattern [81,82]. The exact subcellular structure at the merozoite apex to which CyRPA localizes, and how this structure is implicated in merozoite invasion, remains to be investigated.

P. falciparum is a highly polymorphic organism. This is particularly the case for many surface antigens as a result of natural selective pressure by the human immune responses [83]. Most of the asexual blood-stage vaccine candidates evaluated to date, including AMA-1, MSP-1 and MSP-2, therefore have substantial polymorphisms [39–42]. Immunodominance of the polymorphic and variant epitopes may explain the need for repeated exposure over several years to achieve clinical immunity

against the natural polymorphic parasite poulations [84]. Malaria vaccines based on polymorphic antigens run the risk of compromised efficacy due to selection of vaccine-resistant variants [8]. Therefore, it may be desirable to focus on antigens and protein domains with limited polymorphism. Only some of the sera of malaria-exposed adults tested here contained CyRPA specific Abs; natural immunogenicity of CyRPA thus appears to be limited. Our sequence analysis revealed that CyRPA is a highly conserved protein. Only a single non-synonymous SNP was detected among a range of *P. falciparum* isolates. This, together with the low immunogenicity, is an indication that CyRPA is not a major target of naturally acquired immune responses. Furthermore, the identified SNP-associated amino acid dimorphism is not located within the epitope of the growth-inhibitory anti-CyRPA mAbs and their growth inhibitory effect was not affected by this dimorphism (data not shown). From these results we deduce that the protection-associated epitope of CyRPA is entirely free of polymorphisms, suggesting that a CyRPA-based vaccine would target the entire *P. falciparum* population. This represents a clear advantage compared to polymorphic vaccine antigens like AMA-1, where several studies showed significant allele-specificity in the inhibitory activity of anti-AMA-1 antibodies [85–87].

Our data show that CyRPA clearly fulfils three key criteria applied to select asexual blood-stage antigens as vaccine candidates [88]: i) the protein is highly conserved, ii) antibodies against the antigen inhibit parasite growth *in vitro* and iii) are protective in animal models. Since not all CyRPA-specific Abs inhibit parasite growth, testing for presence of total CyRPA-specific antibody titres in human sera is not sufficient to assess association of natural immune responses with protection from malaria, but requires differentiation between inhibitory and non-inhibitory antibodies.

In summary, we have identified a highly conserved merozoite protein, which induces antibodies that inhibit parasite growth *in vitro* and *in vivo*. We suggest evaluating its suitability as candidate antigen for inclusion into a multivalent malaria subunit vaccine. In addition we adopted the improved *P. falciparum* mouse model based on NOD-*scid IL2Rγ*^{null} mice for functional analysis of malaria blood stage antibodies. This model now allows a more systematic and quantitative comparison of the *in vivo* functionality of malaria antigen specific antibodies.

Materials & Methods

Ethics statement

This study was carried out in strict accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzverordnung) of the Swiss Bundesamt für Veterinärwesen. The protocol was ethically approved by the Ethikkommission beider Basel (Permit Number: 2375). Human sera were obtained from adult volunteers after receiving written informed consent. Ethical clearance was obtained from the Ethikkommission beider Basel.

Culture of parasites

P. falciparum strain 3D7 was cultured essentially as described previously [89]. The culture medium was supplemented with 0.5% AlbuMAX (Gibco) as a substitute for human serum [90]. Cultures were synchronised by sorbitol treatment [91]. Erythrocytes for passages were obtained from the Swiss Red Cross (Switzerland).

Isolation of free merozoites

To obtain *P. falciparum* merozoites, erythrocytes infected with highly synchronous schizont-stage parasites were centrifuged at 700 g for 5 min to separate released merozoites from unruptured schizonts and uninfected erythrocytes. Supernatants containing free merozoites were centrifuged at 3000 g for 10 min to collect merozoites.

Western blot analysis

Blood stage parasite lysates were prepared essentially as described previously by saponin lysis of *P. falciparum* 3D7-infected erythrocytes [89]. In brief, cultured parasites were washed once with PBS. Pelleted infected red blood cells were lysed in 20 volumes of 0.06% (w/v) saponin in PBS for 20 min. Parasites were washed and the final pellet was resuspended in 3 volumes of PBS and stored at −80 °C until further use.

Cell lysate of transfected HEK cells were prepared as described previously [57].

For SDS-PAGE cell- or parasite lysate was resolved on precast 4-12% gradient gels (NuPAGE® Novex 4-12% Bis-Tris Gel, Invitrogen) with MES running buffer according to the manufacturer's directions. The proteins were electrophoretically transferred to nitrocellulose membrane using a dry-blotting system (iBlot, Invitrogen). After blocking the membrane, specific proteins were detected with appropriate dilutions of anti-CyRPA mAbs [57] or anti-hexa-his-tag mAb [57] followed by horseradish peroxidase-conjugated goat anti-mouse IgG mAb (Kirkegaard & Perry Laboratories). Blots were developed using ECL Western blotting detection reagents (ECL Western Blotting Substrate, Pierce).

Immunofluorescence staining of infected erythrocytes and free merozoites

For indirect immunofluorescence microscopy, smears of infected red blood cells were fixed in 60% methanol and 40% acetone for 2 min at −20°C and blocked with 1% BSA in PBS. Isolated free merozoites were spotted and fixed onto L-lysine coated multitest glass slides as described previously [92]. Cells were probed with the following primary or secondary antibodies: biotin-labeled mouse anti-CyRPA mAb c06 [57], Alexa488-labeled mouse anti-RAP-1 5-2 mAb [93], Alexa488-labeled mouse anti-AMA-1 DV5a mAb [92], Alexa488-labeled mouse anti-MSP-1 MC7.2 mAb (unpublished), Alexa488-labeled mouse anti-GAPDH 1.4a mAb [94], anti-MSP-2 rabbit serum (Malaria Research and Reference Reagent Resource Center (MR4), MRA-318), anti-MSP-4 rabbit serum [95], anti-MSP-5 rabbit serum [81], Alexa568-labeled streptavidin (Invitrogen) and Alexa488-labeled chicken anti-rabbit IgG (H+L) (Invitrogen). The slides were mounted in mounting medium containing DAPI (ProLong Gold antifade reagent with DAPI, Invitrogen). Fluorescence microscopy was performed on a Leica DM-5000B using a 60x oil immersion objective lens and documented with a Leica DFC300FX digital camera system. Images were processed using Leica Application Suite and Adobe Photoshop® CS3.

In vitro growth inhibition assay

In vitro growth inhibition assays with *P. falciparum* strain 3d7 were conducted essentially as described [92]. Each culture was set up in triplicate in 96-well flat-bottomed culture plates. Viable parasites were stained with hydreothidine and analysed in a FACSscan flow cytometer (Becton Dickinson, San Jose, Calif.) using

CellQuest software. A total of 30'000 cells per sample were analysed. Percent inhibition was calculated from the mean parasitemia of triplicate test and control wells; (control – test)/(control/100).

In vivo growth inhibition assay

Antibodies were tested in the murine *P. falciparum* model essentially as described [66]. The only modification implemented was that daily human blood injections (0.75ml) were administered by the intravenous route instead of the intraperitoneal route. Once parasitemia reached 0.87 ± 0.12% mice received a single dose of 0.5, 2.5 or 5 mg monoclonal antibody in PBS by intravenous injection. Thereafter parasitemia was measured for the next five days. To monitor serum levels of administered Abs, serum samples were taken just before, one day and five days after mAb injection.

For the transfer experiment blood cells of mice having preciously received 2.5 mg anti-CyRPA mAb c12 were washed with 0.9% NaCl and used to infect naïve human erythrocyte engrafted NOD-*scid IL2Rγ*^{null} mice.

ELISA

Detection of CyRPA-specific Abs in human sera by ELISA

For the analysis of human sera for presence of CyRPA-specific antibodies, plates were coated with 10 µg/ml purified recombinant CyRPA protein. After blocking, plates were incubated with dilutions of human serum. Alkaline phosphatase conjugated goat anti-human IgG F(ab')$_2$ (Jackson ImmunoResearch Laboratories Inc.) was used as secondary antibody and *P*-nitrophenyl phosphate was used as substrate (Sigma). The OD of the reaction product was recorded at 405 nm with a microplate absorbance reader (Sunrise Absorbance Reader, Tecan).

Antibody competition ELISA

Plates were coated with 10 µg/ml purified recombinant CyRPA protein. After blocking, plates were incubated with 10 µg/ml, 1 µg/ml or 0 µg/ml of different anti-CyRPA mAbs (c02, c04, c05, c06, c08, c09, c10, c12, c13). After 30 min different biotinylated anti-CyRPA mAbs (c02, c04, c05, c06, c08, c09, c10, c12, c13) are added to each well resulting in a concentration of 1 µg/ml of labelled antibodies.

Horseradish peroxidase conjugated streptavidin (GenScript Corporation) was used as detecting agent and TMB substrate (Pierce Western Blotting Substrate, Pierce) was used for development. The reaction was stopped with 0.5M H_2SO_4 and the optical density (OD) was measured at 450 nm.

Culture of eukaryotic cells

The human embryonic kidney cell line 293 HEK was obtained from the American Type Culture Collection (CRL-1573, ATCC). 293 HEK cells were cultured in DMEM supplemented with 10% foetal calf serum, glutamine and penicillin/streptomycin at 37°C in a humidified incubator.

Expression of CyRPA protein fragments on HEK cells

DNA sequences that encode fragments of CyRPA protein were amplified by PCR from a plasmid containing the codon-optimized sequence of *PFD1130w*. Primers used for amplification were: Fr26-251; 3910 & 3913, Fr127-352; 3912 & 3905, Fr26-142; 3910 & 3911, Fr127-251; 3912 & 3913, Fr236-352; 3886 & 3887, Fr26-181; 3910 & 3939, Fr74-181; 3938 & 3939, Fr74-251; 3938 & 3913 (Table S1). Amplifications were performed using FIREPol DNA polymerase (Solis Biodyne) according to the manufacturers protocol with the following profile: 5 min 95°C; 30 x (30 s 95°C, 30 s 60°C, 2 min 72°C); 6 min 72°C. The amplicons were digested with restriction endonucleases NheI and NotI (New England Biolabs), and then ligated into a pcDNA3.1-based expression vector. This expression vector allows surface expression of a protein of interest. It contains the secretion signal of bee-venom mellitin, a cloning site for the protein of interest, a FLAG-tag, a transmembrane domain of mouse glycophorine-A and a hexa-his tag. The two tags are positioned just before and after the transmembrane domain to facilitate verification of the extracellular localisation of the recombinantly expressed antigens. 293 HEK cells were transfected with the different expression vectors using JetPEI^{TM} transfection reagent (PolyPlus) according to the manufacturer's protocol. One to two days later, transient transfectants were used for Western blot analysis or immunefluorescence staining

Immunofluorescence staining of living transfected HEK cells

Immunofluorescence staining of live HEK cells was performed in chamber slides (8-well chamber-slide, Lab-Tek™, Nunc™). 12'000 cells/well were seeded and four days later transfected with different expression vectors. The following day immunofluorescence stainings were performed by incubating the wells with 100 µg/ml mAb diluted in IMDM for 30 min on ice. After washing, cells were fixed for 30 min with 4% formaldehyde in PBS. After washing, cells were incubated for 30 min with Cy3-labelled goat anti-mouse IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories) diluted in PBS and washed four times with PBS. Slides were mounted with mounting solution containing DAPI (ProLong® Gold antifade reagent with DAPI, Invitrogen). Stainings were assessed by fluorescence microscopy as described above.

Bacterial strains and media

E. coli strain Top10 (TOP10 Chemically Competent *E. coli* Cells, Invitrogen) was used for the amplification of plasmids. *E. coli* strain BL 21 Star (DE3) (Invitrogen) was used for recombinant expression of CyRPA protein. Bacteria were grown in LB medium containing 100 µg/ml ampicillin.

Recombinant protein expression and purification

The CyRPA protein (without hypothetical secretion signal peptide and GPI attachment signal sequence; amino acids 26-352) was recombinantly expressed in *E. coli* using the pET28a expression system (Novagen, modified to contain an ampicillin selection cassette). Briefly, a PCR product of CyRPA was generated from a plasmid containing the sequence of *PFD1130w* using primer 3986 and 3941 (Table S1). The amplicon was digested with restriction endonucleases NcoI and XhoI (New England Biolabs) and cloned into NcoI and XhoI sites of pET28a. Protein expression in *E. coli* BL 21 Star (DE3) (Invitrogen) was induced by addition of 1mM isopropyl thiogalactoside (IPTG) (Calbiochem). The recombinant protein was purified from inclusion bodies using denaturing conditions (8M urea, 500mM NaCl, 20mM Tris-HCl, 5mM Imidazole) by Ni-NTA chromatography. Cells were induced for 2 hours at 37°C and were harvested by centrifugation and lysed by sonication. Insoluble material was pelleted by centrifugation, resuspended in binding buffer (8M urea, 500mM NaCl,

20mM Tris-HCl, 5mM Imidazole) and sonicated. The lysate was cleared by centrifugation and the supernatant was loaded onto a Ni-NTA column. The column was washed with binding buffer and the hexa-histidine-taged recombinant protein was recovered using elution buffer (8M urea, 500mM NaCl, 20mM Tris-HCl, 500mM imidazole). The purity and integrity of the purified protein was analyzed by SDS-PAGE and the protein concentration was determined by measuring OD_{280}. The purified recombinant protein was identified as the expected CyRPA protein by Western blot analysis with CyRPA-specific monoclonal antibodies.

Sequencing the CyRPA gene of different strains

Genomic DNA was prepared from *P. falciparum* strains 3D7, MAD20, FC27, RFCR3, W2met, Hb3, Ro-33, 7G8, K1 FCR3, ITG2F6, FVO, IFA4, IFA6, IFA10, IFA12, IFA18 and IFA19 and used for PCR amplification of *PFD1130w*. *PFD1130w* was amplified in three overlapping fragments with the following primer combinations: 3888 & 3899, 3896 & 3903, 3900 & 3891 (Table S1). Amplifications were performed using DreamTaq DNA polymerase (Fermentas Life Sciences) according to the manufacturers protocol with the following profile: 5 min 95°C; 35 x (30 s 95°C, 30 s 60°C, 2 min 72°C); 6 min 72°C. Amplicons were purified and subjected to direct sequencing (Macrogen, Seoul, South Korea). Sequences were analyzed using AutoAssembler 1.4.0 software (Applied Biosystems, PerkinElmer).

Accession numbers for all genes/proteins mentioned in the paper.

Sequence of genes and proteins mentioned in this study can be found on PlasmoDB (http://plasmodb.org/plasmo/) with following accession numbers: *P. falciparum* CyRPA (PFD1130w), *P. vivax* CyRPA (PVX_090240), *P. knowlesi* CyRPA (PKH_052740), *P. falciparum* MSP-1 (PFI1475w), *P. falciparum* MSP-2 (PFB0300c), *P. falciparum* MSP-3 (PF10_0345), *P. falciparum* MSP-4 (PFB0310c), *P. falciparum* MSP-5 (PFB0305c), *P. falciparum* AMA-1 (PF11_0344), *P. falciparum* EBA-175 (MAL7P1.176), *P. falciparum* GLURP (PF10_0344), *P. falciparum* SERA5 (PFB0340c), *P. falciparum* RH4 (PFD1150c), *P. falciparum* RH5 (PFD1145c), *P. falciparum* SURFIN4.2 (PFD1160c), *P. falciparum* GAPM2 (PFD1110w), *P. falciparum* PSOP12 (PFE0680), *P. falciparum* GAPDH (PF14_0598).

Acknowledgements

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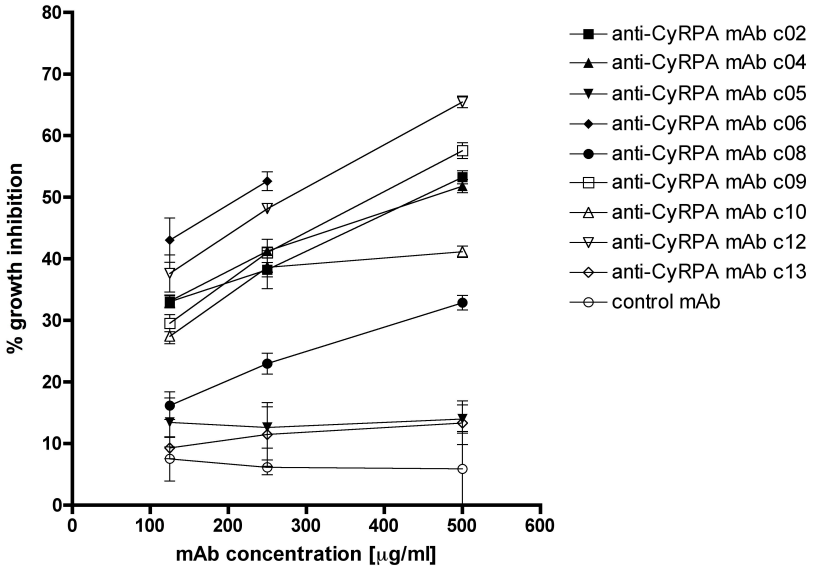
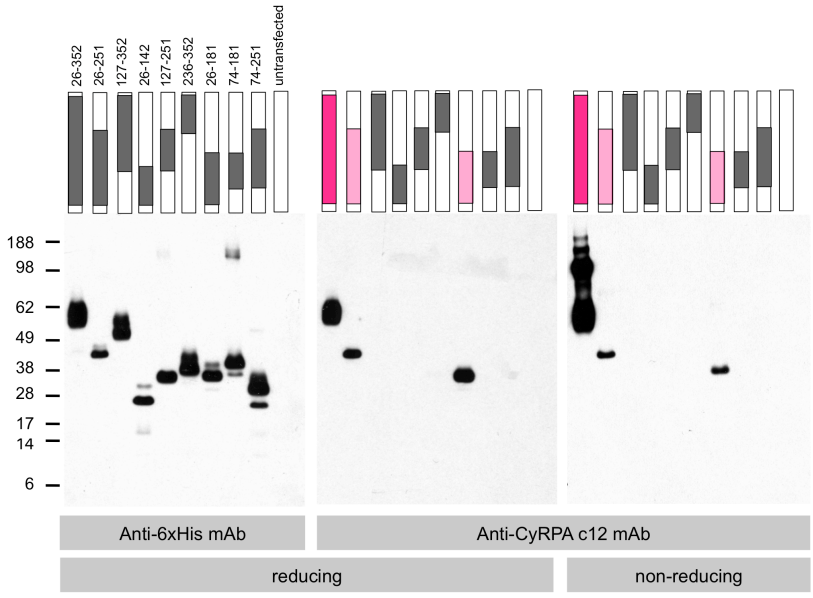
Supporting Information



Supplementary Figure 1. Some, but not all anti-CyRPA mAbs inhibit parasite growth *in vitro*. Synchronous *P. falciparum* 3D7 blood stage parasites were cultured for two cycles in the presence of different concentrations of purified anti-CyRPA mAbs. Percent parasite growth inhibition was calculated against the parasitemia of PBS control wells. Anti-6xHis-tag mAb was used as negative control mAb. Each symbol represents the mean of a triplicate experiment, and error bars indicate the standard deviation.



Supplementary Figure 2. Reactivity pattern of anti-CyRPA mAb c12 with fragments of the target antigen. Lysates of HEK cells transiently transfected with expression plasmids encoding different CyRPA protein fragments were probed with anti-CyRPA mAbs. All nine mAbs recognized fragment 26-352 (dark pink), and all except mAb c10 recognized fragment 26-251 and fragment 26-181 (light pink) (data not shown). As an example, Western blots with anti-CyRPA mAb c12 are shown in the middle and right panel. As positive control lysates were probed with anti-6xHis-tag mAb (left panel). Relative molecular masses in kDa are indicated on the left.

Supplementary Table 1. Oligonucleotide sequence of used primers.

Primer Name	Sequence (5' to 3')
3888	AAAATTTTGTAGGAAATGTTGAGAA
3899	AAAACGATGACAAGTCATATCCTTC
3896	TGTTCCTTGTATTCGTGATATGTTTT
3903	TCATCATTTAATGTAGAAACATCTTGA
3900	TGACAATTACAAATTAGGTGTGCAA
3891	CAAATATGATGATTGTTGAATGGTT
3910	CCAATGCTAGCGGCATAAATTG
3911	ATAAGATAGCGGCCGCCTTACCGTCATTTGAATAGAACCT
3912	ATACTAGCTAGCCGTAAGAAGGATATGACTTGTCACAG
3913	ATAAGAATGCGGCCGCTTTCACATTATTGATGTTGTCTCCA
3886	GCTAGCGACCTGTCCTTTCACTTTTATGTTG
3887	GCGGCCGCGTTGTAAATGCCCTGGGATG
3905	TCGGTACCTCGCGAATGC
3938	ATACTAGCTAGCGAGACCCATATCTACGTGCAG
3939	ATAAGAATGCGGCCGCCCCGCAGATGAGGAAGTAC
3986	ATGCCATGGGCATAAATTGTGACAGCC
3941	CCGCTCGAGGTTGTAAATGCCCTGGGA
3987	CCGCTCGAGCCCGCAGATGAGGAA

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RESULTS PART 3

Immunoglobulin transfer experiments in *Plasmodium falciparum* infected NOD-*scid IL2Rγ*^{null} mice.

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Summary

Antibodies specific for human malaria blood stage parasites can affect parasite growth in many ways. While various growth inhibitory mechanisms of antibodies can be observed in parasite cultures, their relevance *in vivo* has remained largely elusive. Passive transfer experiments are a good tool to assess the functionality of antibodies *in vivo*, but this approach has in the case of the *Plasmodium falciparum* malaria infection only rarely been used, since the human malaria parasites cannot infect rodent erythrocytes. Here we describe passive antibody protection studies in immuno-compromised NOD-*scid IL2Rγ*^{null} mice engrafted with human erythrocytes and infected with *P. falciparum*. We show that the growth inhibitory effect of antibodies specific for the *P. falciparum* blood stage antigen CyRPA varies with parasite density. Complement depletion experiments demonstrate that the complement system augments the anti-parasitic effect, but is not strictly required for antibody mediated growth inhibition. These results qualify the *P. falciparum* malaria NOD-*scid IL2Rγ*^{null} mouse model as suitable tool for the analysis of the multifaceted mechanisms of antibody-mediated immune protection in malaria .

Introduction

Naturally acquired antibodies specific for malaria blood stage parasites are efficacious in the prevention of malaria disease, as shown by passive transfer experiments in humans [1-4]. The specificity of the antibodies that confer protection against malaria as well as the mechanisms by which antibodies mediate protection are not fully characterized. A variety of mechanisms by which antibodies may potentially prevent disease have been suggested include i) blocking of the sequestration of infected erythrocytes to endothelial cells [5], ii) blocking of rosetting of infected erythrocytes [6-8], iii) opsonisation and destruction of infected red blood cells (iRBCs) [9,10] or merozoites [18,19] by phagocytic cells or neutrophil respiratory bursts [21] iv) complement activation [11-13,20], v) blocking of parasite development within erythrocytes [14,15], vi) neutralization of parasite-derived toxic molecules such as GPI [16,17], vii) agglutination of merozoites preventing merozoite dispersal [22,23], viii) prevention of processing of invasion proteins [24] or ix) blocking of merozoite invasion [15,25-29]. It appears that the mechanism by which antibodies inhibit parasite growth depends on the nature of the antigen.

A range of *in vitro* assays have been developed trying to assess the various mechanisms of antibody-based protection against malaria [8,10,11,20,21,24,30-33]. The most broadly used assays measure the growth inhibitory effects of immune sera or purified antibodies alone [32] or in co-operation with human monocytes [33]. *In vitro* assays are criticized for being very fragile, prone to contaminating parasite-inhibitory substances and of poor reproducibility [34]. Additionally there are contradictory results about the correlation of *in vitro* inhibitory activity of antibodies and protection in animals or humans [35-37]. In this regard, *in vivo* assays for the analysis of antibody functions are considered more reliable [38]. Several different mouse models have been used to investigate the *in vivo* protective role of antibodies specific for antigens of rodent malarial parasite species [39,40]. Assessment of *P. falciparum* specific antibodies either has relied on mice infected with genetically modified rodent malarial parasite species expressing the *P. falciparum* target antigen or on rare and costly primates susceptible to *P. falciparum* [41,42]. Alternatively,

immuncompromised mice engrafted with human erythrocytes, which support infection with *P. falciparum*, permit assessment of the protective role of specific antibodies [43,44]. Experiments using human erythrocyte engrafted BXN mice depleted in tissue macrophages showed growth inhibitory activity of passively transferred human antibodies specific for merozoite surface protein 3 (MSP3) [43]. This effect was dependent on concomitant reconstitution with human monocytes, indicating a monocyte-dependent inhibitory machanism of anti-MSP3 antibodies. Our antibody transfer experiments with human erythrocyte engrafted NOD-*scid IL2Rγ*^{null} mice showed a growth inhibitory effect of mouse antibodies specific for the recently characterized cysteine-rich protective antigen (CyRPA) [44]. In comparison to the BXN mouse model the NOD-*scid IL2Rγ*^{null} mouse model features more consistent infection with *P. falciparum* due to superior engraftment with human erythrocytes [45]. Consequently this model showed improved reproducibility and reduced mouse-to-mouse variation in drug tests as well as antibody transfer experiments [44,45]. Due to this, for the first time a dose dependent inhibitory effect of transferred antibodies could be demonstrated [44].

In the present study we have analyzed the potential role of complement in passive immune protection by anti-CyRPA mAbs. For the analysis of the effect of complement, we complement depleted mice by treatment with cobra venom factor (CVF). CVF is a functional analog of mammalian complement component C3b, the active fragment of C3 [46]. Both CVF and C3b can bind factor B and subsequently form the bimolecular C3/C5 convertases CVF,Bb or C3b,Bb, respectively. The two homologous enzymes exhibit a difference in physico-chemical stability, allowing continuous activation of C3 and C5 by CVF,Bb, leading to serum complement depletion. Furthermore we assessed the influence of the time point of administration on passive immune protection by anti-CyRPA mAbs and the *in vivo* growth inhibitory capacity of a range of monoclonal mouse antibodies specific for three other blood stage antigens considered promising vaccine candidates.

Results

Growth inhibitory effect of anti-CyRPA mAbs in relation to the parasitemia level

It was shown previously that anti-CyRPA mAbs exert a parasite growth inhibitory activity in *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice [44]. Here we assessed the parasite growth inhibitory effect of anti-CyRPA mAbs when administered either before infection of mice, at low parasitemia, or at high parasitemia (Figure 1). The group of mice having received a single dose of 2.5mg anti-CyRPA mAb 12 one day before getting infected with *P. falciparum* developed parasitemia. Parasitemia was 0.37±0.58% when measured for the first time three days after infection and did not significantly increase over the next five days (0.53±0.15% on day 6, T-test comparing day 1 with day 6; p=0.152). Parasitemia was significantly lower compared to the PBS control group from day 3 onwards (0.53±0.15% vs. 6.67±01.35% on day 6, T-test p=0.001). In the group of mice having received a single dose of 2.5 mg anti-CyRPA mAb 12 at a parasitemia of 0.53±0.06%, parasitemia increased only slightly during the next five days to 1.87±0.40%, which was significantly lower compared to the mock treated mice (13.40±3.77, T-test p=0.006). In the group of mice having received the same treatment at a parasitemia of 4.13±0.47%, parasitemia started to drop for the next three days down to 3.10±0.36%. But the following 3 days this effect was outweighed by a mild increase in parasitemia to 5.47±0.12% on day 12.

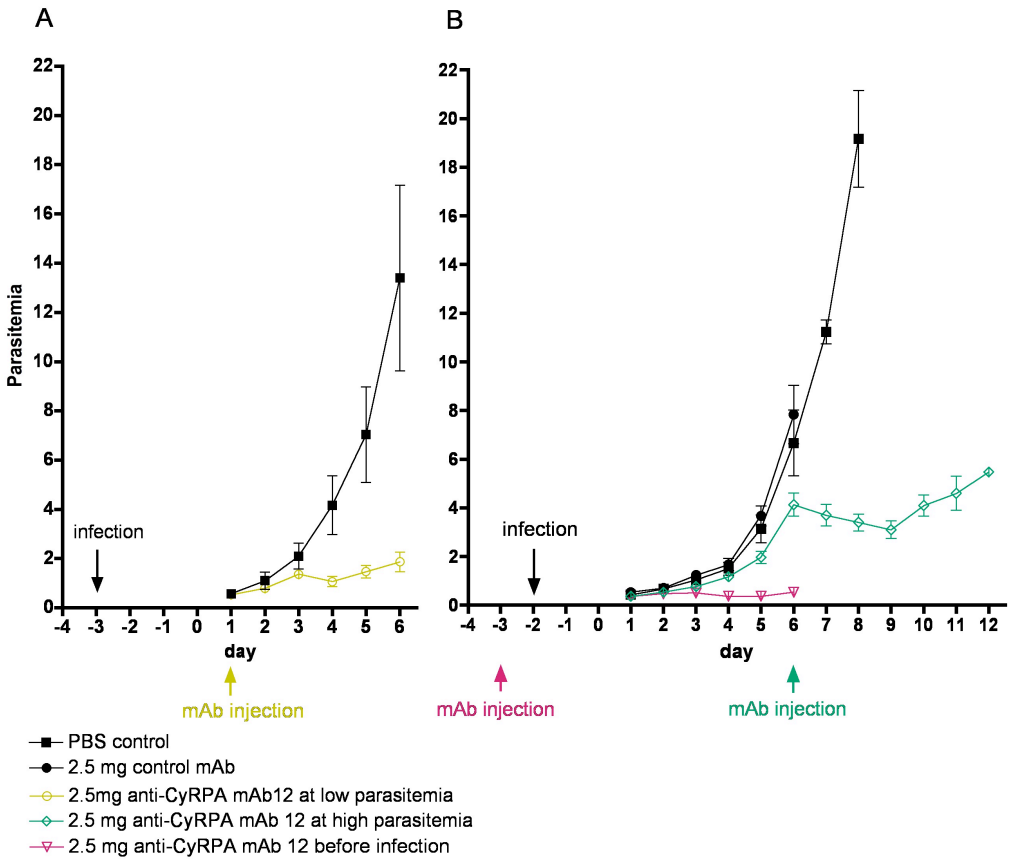

Figure 1. Implication of parasitemia on the *in vivo* growth inhibitory effect of anti-CyRPA mAbs. *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice received 2.5mg purified anti-CyRPA mAb 12 or an isotype/subclass control mAb by iv injection. Arrows indicate the day of antibody administration; one day before infection (pink), at low parasitemia (yellow) or at high parasitemia (green). Data of figure A) and B) were achieved in independent experiments. Values are the mean parasitemia ± SD in peripheral blood of three mice per group.

Role of complement for the *in vivo* parasite growth inhibitory activity of anti-CyRPA mAbs

Anti-CyRPA mAbs inhibit parasite growth not only in a mouse model but also in a parasite growth inhibition assay (GIA) using *P. falciparum in vitro* cultures without addition of phagocytes [44]. Hence anti-CyRPA mAbs have a direct *in vitro* red blood cell invasion inhibitory activity [44], which is independent of additional elements of the immune system, like complement and immune cells. Nevertheless such factors may enhance *in vivo* growth inhibitory activity of anti-CyRPA mAbs. To assess the potential impact of complement, we performed passive immunisations of *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice (Figure 2) depleted of complement by daily intraperitoneal injections of CVF.

Complement depletion per se had no marked effect on the growth of asexual blood stage *P. falciparum* parasites in NOD-*scid IL2Rγ*^{null} mice. On day 6 mean parasitemia in mice depleted of complement was higher compared to non-depleted mice, but this difference was not significant (16.53±2.30% vs. 13.40±3.77%, T-test p=0.287). The dose dependent and statistically significant growth inhibitory effect of anti-CyRPA mAb 12 was observed in both complement-depleted and non-depleted mice. But parasitemia on day 6 was significantly higher in CVF-treated mice compared to non-treated mice, indicating that complement enhances the inhibitory activity of ant-CyRPA mAbs. Treating mice with 2.5mg anti-CyRPA mAb increased the parasite doubling time by 3.07 times compared to the PBS control when complement was present, but only by 2.08 times compared to the PBS/CVF control when complement was depleted.

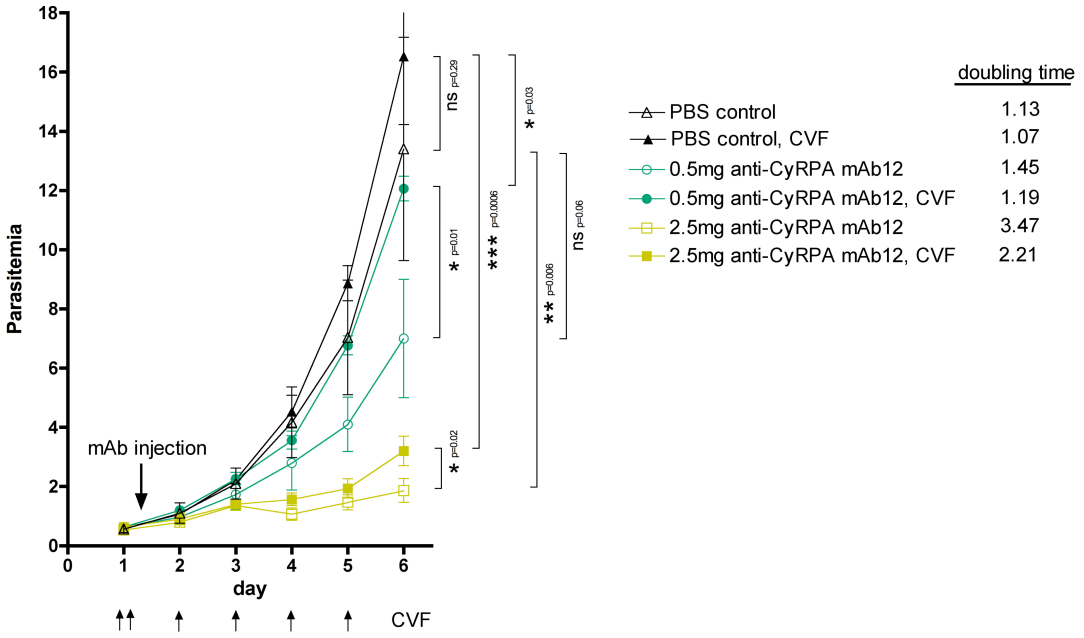

Figure 2. Parasite growth inhibitory effect of anti-CyRPA mAbs in complement depleted mice. *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice received 0.5mg (green) or 2.5mg (yellow) purified anti-CyRPA mAb 12 or just PBS (black) by iv injection. Three groups of mice (closed symbols) were complement depleted by intraperitoneal injections of 5 units cobra venom factor (CVF) 7h and 3h before antibody/PBS injection and daily thereafter (arrows). Values are the mean parasitemia in peripheral blood of three mice per group. Data are means ± SD. Doubling times were calculated from fitted exponential regression lines (R^2=0.720-0.996).

Assessment of the growth inhibitory potential of mAbs specific for the blood stage antigens D13, AMA-1 and MSP-3 in the *P. falciparum in vivo* mouse model

A range of malaria asexual blood stage antigens have been described as targets of *in vitro* growth inhibitory antibodies, independent or dependent of human monocytes. These include the apical membrane antigen 1 (AMA-1), the merozoite surface protein 3 (MSP-3) and D13 [47-49]. Here we assessed monoclonal mouse antibodies specific for these antigens for their *in vivo* growth inhibitory capacities (Figure 3). Mice with a mean parasitemia of 0.64±0.10 received a single intravenous administration of purifed mAbs and parasitemia was measured for the next five days. A statistically significant antibody dependent parasite growth inhibitory effect was measured for the positive control, anti-CyRPA mAb 12, but not for D13-, AMA-1- and MSP-3 specific mAbs (Figure 3).

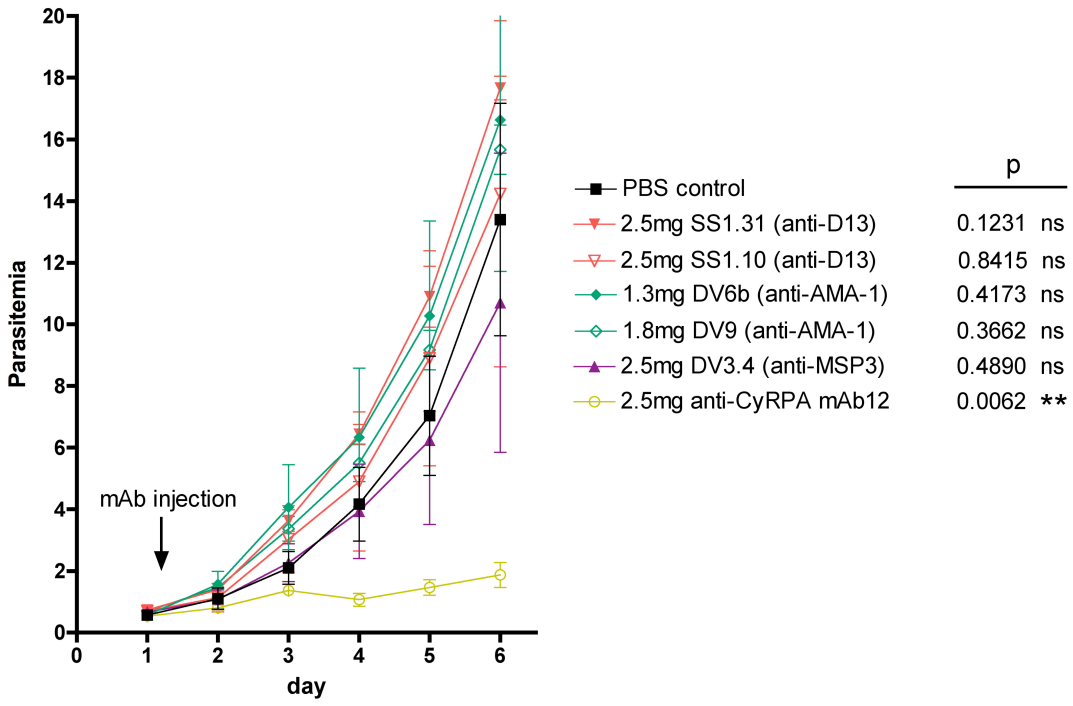

Figure 3. *In vivo* growth inhibitory activity of mAbs specific for different malaria blood stage antigens. *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice received a single intravenous injection of purified mAbs specific for D13 (red), AMA-1 (green), MSP-3 (purple) or CyRPA (yellow). Control mice received mock treatment with PBS (black). Parasitemia was measured over the next five days. Values are the mean parasitemia ± SD in peripheral blood of three mice per group. Shown are p-values for t-tests comparing parasitemias on day 6 of treatment group with the negative control group (PBS). ns; not significant.

Discussion

Passive transfer of immunoglobulin preparations from immune sera in humans was shown to result in a profound but non-sterilizing drop in parasitemia [3,4]. Clearance of parasites and symptoms was as fast or faster than with drugs. Passive immunisation of *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice with monoclonal anti-CyRPA antibodies either resulted in a stagnation in parasitemia when antibodies were administered at 0.5% parasitemia or a transient but moderate decrease in parasitemia when admininstered at 4% parasitemia. In passive immunisation experiments with MSP3-specific human antibodies a more profound decrease in parasitemia has been described [43]. Whether these difference are due to inherent differences of the protective mechanisms of the antibodies or due to profound differences in the animal models used remains to be addressed.

Passive transfer of anti-CyRPA mAbs one day before infection of mice did not prevent establishment of infection but after 5 days had a significant inhibitory effect on the course of parasitemia. In summary our data shows the higher parasitemia, the stronger the growth inhibitory action of anti-CyRPA mAbs. Mechanisms explaining this effect remain to be identified. Additionally, so far only the effect of monoclonal antibodies was assessed. But considering CyRPA as a malaria blood stage vaccine antigen, it will be important to assess the inhibitory capacity of polyclonal CyRPA-specific antibodies. However, the most important question remained to be answered, is whether anti-CyRPA antibodies would also have a parasite growth inhibitory effect in humans. This information would not only reflect the potential of CyRPA as malaria blood stage vaccine candidate, but additionally would disclose the predictive value of the SCID-mouse based *P. falciparum in vivo* model.

Innate immune factors play a crucial role in reducing the parasite load in malaria infections. On the other hand host innate responses were found to contribute to the pathophysiology of severe malaria [50]. One component of the innate immune system found to be important during malaria infections is the complement system. Human malaria patients were shown to have decreased serum concentrations of C3 and C4 and elevated serum concentrations of C3d, suggesting complement

activation by malaria infection [51]. Indeed, erythrocytes infected with *P. falciparum* were shown to activate the alternative complement pathway. In the presence of parasite-specific antibodies, activation was augmented by the classical pathway [11], leading to C3b binding to the infected erythrocyte. Surface bound complement did not result in cell lysis but possibly facilitates opsonization and elimination of the infected cells by phagocytes. Additionally, certain antibodies specifically binding to the surface of merozoites were found to exert a complement dependent parasite growth inhibitory action, probably by causing complement-mediated damage of merozoites [20]. Hence, merozoite antigens may generally be targets of antibody-mediated activation of complement. This assumption is in agreement with the finding that the decrease in serum concentrations of C3 and C4 in malaria patients occurs at the time of merozoite invasion [52,53].

Complement depletion of *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice had no significant effect on the course of infection. This indicates that potential activation of the alternative complement pathway had no marked effect on the parasitemia. The inhibitory effect of anti-CyRPA mAbs was observed irrespective of whether the complement system was intact or depleted. These results, together with the fact that anti-CyRPA mAbs also inhibit parasite growth *in vitro* in absence of additional elements of the immune system [44], prove that the inhibitory mechanism of anti-CyRPA mAbs is independent of complement. However, the *in vivo* inhibitory effect of anti-CyRPA mAbs was diminished by complement depletion, indicating that the complement system in combination with the antibodies exerts some parasite growth inhibition enhancing effect. Anti-CyRPA mAbs were shown to retard merozoite invasion [44]. Consequently hampered merozoites with bound anti-CyRPA mAbs possibly activate the classical complement pathway resulting in the opsonization of merozoites, which could further reduce the invasion success of the merozoites.

Having shown that complement enhances but is not a strict prerequisit for the inhibitory effect of CyRPA antibodies, the question remains open as to whether other immune factors are involved in anti-CyRPA mediated effect *in vivo*. Hence, besides inhibiting merozoite invasion, CyRPA antibodies conceivably promote

opsonisation and destruction of merozoites by phagocytic cells [18,19] or by neutrophil respiratory bursts [21].

Even though antibodies specific for AMA1, MSP3 and D13 were shown previously to inhibit parasite growth *in vitro* [47-49], a range of monoclonal antibodies specific for these antigens tested by passive immunsiation of *P. falciparum* infected NOD-*scid IL2Rγ*^{null} mice showed no growth inhibitory effect *in vivo*. A possible explanation for this discrepancy could be that the tested mAbs have the wrong fine specificity. For several blood stage antigens it has been shown that the fine specificity of the antibodies is crucial for their functionality [54,55]. Notwithstanding the above, the remarkable results obtained with antibodies specific for the merozoite protein CyRPA adverts the exceptional characteristics of this protein and highlights the potential of CyRPA as malaria blood stage vaccine antigen.

Materials and Methods

In vivo growth inhibition assay

Antibodies were tested in the murine *P. falciparum* model essentially as described [56]. The only modification implemented was that daily human blood injections (0.75ml) were administered by the intravenous route instead of the intraperitoneal route. Mice received a single dose of monoclonal antibody in PBS by intravenous injection either one day before infection or when parasitemia was approximately 0.5 or 4%. To deplete complement mice received 5 units of purified cobra venom factor (CVF, Quidel) in 200 µl sterile saline 7 and 3 hours before infection and daily thereafter.

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RESULTS PART 4

Pfs12p/PFF0620c, a 6-cysteine protein expressed in gametocytes and sporozoites.

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Summary

Among protein classes of *Plasmodia* the family of 6-cysteine proteins has gained major interest in malaria research. 6-cysteine proteins are characterized by the presence of one or several s48_45 domains, containing six positionally conserved cysteine residues, which are predicted to form disulfide bridges resulting in a β-sandwich structure. Various members are characterized as surface ligands involved in cell-cell interaction. Individual 6-cysteine proteins were shown to be implicated in liver stage development, merozoite invasion or parasite fertilization. Of 11 members encoded by the genome of *P. falciparum*, PFF0620c/Pfs12p has not yet been characterized. In the present study we used specific antibodies to look at the expression of PFF0620c at the protein level in different life-cycle stages of *P. falciparum*. By immunofluorescence staining protein expression of PFF0620c was detected in stage I to stage V gametocytes and salivary gland derived sporozoites. However, partly conflicting results attained by western blot analysis of stage specific lysates calls for further experiments. The tentative stage specific expression of PFF0620c in gametocytes and sporozoites suggests a possible role in fertilization, penetration of mosquito gut lining, sporozoite motility or hepatocyte invasion. In this regard, PFF0620c-specific antibodies may be applied for transmission and hepatocyte-invasion blocking experiments.

Introduction

Malaria is caused by an infection with a eukaryotic protist of the genus *Plasmodium.* The parasite undergoes a complicated life cycle involving a mosquito of the anopheles genus as primary host and transmission vector and a vertebrate as secondary host. For the major part of the life cycle the parasite remains intracellular, developing within hepatocytes and erythrocytes of the vertebrate host. Only the merozoite, the form that invades erythrocytes, the gamete, developing from intracellular gametocytes upon ingestion by a mosquito, the ookinete and oocyst that result from gamete fertilisation, and the sporozoite, the form that is introduced by a mosquito bite and subsequently invades liver cells, are extracellular. These extracellular stages undergo highly specific cell-cell interactions; merozoites invade erythrocytes, sporozoites invade hepatocytes, male and female gametes fuse for fertilisation and ookinetes penetrate the gut lining of the mosquito. All these processes depend on highly specific interactions of cell-surface proteins. As a consequence, surface proteins and proteins released to the cell surface from apical secretory organelles upon invasion of extracellular stages are of major scientific interest, as they present the key to understand the complex processes of parasite-host/parasite-parasite cellular interactions. Besides, these molecules present the Achilles' heel of malaria infection. Therefore it is no surprise that most malaria vaccine antigens under development are surface proteins or proteins released from apical secretory organelles of extracellular stages [1].

Among protein classes of *Plasmodia* the family of 6-cystein proteins has gained major interest in malaria research. Members of the 6-cysteine protein family are exclusively found in Apicomplexan species. 6-cysteine proteins are characterized by the presence of one or several s48_45 domains. These domains are approximately 120 amino acids in size and contain six positionally conserved cysteine residues, which are predicted to form disulfide bridges resulting in a β-sandwich structure [2]. The *Plasmodium* genomes encode 11 members of this protein family. All of them have a secretion signal peptide and 8 are predicted to be GPI anchored [3-5], rendering them hypothetical surface proteins. For 10 members it was shown

experimentally that they locate to the parasite surface or within apical secretory organelles. Additionally they were found to be expressed exclusively in one specific extracellular parasites stage [3,6-15]. Consequently they got considered to play an important role in cell-cell interactions. This was shown for the gamete surface protein P48/45, the male gamete surface protein P230 and the female gamete surface protein P47, which were found to be essential for parasite fertilization [6,16,17]. Additionally, antibodies specific for P48/45 [12,18,19] and P230 [20-24] were shown to prevent zygote formation and thus block transmission of the parasites. Furthermore, deletion of the sporozoite proteins P52/P36p and/or P36 resulted in aborted development in hepatocytes [9,25-27]. Immunisation with these mutant sporozoites induced protective immune responses against challenge with wild-type sporozoites in rodent models [9,25]. Four 6-cysteine proteins expressed in late asexual stages, P12, P38, P41 and P92, were found to localize to the merozoite surface or to apical secretory organelles [3,5]. Pf92 was found to be refractory to genetic deletion, suggesting that it plays an important role in blood-stage development [28]. For all four 6-cystein proteins expressed in merozoites erythrocyte binding peptides were identified, which inhibited merozoite invasion *in vitro*, suggesting a possible role of these proteins during erythrocyte invasion [29,30]. Taken together, studies of different 6-cystein proteins either proved or suggested a function in cell-cell interaction. Accordingly some of them are considered promising malaria vaccine candidate antigens [24,31].

However, there is one member of the predicted 6-cystein-protein family of *Plasmodium falciparum* that has not yet been characterized at all; PFF0620c, sometimes referred as Pfs12p due to its close proximity to Pfs12. No transcipt data is available for PFF0620c except for asexual blood stages, where transcript levels are consistently low. But by mass spectrometry the protein has been detected in oocyst derived *P. falciparum* sporozoites [32]. By contrast, its ortholog in *P. berghei*, PBANKA_011110, was detected in gametocytes [11]. In order to characterize this protein in more detail we have generated monoclonal antibodies specific for PFF0620c [33]. In the present study we use specific antibodies to look at the

expression of PFF0620c at the protein level in different life-cycle stages of *P. falciparum*.

Results

PFF0620c is a member of the 6-cysteine protein family

ORF *pff0620c* is positioned on chromosome 6 and is predicted to encode a protein with an N-terminal secretion signal peptide, two s48_45 domains and a GPI-anchor [34-37,5]. Orthologs of PFF0620c are present in all *Plasmodium* genomes available to date; *P. vivax*, *P. knowlesi*, *P. berghei*, *P. yoelii* and *P. chabaudi* [38]. Comparison of these protein sequences revealed conservation with 37-50% identity (Figure 1). In contrast to its orthologs the second s48_45 domain of PFF0620c is interrupted by 40 amino acid long asparagine/aspartic-acid low complexity region.

PFF0620c is highly conserved

Sequencing of the *pff0620c* sequences of 17 *P. falciparum* strains has identified two synonymous and five non-synonymous single nucleotide polymorphisms (SNPs) [39-41]. Three non-synonymous SNPs result in asparagine/aspartic acid dimorphisms within the asparagine/aspartic acid stretch, one SNP results in a leucine/phenylalanine dimorphism at the very C-terminus of the GPI-attachment signal sequence and one results in a glutamic acid/lysine dimorphism within the N-terminal s48_45 domain (Figure 1).

PFF0620c is expressed in gametocytes and sporozoites

No transcipt data is available for PFF0620c except for asexual blood stages, where transcript levels are consistently low [42]. By mass spectrometry the protein has been detected in oocyst derived *P. falciparum* sporozoites [32]. But its ortholog in *P. berghei*, PBANKA_011110, was detected in gametocytes [11]. To further assess protein expression we used PFF0620c-specific mouse mAbs for immunofluorescence staining of different parasite stages of *P. falciparum*. Gametocytes, from stage I through to stage V (Figure 2A), as well as salivary gland derived sporozoites (Figure 2C) were stained by anti-PFF0620c mAbs. With asexual blood stage parasites no staining was observed, except a very faint staining of schizonts. In contrast to Pfs25, a marker for late stage gametocytes and gametes, PFF0620c was already detected in

MUSCLE (3.7) multiple sequence alignment

Figure 1. Amino acid sequence alignment of PFF0620c with its orthologs. Full-length putative orthologs of *P. falciparum* PFF0620c are found in the genomes of the human plasmodial species *P. vivax* (PVX_113780) and *P. knowlesi* (PKH_116310) and in the genomes of the rodent plasmodial species *P. chabaudi* (PCHAS_011170), *P. berghei* (PBANKA_011110) and *P. yoellii* (PY03099). Sequence identities are 37-50%. "*"; identical, ":"; conserved substitutions, "."; semi-conserved substitution. Positionally conserved cysteine residues are shaded in grey. Amino acid residues of the predicted secretion signal sequences are colored in pink. Residues of predicted s48-45 domains are colored in blue. Predicted ω-sites are colored in red. Among 17 *P. falciparum* strains four amino-acid dimorphisms were found within PFF0620c (shaded in yellow).

stage I gametocytes and expression intensity increased marginally during gametocytogenesis.

Correspondingly, expression of PFF0620c in different life-cycle stages was assessed by Western blot analysis of cell lysates using anti-PFF0620c specific mAbs (Figure 3). With gametocyte lysate a discrete 38kDa band was detected (red arrow), corresponding exactly to the molecular weight of PFF0620c predicted form the amino acid sequence without signal peptide and GPI-anchor attachment signal. For asexual lysates a very faint band was detected, which in the case of strain 3D7 was at the same size as the band with 3D7 gametocyte lysates, and a little bit lower in the case of strain K1 (approximately 35kDa) (blue arrow, Figure 3A). In K1 trophozoite lysate an additional band of approximately 120kDa was detected (green arrow). The 38 and 35 kDa bands were not detected when proteins had been reduced (Figure 3C). In contrast, the 120kDa band was visible under reduced and non-reduced conditions. In lysate of salivary gland derived sporozoites no band was detected. Western blot analysis of asexual blood stage lysates with mAbs specific for Pfs48/45, a marker for early gametocytes, indicated that asexual blood stage lysates of neither the gametocyte producing strain 3D7 nor the non-producing strain K1 contained traces of gametocytes (Figure 3B). Correspondingly, presence of the major sporozoite surface protein (CSP) was detected in the sporozoite lysate (Figure 3B).

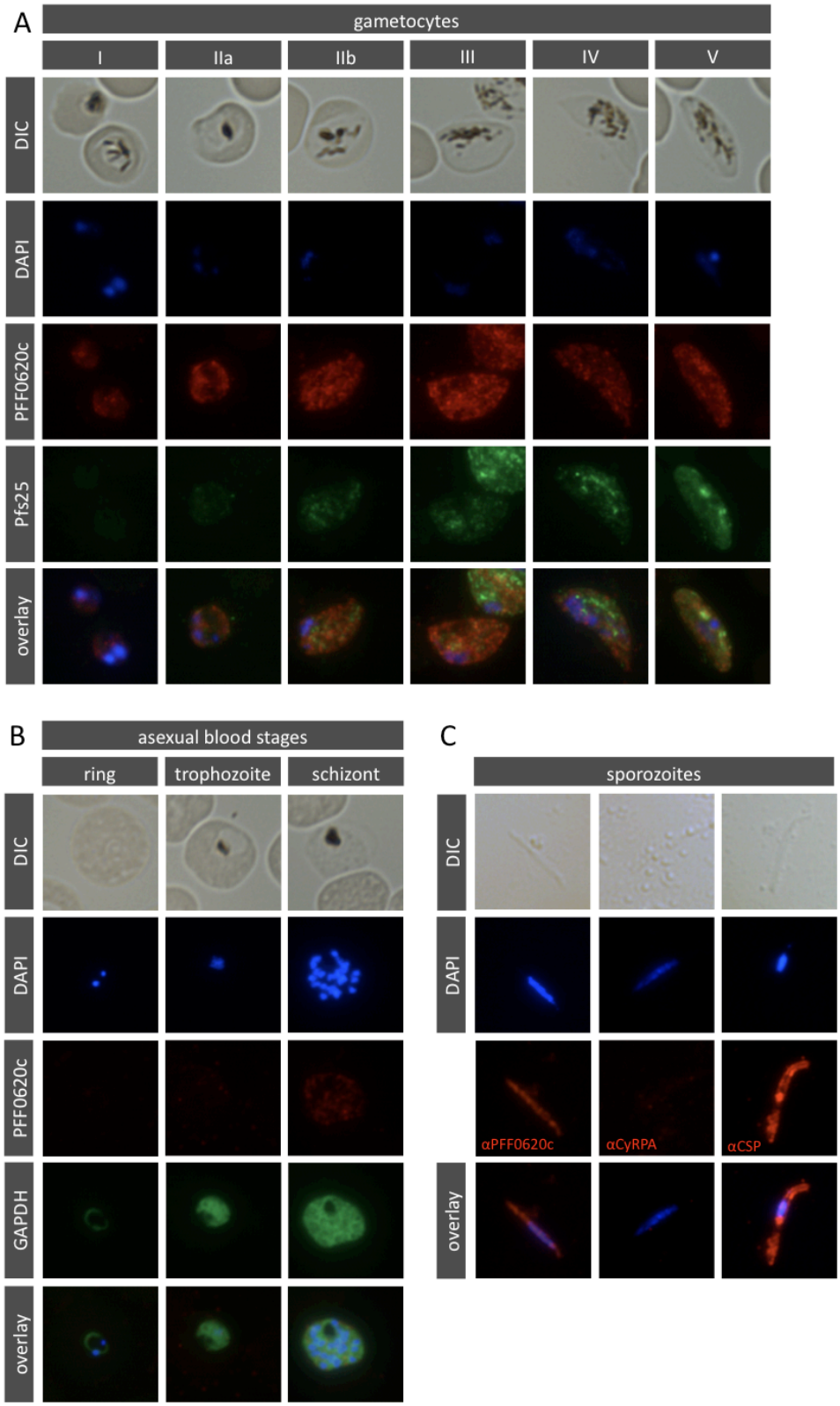

Figure 2. Detection of PFF0620c expression in different life-cycle stages of *P. falciparum* by immunofluorescence staining. Acetone-methanol fixed stage I, II, III, IV and V gametocytes (A), asexual blood stages (B), and salivary gland derived sporozoites (C) were probed with PFF0620c-specific mAb c29 by indirect immunofluorescence staining. Gametocytes were costained with anti-Pfs25 rabbit sera. Asexual ring, trophozoite and schizont stages were costained with anti-GAPDH mAb. Sporozoites were stained with PFF0620c-specific mAb c29 or anti-CSP mAb.

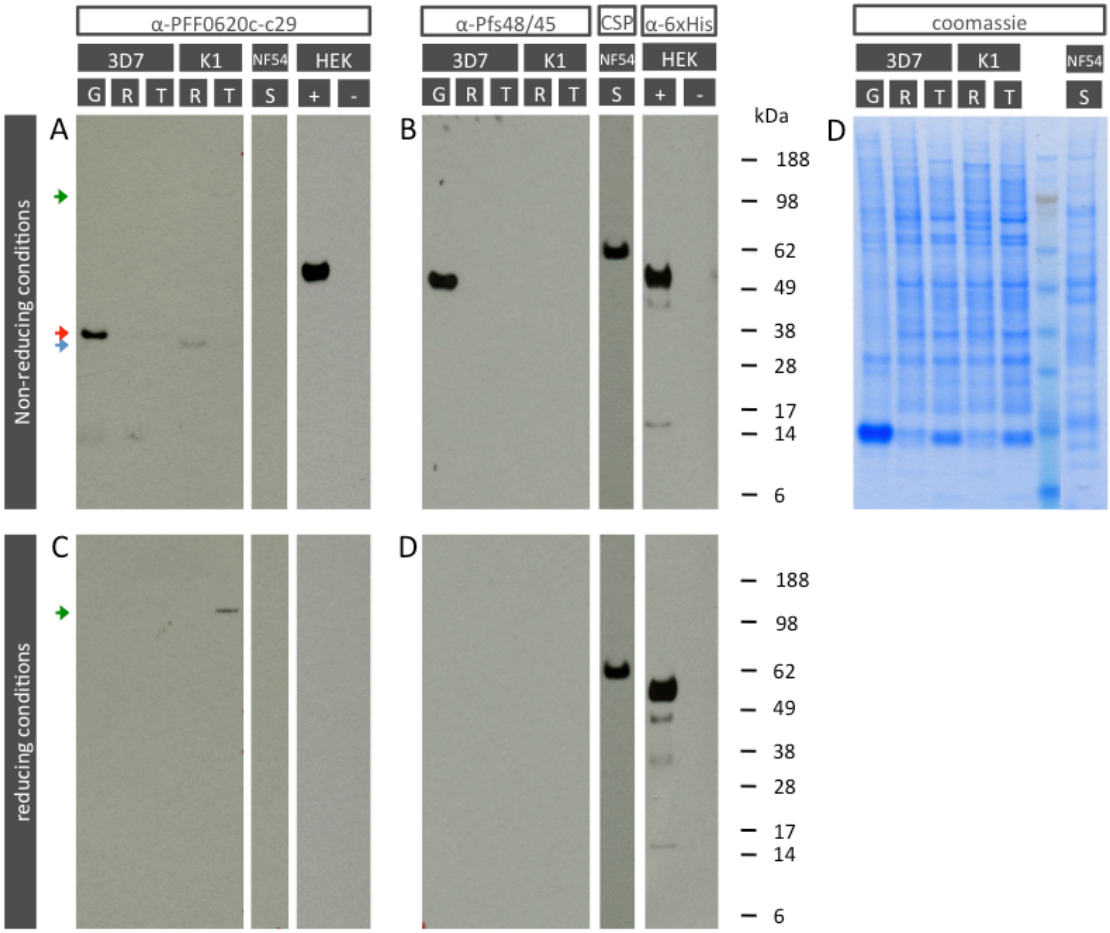

Figure 3: Detection of PFF0620c expression in different life-cycle stages of *P. falciparum* by Western blot analysis. Either reduced (C) or non-reduced (A) protein lysates of gametocytes (G), asexual ring (R), trophozoite (T) stages of either a gametocyte high producing strain (3D7) or a non-producer strain (K1), and salivary gland derived sporozoites (S) were probed for expression of PFF0620c by Western blot analysis using anti-PFF0620c mAb c29. As positive control non-transfected (-) and PFF0620c-expressing (+) HEK cell lysates (HEK) were used. Monoclonal antibodies specific for Pfs48/45 (early gametocyte marker), for CSP (sporozoite marker), or an anti-6xHis mAb were used as positive controls (B, D). For all parasite lysates equal amounts of total protein was loaded per lane and verified by coomassie staining (D).

Discussion

In the present study the expression of PFF0620c, a previously unstudied member of the 6-cysteine protein family, in different *P. falciparum* life stages was assessed using specific mAbs. Our immunofluorescence staining results revealed expression of PFF0620c in gametocytes, sporozoites and schizonts. Expression was highest in gametocytes, lower in sporozoites and borderline in schizonts. Accordingly a discrete band of the expected size was detected in gametocyte lysates by Western blot analysis. In asexual blood stage lysates of strain 3D7 a very weak band of the same size was detected. This does not correlate to immunofluorescence staining results, where only a very faint staining of schizonts was observed. Contamination of asexual blood stage lysate with gametocytes could be excluded, as western blot with an early gametocyte marker was negative. Interestingly in asexual blood stages of strain K1 no corresponding band, but a slightly lower band was detected, which could indicate, that PFF0620c of strain K1 has a lower molecular weight compared to 3D7. Another explanation could be that anti-PFF0620c mAbs cross-react with an asexual blood stage antigen, possibly another member of the 6-cysteine protein family. The 38 and 35kDa band appear only with non-reduced parasite lysates. The same calls true for the 55kDa band with recPFF0620c-expressing HEK cell lysate. This indicates that the anti-PFF0620c mAb recognizes a conformational epitope, which is lost upon disulphide bond destruction. In contrast, the 120kDa band detected with trophozoite lysate of strain K1 is also detected with reduced lysate, suggesting that this band derives from PFF0620c non-related cross reactivity. In sporozoites PFF0620c was detected by immunofluorescence staining but not with western blot analysis. One possible explanation could be that the sporozoite lysate was too diluted. This could hold true even though we aimed for equal loading of total protein for lysates of different parasite stages, as this preparation is a crude extract from mosquito salivary glands, not only containing sporozoites. Discrepancies in Western blot and immunofluorescence results could be explained by PFF0620c-unrelated crossreactivity of the mAbs or by stage dependent protein modifications differentially influencing epitope recognition by the Abs under Western blot or cell staining conditions.

Eight members of the 6-cystein protein family, including PFF0620c, form head to tail arranged tandem paralog pairs within the genome [43]. In contrast to all other pairs, PFF0620c and its tandem pair Pfs12 (PFF0615c) are expressed at different time points of the parasite life cycle; PFF0620c being mainly expressed in gametocytes and sporozoites, whereas Pfs12 is expressed in merozoites [3,5]. This shows that expression of tandem-arranged genes encoding 6-cystein proteins is not coercively co regulated.

PFF0620c is a highly conserved protein; among 17 strains only five non-synonymous single nucleotide polymorphisms were identified in the PFF0620c encoding sequences [39-41]. Three of them are asparagine/aspartic acid dimorphisms within the asparagine/aspartic acid stretch and one is positioned at the C-terminus of the GPI-attachment signal sequence. Therefore the only SNP possibly having conformational or functional effects is the glutamic acid (negatively charged)/lysine (positively charged) dimorphism within the N-terminal s48_45 domain. High sequence conservation could speak for absence of immune pressure on PFF0620c or alternatively for a function of PFF0620c requiring high conservation. In case that continuative research identifies PFF0620c as target of protective immune responses, rendering PFF0620c a potential vaccine antigen, low polymorphism would reduce the risk of allele specific resistance.

As expression of PFF0620c was detected in gametocytes and salivary gland derived sporozoites, it is highly probable that the protein is also expressed in intermediate stages, meaning gametes, ookinetes and oozysts. Therefore possible functions of PFF0620c comprise fertilization, penetration of mosquito gut lining, sporozoite motility or hepatocyte invasion. Identification of the function of PFF0620c calls for the analysis of mutant parasites or the conduction of transmission and hepatocyte-invasion blocking experiments with PFF0620c-specific antibodies.

Materials and Methods

Culture of *P. falciparum*

P. falciparum strain 3D7 was cultured essentially as described previously [44]. The culture medium was supplemented with 0.5% AlbuMAX (Gibco) as a substitute for human serum [45]. Cultures were synchronized by sorbitol treatment [46]. Erythrocytes for passages were obtained from the Swiss Red Cross (Switzerland). Gametocytes of 3D7 *P. falciparum* were generated *in vitro* by using a modification of a method previously described [47].

Immunofluorescence staining of *P. falciparum* blood stages and sporozoites

For indirect immunofluorescence microscopy, smears of infected red blood cells or air-dried salivary gland sporozoites (*P. falciparum* strain NF54) attached to microscope slides were fixed in 60% methanol and 40% acetone for 2 min at −20°C and blocked with 1% BSA in PBS. Cells were probed with the following primary or secondary antibodies: unlabeled or biotin-labeled mouse anti-PFF0620c mAb c29 [48], mouse anti-CyRPA mAb c06, mouse anti-CSP mAb EP9 [49], Alexa488-labeled mouse anti-GAPDH 1.4a mAb [50], anti-Pfs25 rabbit serum (obtained through the Malaria Research and Reference Reagent Resource Center MR4 (MRA-38), deposited by C. Gowda), Alexa568-labeled donkey anti-mouse IgG (Invitrogen), Alexa568-labeled streptavidin (Invitrogen) and FITC-labeled goat anti-rabbit IgG. Slides were mounted in mounting medium containing DAPI (ProLong Gold antifade reagent with DAPI, Invitrogen). Fluorescence microscopy was performed on a Leica DM-5000B using a 60x oil immersion objective lens and documented with a Leica DFC300FX digital camera system. Images were processed using Leica Application Suite and Adobe Photoshop® CS3.

Western blot analysis of *P. falciparum* blood stages, sporozoites and transfected HEK cells

Parasite and HEK cell lysates were prepared and western blot was performed as described previously [33]. Membranes were probed with the following primary and secondary antibodies: anti-PFF0620c mAb c29 [33], anti-Pfs48/45 mAb (obtained

through the Malaria Research and Reference Reagent Resource Center MR4 (MRA-316), deposited by LH Miller, A Saul), anti-CSP mAb EP9 [49], anti-6xHis mAb [33] and horseradish peroxidase conjugated anti-mouse IgG mAb (GAM/IgG (γ-chain)/HRP, Kirkegaard & Perry Laboratories).

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DISCUSSION

It was May 14, 1796, when Edward Jenner inoculated the 8-year-old boy, James Phipps, with matter from a cowpox lesion. Two months later, the boy was challenged with matter from a fresh smallpox lesion - no disease developed [1,2].

Although the Royal Society did not accept his work for publication, it stands for the beginning of vaccination. It was not until a century later that it was discovered that infections are caused by microbes. It was Luis Pasteur who started to rationally develop vaccines by generalizing Jenner's idea to the basic principle: isolation, inactivation and injection of the causative agent [3]. Following these principles, vaccines against rabies, polio, measles, mumps and rubella were developed. In the course of the 20th century another strategy, the so-called 'subunit vaccine approach', was applied for the development of vaccines, mainly against bacterial infections. Isolation, inactivation and injection of bacterial toxins ensued vaccines against diphtheria and tetanus. In contrast, for vaccines against *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*, among others, capsular polysaccharides were applied. These and other vaccines allowed protection against many once lethal diseases. Together with the discovery of antibiotics, these developments are THE milestones in the history of medicine. However, for some infectious diseases these conventional approaches have so far not been crowned by success. Consequently, new approaches for vaccine development need to be considerer. Remarkable progress was achieved by the introduction of adjuvants and new technologies like recombinant DNA and chemical conjugation of polysaccharides to carrier proteins. However, it is the start of the genomic era, which is thought to revolutionize vaccine development.

Reverse Vaccinology; a prospective for malaria vaccine development.

Sequencing of the entire genome of infectious agents now suddenly gives full access to all the proteins that a microorganism can potentially encode. The genome as a whole can therefore be screened, using bioinformatics approaches, to identify genes with the desired properties. This approach, referred to as reverse vaccinology, is expected to identify vaccine targets in a more rational and faster way compared to traditional methodologies [4]. Criteria to select hypothetical proteins from the genome for subsequent experimental assessment are versatile and depend on the disease and the type of vaccine that is aspired. Possible selection criteria include surface expression, homology to known targets of protective immunity or, more generally, the prediction of being implicated in motility, adhesion, invasion, secretion, signalling, immune evasion or toxicity. Integrating comparative genome analysis may further help to identify candidates associated with virulence, which are conserved among the pathogen population or have no homology to self-antigens. Additional criteria could incorporate information from functional genomics, like transcription analysis or from proteomics. The prime example, demonstrating the potential of reverse vaccinology, was the development of a novel vaccine against *Neisseria meningitidis* serogroup B by Chiron (now Novartis) launched in 2000 [5,6]. Using computer analysis, hypothetical cell-surface proteins or homologs of known proteins associated with virulence and pathogenesis were selected from the entire genome. These hypothetical proteins were recombinantly expressed, purified and used to immunize mice. Immune sera were then tested in ELISA, FACS and Western blot analysis in order to confirm their surface-exposure. Immune sera were also tested in complement-mediated bactericidal assays for protective activity. Finally, positive hits were tested for presence and conservation in a panel of *N. meningitidis* variants [7]. In the end, the five most promising candidates were combined for vaccine formulation. The vaccine has been tested in phase III clinical trials, which showed that the vaccine induces broad protective immunity [8].

Decades of malaria research have identified only a few promising vaccine candidates. To date, solely two vaccines were able to induce up to 50% protective efficacy [9,10]. This clearly is a milestone in malaria research, but, on the other hand, it is an anticlimax considering the invested time and efforts. Explanations for the rather modest progress in malaria vaccine development may be manifold - partly political, but mainly scientific in nature. Most of the advanced vaccine candidates are among the proteins discovered in the early days of molecular analysis of the malaria parasite in the 1980's [11]. Current candidates represent less than 0.5% of the entire genome. Bearing in mind that the malaria parasite expresses more than 5'000 different proteins during its life cycle, calls for the selection of new candidates based on more rational criteria have been raised [12]. This leaves us to ask how to select those antigens, which mediate protective immunity?

In 2002 the genome sequence of *P. falciparum* has been completed [12]. This was a prerequisite for the elucidation of the *P. falciparum* transcriptome and proteome [13–18]. Together, this information presents an opportunity to use high throughput approaches to identify novel vaccine antigens. There are a few studies that applied the principle of reverse vaccinology for malaria [19]. Intended as a community resource to aid malaria vaccinologists, a database (MalVac) containing information of known vaccine candidates and predicted adhesins was established [20]. The database houses information on 161 *P. falciparum* proteins that were analysed for features important from the view of vaccinology; predictions for orthologs, paralogs, transmembare topologies, beta helix supersecondary structural motifs, subcellular localization, similarity with human proteins, antigenic regions, conserved domains, B- and T-cell epitopes and allergens are summarized [20]. There are at least two studies applying the combination of proteomics and serological analysis known as SERPA (serological proteome analysis) for identification of novel potential vaccine candidates. Doolan et al. used a protein microarray spotted with 250 *P. falciparum* proteins expressed in an *E. coli* based cell-free *in vitro* transcription/translation system to test human sera from volunteers immunized with radiation-attenuated sporozoites, which were either protected or non-protected to experimental challenge [21]. Three novel antigens recognized by protected but not by unprotected

volunteers may be of interest for vaccine development. In contrast, Crompton et al. used a protein microarray spotted with 1204 proteins selected based on stage-specific expression, subcellular localization, secondary protein structure or documented immunogenicity to analyze sera from malaria exposed Malian children [22]. For 49 proteins antibody reactivity was significantly higher in protected vs. susceptible children. These included the vaccine candidates STARP, LSA-1 and RESA but did not include MSP-1, MSP-2, AMA-1, LSA-3 or CSP. Furthermore, available genome, transcriptome and proteome data was used to identify protein function or to identify subsets of proteins likely to be involved in merozoite invasion or to be merozoite surface proteins [23–25]. Although all these studies identified proteins with highly interesting attributes for malaria vaccine antigens, studies did not progress to evaluate whether these proteins indeed are targets of protective immunity. And that presents the crux of the matter; eventually candidate antigens need to be tested experimentally for their potential of inducing protective immunity. Consequently, the success of reverse vaccinology stands and falls with the availability of a medium-throughput system to test for protective immunity, which, most critically, has to correlate with protection in humans. Although various *in vitro* assays and animal models to assess functional activity of *P. falciparum* specific immune factors have been described, to date, none of them has reliable predictive potential. The reason for this is that mediators of naturally acquired as well as RTS,S induced protective immunity are still not entirely understood.

Also the dissertation in hand followed the principle of reverse vaccinology to identify novel malaria vaccine antigens. From the *P. falciparum* genome we selected potential targets of protective humoral immunity, meaning hypothetical proteins predicted to be on the surface of extracellular parasite stages. More precisely, we selected proteins predicted to encode an N-terminal secretion signal and a C-terminal GPI-attachment signal. From these we selected based on transcriptome and proteome data proteins with specific expression in one of the extracellular parasite stages and/or with predicted domains associated with surface ligands. We did not aim at establishing a comprehensive list of potential vaccine candidates but to select

a handful of promising proteins, subject them to detailed characterization and to assess their actual vaccine potential.

To this end we generated monoclonal antibodies specific for selected candidates. In contrast to knockout mutant strategies, antibodies can be used to assess a variety of protein features like protein expression, processing, subcellular localization, protein function and moreover to appraise the potential impact of humoral immunity directed towards this target candidate antigen. Here, the generated antibodies were used to characterize the target proteins in regard of their stage specific abundance and subcellular localisation and for functional *in vitro* and *in vivo* assays. Depending on the protein this may include *in vitro* assays for asexual growth inhibition, merozoite invasion inhibition, sporozoite invasion inhibition, inhibition of sporozoite gliding motility or *in vivo* passive immunisation and transmission blocking assays.

Most reverse vaccinology approaches involve recombinant expression of *in silico*-selected candidates in *E. coli* or *E. coli*-based cell-free *in vitro* expression systems either for the generation of specific immune sera, or as probe for protein microarrays to analyze immune sera [21,22]. Rather unproblematic for bacterial pathogens, recombinant expression of plasmodial proteins in *E. coli* can be troublesome as proteins may not be folded or glycosylated appropriately. This is especially problematic for surface proteins, which are prone to contain complex folds with numerous disulfide bonds and glycosylation sites. The use of misfolded proteins for immunisation may lead to induction of antibodies incapable of recognizing the endogenous protein, or to non-reactivity when used for the assessment of human immune sera resulting, in both cases, in false negative results. To overcome this difficulty, we developed a eukaryotic, cell-based expression system, which expresses recombinant proteins on eukaryotic cell surfaces [26]. Thereby, we favoured correct folding of the protein. We used these expressing cells to immunize mice as well as for selection of specific monoclonal antibodies, which entailed generation of antibodies specific for conformational epitopes and capable of binding to the endogenous protein in native conformation. Such high quality antibodies are pivotal for functional *in vitro* or *in vivo* analysis. For three hypothetical surface proteins we were able to generate antibodies binding the endogenous

protein in a native conformation. For two of the three, we could further show that monoclonal antibodies recognize conformational epitopes. A great outcome considering the complex folding of PFF0620c, a member of the 6-cystein protein family, as well as for PFD1130w/CyRPA which contains 12 cysteine residues. The significance of our strategy is demonstrated by the fact that, in contrast to sera from mice immunised with purified recombinant CyRPA produced in *E. coli* (data not shown), anti-CyRPA antibodies generated by immunisation and selection with surface-expressing eukaryotic cells showed parasite growth inhibitory activity.

Although time consuming to generate, using monoclonal antibodies rather than polyclonal immune sera for functional assays offers the advantage of discriminating functionality of antibodies specific for particular epitopes. This can be of immense impact, as the functional activity of antibodies can largely depend on their fine specificity. This has been shown for the growth inhibitory activity of antibodies specific for the blood stages antigens MSP-1 and AMA-1 [27,28]. Akin, we found that antibodies specific for CyRPA can be growth inhibitory or neutral depending on their unique specificity. Hence, by using polyclonal antibodies we possibly could have missed the potential of CyRPA as vaccine target.

Taken together, we applied the principle of reverse vaccinology for the identification of novel malaria vaccine candidates. By a low-throughput, but high quality approach, surface proteins of extracellular stages were identified and characterized. To date, a series of *in-silico* predicted candidates still await generation of specific antibodies. For two candidates, the merozoite protein PF14_0325 and the gametocyte and sporozoite expressed 6-cysteine protein PFF0620c, characterization is ongoing. For a third candidate, CyRPA, we could show by passive immunisation studies that this protein is a target of merozoite invasion inhibitory antibodies and consequently has potential as subunit component for a malaria blood stage vaccine. By continuing this strategy, we expect to identify a range of potential vaccine antigens from various *P. falciparum* stages. Thereby, we hope to increase the choice of vaccine antigens promoting the rational selection of the best candidates for the formulation of an effective multivalent, multi-stage malaria subunit vaccine.

Immunodominant versus conserved; implications for malaria vaccine development.

Malaria parasites are highly polymorphic [29–31]. The underlying mechanism is the coevolutionary "arms race" between the host's immune system and the evasion strategies of the parasite, which has left characteristic traces of selection in either genome. Proteins under immune pressure can undergo natural balancing selection, meaning frequency-dependent selection with a rare-allele advantage, leading to extensive sequence polymorphisms [32]. Especially parasite proteins that are accessible to the host immune system, mainly proteins expressed on the surface of the extracellular stages or proteins transported to the surface of the host cell, are highly polymorphic [30,33]. Current malaria blood stage vaccine antigens are merozoite surface proteins or proteins secreted from apical secretory organelles upon invasion. Hardly surprising, these proteins mostly comprise substantial polymorphisms [34]. Extensive polymorphism has been observed for MSP-1 in isolates from different geographical areas. Its gene has a mosaic and heterogeneity structure with allelic recombination demonstrated in isolates from different countries [35]. However, reduced nucleotide diversity was noted in a region encoding the 19-kDa C-terminal epidermal growth factor-like domain, a prominent vaccine target [35]. In contrast, for AMA-1 all of the sequence diversity is found in the form of SNPs. The analysis of 506 human *P. falciparum* infections in Mali identified 214 unique AMA-1 haplotypes [36]. In contrast, the asexual blood stage antigen CyRPA identified in this thesis is highly conserved; DNA sequencing of a selection of 18 *P. falciparum* strains from different geographic regions identified only two distinct CyRPA haplotypes differing by a single amino acid dimorphism. Furthermore we could demonstrate that variants are likewise affected by growth inhibitory anti-CyRPA mAbs, indicating that this dimorphism is of no functional importance.

Polymorphisms can only be associated with balancing selection if integrated with information about allele frequencies. By doing so, balancing selection was shown to be the driving force for polymorphisms of MSP-3, AMA-1, eba-175 and SERA-5 [37–

41]. These predictions were supported by immuno-epidemiological studies showing that antibody responses are allele-specific and associated with protection [37,42–44]. As a result, genome-wide analysis for signatures of balancing selection is thought to discover new vaccine candidates [33,41,45,46]. In contrast to the approach of identifying immunodominant antigens by the analysis of immune sera, this strategy offers the advantage of identifying those immunodominant antigens, which truly are targets of protective immune mechanisms. However, as immune selection acts upon phenotypic variation - a product not solely of allelic variation but also epigenetic regulation of transcription or translation - this approach potentially misses some protective antigens [46]. In the case of CyRPA we identified strong conservation although it is a target of protective antibodies. This could indicate that either CyRPA does not experience selective pressure or that other evasion strategies, which do not involve diversification apply for this protein. Features of CyRPA indicating its moderate immunogenicity are the rather low CyRPA-specific titres of sera of malaria exposed individuals and its presumably low abundance; western blot or immunofluorescence-staining signals with specific antibodies were of moderate intensity compared to major merozoite proteins. Furthermore, in released merozoites CyRPA comprises an intracellular localization and is thought to only be accessible by inhibitory antibodies during the short period of invasion. *In vitro* as well as *in vivo*, CyRPA-specific mAbs exerted a strong growth inhibitory effect but did not clear infection. This presumably indicates that the CyRPA-dependent function is not entirely blocked by the antibodies. But as *P. falciparum* was described to relay on redundant invasion pathways, we cannot exclude that the antibody mediated blocking of CyRPA possibly is compensated by alternative pathways and therefore presents another potential mechanism of evading immune pressure [47–49]. But this is rather unlikely, as mice infected with parasites from anti-CyRPA mAb-treated mice remained sensitive to treatment with the same antibody.

Although proteins under balancing selection are likely to be targets of protective immune responses, they consequently may be problematic for the development of a broadly effective vaccine. Immunisation with a certain variant potentially induces allele-specific immunity and therefore targets only a certain fraction of the entire

parasite population. Besides being only partly effective, vaccination with a single allele could lead to an increased frequency of variants not targeted by the vaccine, as was observed with pneumococcal vaccines; following the introduction of the seven-valent conjugate pneumococcal vaccine (PCV7) higher colonisation and invasion rates of non-vaccine serotypes emerged [50,51]. In the case of malaria, results of a phase 2 trial of a multi-antigen blood stage vaccine detected selection for clinical infections with non-vaccine type variants [52]. The issue of vaccine resistant malaria may possibly be resolved by including several alleles into a multivalent vaccine formulation or by designing chimeric antigens eliciting cross-reactive responses [53]. Such an approach would not be required for a vaccine based on the conserved CyRPA, which would likely target the entire parasite population.

A way to overcome the problems associated with antigenic diversity may be to rely on new vaccine targets like CyRPA that are more conserved. But why may some targets of protective immune responses be conserved? One reason can be that binding sites of surface ligands must have a limited scope of variability in order to remain capable of binding to their receptors. Furthermore, as a mechanism of immune evasion conserved antigens seem to be selected by evolution to be poorly immunogenic. Molecular reasons behind the poor immunogenicity are not entirely clear. Besides being of low abundance, considered mechanisms are that certain conserved epitopes cannot be recognized by the B or T cell germline repertoire, or that epitopes are hard to reach [54,55]. For other infectious diseases with the issue of immune evasion, like HIV and influenza, conserved protective epitopes could be identified. Universal neutralizing antibodies have been isolated against the conserved region of hemagglutenin of influenza [56]. However, such antibodies are rare, have low affinity, and cannot be induced in large quantities during infection or vaccination [57]. Akin, a broadly neutralizing monoclonal antibody against the CD4 binding site of the HIV envelope protein gp120 has been identified [58]. Yet, the antibody was shown to bind the epitope only with its heavy chain, because the epitope is recessed and not easily accessible [59]. Cryptic B-cell epitopes have also been described for malaria antigens including MSP2, CSP and TRAP [60–62].

Cryptic antigens/epitopes can only be useful for vaccine development if a qualitatively or quantitatively superior immune response can be induced upon vaccination compared to natural infection. Different strategies to convert conserved immunosilent epitopes into immunodominant ones have been described [60–64]. If presented in a non-natural configuration, cryptic epitopes can sometimes induce immune responses that surprisingly can recognize the native protein [63,64]. For example, invasion-blocking antibodies are induced by an immunologically cryptic epitope located in the N-terminal region of CSP, but only when it is presented in the absence of the immunodominant rest of the protein [62]. On the other hand immunisation with chimeric peptides was necessary to induce immune responses that target certain otherwise cryptic motifs of MSP2, CSP and TRAP [60,61]. In the studies described here, monoclonal antibodies specific for CyRPA inhibited merozoite invasion depending on their unique specificity. These antibodies were generated by immunisation of mice with recombinantly expressed protein. Consequently, if anti-CyRPA antibodies can be invasion inhibitory or non-inhibitory a CyRPA-based vaccine component should be designed in a way that favours induction of inhibitory compared to non-inhibitory responses. This possibly implies the design of protein surface loops comprising primarily epitopes associated with protection [65].

Prospects of parasite growth inhibitory vaccines.

Potential of the *P. falciparum* NOD/*scid-IL2Rγ*^{null} mouse model for assessment of antibody-mediated effector mechanisms.

The identification of robust correlates of protection and assays that measure functionally relevant immune responses will be of enormous benefit for the development of malaria vaccines. We describe for the first time the use of the *P. falciparum* NOD/*scid-IL2Rγ*^{null} mouse model for antibody transfer experiments. This model produces very consistent and reproducible infections with *P. falciparum* and therefore allowed the demonstration of a dose-response relationship of administered antibodies. Consequently, we propose this model for the quantitative comparison of the relative inhibitory potency of *P. falciparum* specific antibodies *in*

vivo. However, it remains to be shown whether antibody-mediated parasite growth inhibition measured in the *P. falciparum* SCID-mouse model can be translated to human *P. falciparum* infections. The assessment of the predictive potential is a crucial step towards the use of results generated by this model as surrogate marker for protection. Transfer experiments in humans with antibodies validated in the SCID-mouse model and subsequent parasite challenge present a possible approach to elucidate the predictive potential. But the use of very costly clinical trials solely for concepts validation may not be realistic.

In *in vitro* assays malaria blood stage-specific antibodies were demonstrated to interfere with parasite growth by various modes of action. Antibodies specific for merozoite surface proteins were shown to not only block merozoite invasion but also to mediate agglutination of merozoites or to induce monocytes to release mediators that block division of intraerythrocytic parasites [66–68]. Furthermore, antibodies bound to infected erythrocytes or free merozoites were measured to induce phagocytosis, neutrophil respiratory bursts or activation of the complement system [69–73]. In addition, antibodies specific for PfEMP1 were demonstrated to block resetting and sequestration of infected erythrocytes to endothelial cells [74,75]. Hence, an interesting question left unanswered is whether the *P. falciparum* NOD/*scid-IL2Rγ*^{null} mouse model can be used for the assessment of the various antibody mediated mechanisms. Such studies may shed light on the relevance of different mechanisms for *in vivo* protection.

Relevance of antibody mediated *in vitro* growth inhibition for immunoprotection in humans.

We demonstrated in our *in vitro* studies that antibodies specific for *P. falciparum* CyRPA protein can inhibit asexual parasite growth by interfering with merozoite invasion. However, the predictive potential of *in vitro* functional assays, including growth inhibition assays with antibodies, for human malaria infection is ambiguous. Criticism has been raised after field trials with vaccine formulations that induced antibodies capable of inhibiting *in vitro* growth but failed at conferring protection from disease [76,77]. Several studies demonstrated that antibodies from semi

immune individuals transferred to non-immune individuals induced a rapid drop in parasitemia and vanishing of clinical symptoms. However, the transferred antibodies that conferred protection inhibited *in vitro* parasite growth only in cooperation with human monocytes [78]. These findings cast doubts on the relevance of *in vitro* growth inhibitory activity of antibodies by themselves for *in vivo* protection. Recently, the relationship between *P. falciparum* growth rates in humans and *in vitro* growth inhibition activity was assessed. Malaria-naive volunteers were immunised with AMA1-C1 adjuvanted with Alhydrogel, CPG 7909 and subsequently challenged by intravenous inoculation with *P. falciparum* infected erythrocytes [79]. Although no clinically relevant vaccine effect was observed in the small numbers of subjects in this study, a significant association between *in vivo* parasite multiplication rate and vaccine-induced *in vitro* growth inhibitory activity was observed. This is the first evidence that the *in vitro* assay of parasite growth inhibitory activity can be a valid surrogate marker for malaria vaccine efficacy in the selection of candidate blood-stage vaccines. Consequently, invasion inhibitory antibodies specific to other blood stage antigens, like CyRPA, may also have an impact on parasite growth in human infections.

If antibodies specific for CyRPA can be shown to control parasitmia in humans, too, such antibodies could theoretically be used as therapeutics for malaria. However, antibody-based therapeutics are considered too expensive for malaria, a disease mainly affecting poor populations. Antibody-based therapeutics currently undergo a revival and thereby entailed major technical advances in antibody design and production [83]. But even if costs are brought down substantially, antibody-based therapies are unlikely to be applicable as standard treatment of malaria. But antibody-based prophylaxis for non-immune individuals travelling to affected countries might be realizable. Appliance of CyRPA-specific mAbs for prophylaxis may only be possible if antibodies prove to prevent establishment of blood stage infection after an infectious mosquito bite. In comparison to antimalarials, passive administration of human immunoglobulins from hyperimmune sera conferred a faster drop in parasitemia and cease of symptoms [81,82]. Therefore antibody-based therapy may be conceivable for the treatment of severe malaria in hospital settings.

Considerations on the potency of a CyRPA-based malaria vaccine.

The *in vitro* as well as *in vivo* growth inhibitory effect of anti-CyRPA antibodies was found to be dose dependent. Although lower doses showed some growth inhibitory effects, only doses of around 2.5 mg CyRPA-specific antibody considerably inhibited parasite growth in mice. The protective effect of a hypothetical CyRPA-based vaccine may therefore depend on the ability of inducing and maintaining titres of inhibitory antibody in the mg/ml range. For most human vaccines specific antibody concentrations from <1 ug/ml to ~200 ug/ml are sufficient to confer protection. However, it is feasible to induce higher concentrations of specific antibodies, as shown by a phase I/IIa study of RTS,S adjuvanted with AS02A; serum concentrations of >9 mg/ml specific antibody could be induced in malaria-naïve adults [80]. CyRPA-specific antibodies are expected to interfere with merozoite invasion independent of whether parasites infect human erythrocytes in engrafted SCID-mice or in the human host. But since the parasite growth rate may be different in the human infection compared to the mouse model the specific efficacy of inhibitory antibodies may also differ. Furthermore, polyclonal antibodies induced by active immunisation may have higher affinities compared to monoclonal antibodies. Hence, vaccine induced antibody concentration required for protection of humans may be lower than the concentration of mAbs required for growth inhibition in the SICD-mouse model. Furthermore our mouse experiments showed that parasite density influences the growth-inhibitory effect of anti-CyRPA antibodies. Deducing a similar association in human infections, a CyRPA-based vaccine is expected to most effectively control low-level parasitemia.

Conclusion

In this thesis, we anticipated the rational discovery of novel malaria subunit vaccine candidates. Our strategy was based on the selection of hypothetical parasite proteins that are accessible to the host immune surveillance by antibodies, and their subsequent functional characterization using specific monoclonal antibodies. Our findings lead to the following key conclusions:

1. The developed whole cell-based immunization and hybridoma selection strategy for the generation of mAbs efficiently generated mAbs reactive with the native endogenous antigens. This strategy may be applicable for a wide range of cell-surface proteins.

2. One of the characterized candidate proteins, CyRPA, is the target of growth inhibitory antibodies. Together with its high sequence conservation, this renders CyRPA a potential subunit component for a malaria blood stage vaccine.

3. As at least one selected protein candidate proved indeed to be a target of protective immune mechanisms affirms that the applied *in silico* selection criteria are appropriate for the identification of novel vaccine targets.

4. Passive immunisation in a *P. falciparum* NOD/*scid*-*IL2Rγ*^{null} mouse model was found to measure dose-dependent effects. Therefore, this model can be used for comparison of the relative inhibitory potency of malaria asexual blood stage specific antibodies *in vivo*.

By continuing application of the developed strategy we anticipate the identification of additional potential vaccine antigens from various *P. falciparum* stages. Thereby, we hope to promote the rational selection of auspicious candidates for the formulation of an efficient multivalent, multi-stage malaria subunit vaccine.

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- Experimental PhD thesis on the identification and characterisation of potential vaccine candidates of *Plasmodium falciparum* in the lab of Prof. G. Pluschke at the Swiss Tropical and Public-Health Institute, University of Basel
  2007 – present

Publications

- **Anita M. Dreyer**. (2007). *Detection and characterisation of antigens by mass spectrometry*. Master Thesis, University of Basel, Switzerland

- **Anita M. Dreyer**, Jeremy Beauchamp, Hugues Matile and Gerd Pluschke (2010) *An efficient system to generate monoclonal antibodies against membrane-associated proteins by immunisation with antigen-expressing mammalian cells.* BMC Biotechnology, 15;10:87.

- **Anita M Dreyer**, Hugues Matile, Petros Papastogiannidis, Jolanda Kamber, Till Voss, Sergio Wittlin, Gerd Pluschke (2011) Passive immunoprotection of *Plasmodium falciparum* infected mice designates the Cysteine-Rich Protective Antigen as candidate malaria vaccine antigen. Submitted.

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