Universiteit Leiden
The Netherlands

Towards a blood stage malaria vaccine, dealing with allelic polymorphism in the vaccine candidate apical membrane antihen 1

Kusi, K.A.

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Chapter 1

General introduction

General introduction

Malaria is an infectious disease that is prevalent in the tropical and some temperate areas of the world. About half of the world's population, living in some 106 countries and territories, were believed to be at risk of infection with malaria parasites in the year 2009 [1]. Current malaria control strategies involve preventive measures such as targeting the vector with insecticides, use of insecticide-treated bed nets and therapeutic interventions with various drug formulations that target the parasite. However, the emergence of insecticide and drug-resistant strains of the mosquito vectors and parasites respectively [2-6] continue to undermine these control efforts. The greatest risk of disease and fatality in *Plasmodium falciparum*-endemic areas occurs in children under 5 years and in first-time pregnant women (and their foetuses), with the majority of fatal cases occurring in sub-Saharan Africa [1]. The disease, especially in its severe forms, has a great economic burden as it usually leaves its victims incapacitated and sometimes bed-ridden. The development of an effective malaria vaccine therefore remains an important objective for public health and safety, as none of the currently available tools has proved fully efficacious. An effective malaria vaccine would add to the existing arsenal for the control and subsequent elimination of malaria globally.

The parasite, disease pathology and symptoms

Malaria is caused by apicomplexan parasites of the genus *Plasmodium* and is transmitted by female mosquitoes of the genus *Anopheles*. Four major parasite species are known to cause disease in humans, namely *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae. Plasmodium knowlesi*, a well-known simian parasite species, has also been recently implicated in potentially life-threatening human infections [7-10]. Malaria parasites have a complex life cycle that takes place within a primary (vertebrate) host and a secondary (insect vector) host (Figure 1). Generally, the human-infective sporozoite stage develops within the mosquito gut and is injected from the salivary glands into the blood stream when the mosquito takes a blood meal. Sporozoites migrate to and develop within liver cells, and upon release as free merozoites after 6 – 10 days, enter the blood phase where they cyclically invade red blood cells. *P. vivax* and *P. ovale* infections are unique in that some liver stages develop into dormant stages known as hypnozoites which can be later activated to cause disease relapse without new infection through mosquito bites. The blood stages of most human *Plasmodium* species have a 48-hour cycle, with *P. malariae* (72 hours) and *P. knowlesi* (24 hours) being exceptions.

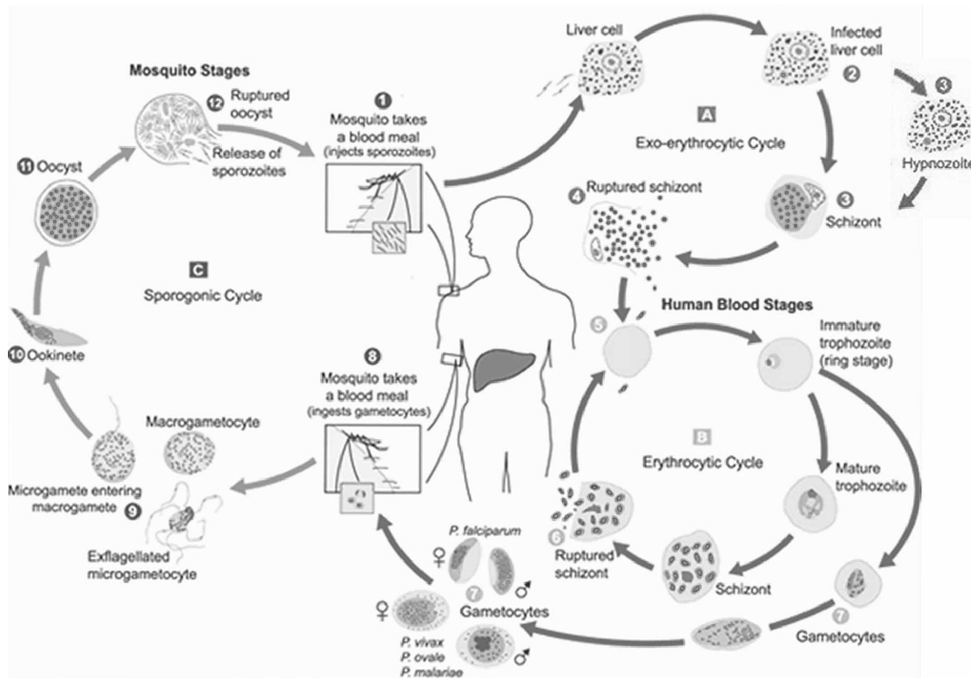

Figure 1. Life cycle of the human *Plasmodium* species. The asexual liver (A) and blood (B) stages occur in the primary host (humans), while the sexual stages (C) occur in the secondary host (mosquito). Source: http://www.uni-tuebingen.de/modeling/Mod_Malaria_Cycle_en.html, with slight modification.

Some parasites develop into the mosquito-infective male and female gametocyte stages in the bloodstream, which after being taken up by mosquitoes during a blood meal fertilize and perpetuate the entire cycle.

Blood stage infection with the parasite is responsible for such non-specific clinical symptoms as nausea/vomiting, sweating, headaches, muscular/joint pains and fatigue in the vertebrate host. Fever/chills experienced by an infected person are associated with the periodic release of toxins/pyrogens and other inflammatory mediators alongside merozoites following the synchronous lyses of infected red blood cells.

Of the species known to cause disease in humans, *P. falciparum* infection is the most severe and usually results in disease complications. *P. vivax* (and *P. ovale*) infections can also cause disease with relapses that are especially difficult to treat. While all other species have an erythrocyte developmental age restriction, *P. falciparum* (and *P. knowlesi*) infects erythrocytes of all ages and can thus attain very high parasitaemias *in vivo*. This, together with the ability of red cells that contain developing trophozoites/schizonts to sequester in the microvasculature of vital organs by adhering to endothelial cells, and also form rosettes with uninfected erythrocytes, makes *P. falciparum* highly virulent. Cytoadherence and red cell rosettes are believed to cause blockage of small blood vessels in vital organs such as the brain, resulting in ischaemia. Disease complications include

severe anaemia, cerebral malaria, respiratory distress, lactic acidosis, multiple organ pigmentation and organ failure, resulting in death in extreme cases. Although parasite sequestration and rosetting have been implicated in these disease complications, a significant portion of clinical symptoms are also due to immuno-pathology resulting from the host's response to the presence of parasites [11,12].

Immunity to malaria

People living in malaria-endemic areas usually develop natural immunity to clinical malaria in an age- and exposure-dependent manner after a number of parasite infections [13,14]. Development of immunity is believed to be faster in high disease transmission areas compared to low transmission areas [14-16]. Infants and young children are most especially susceptible to disease, and usually suffer a number of clinical episodes before achieving a level of protection from severe disease, although they may continue to harbour parasites and suffer mild disease. Even in adults who have had several parasite encounters, acquired clinical immunity is believed to be partial and short-lived, and its maintenance requires periodic exposure to low-level parasitaemia [14,17].

The mechanisms underlying protective immunity to parasite infection and clinical disease in humans are not fully understood. Studies conducted *in vitro,* in animal models and in humans point to the involvement of both cellular and humoral immune effectors in a parasite stage-specific manner [14,18], and include both innate and adaptive immune mechanisms. Innate immune mechanisms involving monocytes, neutrophils, macrophages, natural killer (NK) cells, γδT cells and natural killer T (NKT) cells as well as their soluble secretory products have all been shown to play a role in parasite killing and protection from disease. These cells either inhibit parasite growth or mediate parasite clearance through phagocytosis and cytotoxicity [19-21]. Secreted factors such as nitric oxide (NO) kill parasites by inhibiting key enzymes, while factors such as TNF-α and IFN-γ augment the parasite killing functions of immune cells in pre-erythrocytic and erythrocytic stages [18,21-26]. The innate arm of immunity also provides the necessary help to achieve an effective adaptive response, and immune effects of the two arms are not mutually exclusive.

Cell-mediated immunity

Apart from the non-specific effector functions of innate immune cells, parasite antigen-specific T cell responses have also been demonstrated. Both CD4+ and CD8+ T cell responses are elicited by processed parasite antigenic peptides presented in the appropriate MHC context by APCs such as dendritic cells or macrophages along with the appropriate co-stimulatory ligations, and these responses directly or indirectly kill parasites or inhibit their growth [18,27]. Generally, a T helper 1 (Th1)-driven immune response is necessary for initial

control of the acute phase of disease, whilst a Th2-type counterbalance becomes essential for eventual elimination of the parasite and subsequent resolution of disease as well as for curbing immunopathology [17,28]. CD4+ Th1 cells promote a more pro-inflammatory response early during infection, while CD4+ Th2 cells promote anti-inflammatory responses during the chronic phase of infection or during convalescence. While both CD4+ and CD8+ T cell responses are generally believed to be important against liver stages of the parasite [18,22,29,30], the direct cytotoxic activity of CD8+ T cells may have a limited role in controlling blood stage infections since erythrocytes, which harbour the blood stage parasites, do not express MHC molecules and cannot present processed parasite peptides directly to T cells [31]. CD4+ T cells have however been shown to be important for blood stage immunity as through secretion of the appropriate cytokines/proteins, they induce nitric oxide synthesis and provide B cells the needed help for antibody production and class-switching [23,32-35].

CD4+ and CD8+ T cell responses are adaptive responses and therefore induce memory. T cell memory against malarial antigens has been demonstrated [29,36-38]. Primed T cells undergo expansion and maturation during the secondary response to processed and MHC-presented cognate peptides and yield memory and effector T cells. While effector T cells undergo apoptosis after resolution of infection, memory T cells persist and are likely to be long-lived since re-exposure to parasite antigens after some time results in rapid gain of immunity [39,40]. Although other studies have found short-lived malaria-specific T cell responses [41,42], the induction of long-term T cell memory is crucial in vaccine design since vaccine effectiveness depends on the rapid recall of previously encountered antigens. These notwithstanding, protective immunity especially against acute malaria may ultimately depend on the maintenance of high levels of the relevant effectors rather than on generating effectors from resting memory cells [40].

Regulatory T cells (Tregs) are also important immune effector cells that can greatly influence the course of an infection. An appropriate and timely induction of Tregs is required to regulate the induced anti-parasite responses and prevent excessive immune elaboration. Excessive responses resulting from the lack of control of the immune response to parasites may cause serious immunopathology, whilst an early induction of regulatory mechanisms may elicit tolerance to parasites and render parasite control by the immune system ineffective [28,43]. Guilbride et al. [44] have recently suggested that sporozoites that remain in the skin following inoculation by the mosquito vector may induce parasite-specific regulatory T cells within the skin and these may subsequently thwart the development of protective immunity against infection. B cells with general immune suppressive/regulatory functions (Bregs) have also been described mostly in mice (Reviewed in [45]), but there is very little human data and currently no data on the role of Bregs in malaria.

Antibody-mediated immunity

Antibodies are secreted by B cells and have very important effector functions in the adaptive immune system. Immature B cells released from the bone marrow usually express membrane-bound IgM, and develop into naïve B cells in the marginal zone or follicular areas of secondary lymphoid organs such as the spleen and lymph nodes as well as secondary lymphoid follicles in inflamed tissue (Figure 2). Upon encounter with foreign antigen presented by APC and with T cell help, follicular naïve B cells, which are capable of peripheral circulation, differentiate into antigen-specific memory B cells or plasma cells/precursors. These differentiated cells undergo isotype/subclass switching and express IgG, IgA or IgE depending on the antigen stimulus and the cytokine signals received from helper T cells. Memory B cells do not secrete antibodies but rather express membrane-bound immunoglobulins. Marginal zone B cells are non-circulating, develop into short-lived plasma cells without T cell help upon antigen contact and do not usually differentiate into memory cells [47,48]. A small proportion of antibody-secreting/plasma cells usually resides in secondary lymphoid organs and secretes antibodies. The majority of these cells, depending on the chemokine receptors they express, migrate as plasma cell precursors called plasmablasts either to tissue sites of infection or into the bone marrow and actively secrete antibodies specific for the cognate antigen [46]. Inflammatory mediators in inflamed tissues act as survival signals, and plasma cells in these tissues are short-lived as they undergo apoptosis upon resolution of inflammation/infection, while long-lived plasma cells can reside in the bone marrow for months to decades [46,49-51].

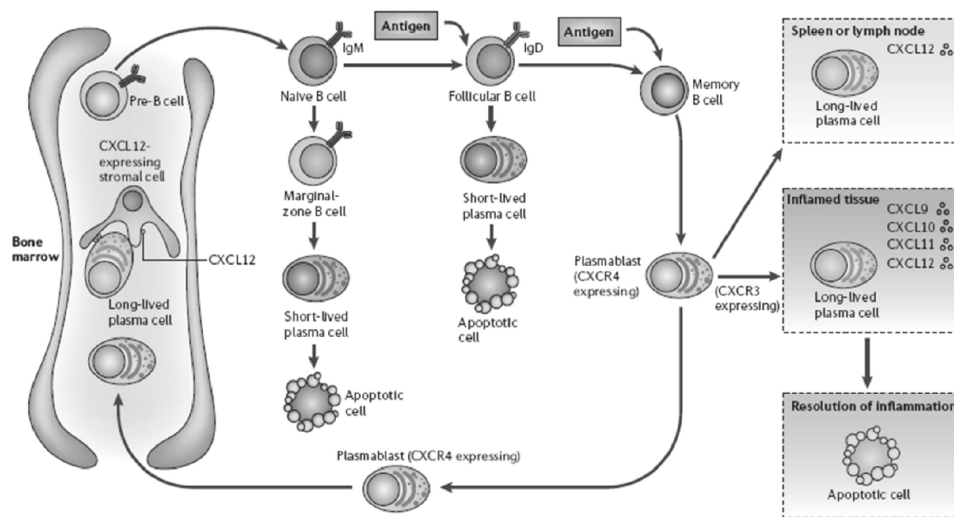

Figure 2. Maturation, activation, differentiation and survival of B-lymphocytes.
Source: Radbruch et al. [46].

Upon secondary contact with the same or similar antigen in germinal centres of secondary lymphoid organs, memory B cells rapidly expand and undergo affinity maturation through somatic hypermutation in the variable regions of immunoglobulin genes [52,53]. The antibodies rapidly produced after these events show higher specificity/affinity for the cognate antigen. Antibodies recognise and bind to specific determinants known as epitopes on an antigen. Antibody epitopes are usually conformational in nature, although linear epitopes occur. Antibodies specific for one antigen may cross-react with other antigens to varying extents if these other antigens share epitopes with the antibody-inducing antigen.

Anti-malarial antibodies play a major role in controlling blood stage infection/clinical disease symptoms. This was demonstrated in passive transfer experiments decades ago, in which gamma globulins isolated from the hyperimmune serum of adults were able to control clinical symptoms and parasitaemia in children [54-57]. Anti-malarial antibodies are usually directed against free merozoite surface antigens or parasite antigens expressed on the surface of infected erythrocytes, and are mainly of the IgG isotype. The extent of anti-disease protection has been shown to correlate with levels of antibody against asexual stage parasite antigens and is dependent on antibody isotypes, with IgG1 and IgG3 being most important in humans [58-65]. In normal healthy individuals the total IgG pool consists on average of 67% IgG1, 22% IgG2, 7% IgG3 and 4% IgG4 [66]. Predominant expression of IgG1, IgG3 or both subclasses in response to malaria infection is believed to be dependent on the type of antigen, cumulative exposure to the antigen as well as on the infected person's age [65,67]. High levels of antibodies against pre-erythrocytic parasite antigens have also been associated with a reduced risk of developing clinical malaria in endemic areas [68,69]. Protective anti-malarial antibodies are known to mediate anti-parasite effector functions such as the inhibition of erythrocyte invasion [70-72], inhibition of cytoadherence [73,74], neutralisation of parasite toxins [75,76] and participation in antibody-dependent cytotoxicity and antibody-dependent cellular inhibition [77-79].

Despite the crucial role antibodies play in the resolution of infection, anti-malarial antibodies, either acquired naturally or induced by vaccination are rapidly lost after resolution of infection in comparison with other infections/immunisations [80]. Short-lived protective responses have been partially attributed to immune evasion mechanisms of the parasite. Interference with the maturation and antigen presenting functions of dendritic cells, the first stage in inducing memory and effector B and T cells, as well as the active deletion of malaria-specific memory and effector cells have been demonstrated [81-84]. Long-term protection from disease depends on the maintenance of immune memory to infection, such that subsequent encounter with parasites induces a rapid recall response. There are conflicting reports on the longevity of memory to malaria infection in endemic areas. While some researchers have reported

short-lived memory and antibody responses in the absence of continued exposure to low-level parasitaemia [85-87], others have recently identified populations with stable levels of anti-malarial antibodies and memory B cells in the absence of recurring infections [88,89]. Some other studies have shown that there is an age-dependent transition from short-lived to long-lived antibody responses in endemic areas [90,91]. Despite these varied findings, protective immunity especially against acute malaria may ultimately depend on the maintenance of optimal levels of the relevant effectors (here plasma cells and/or antibodies) and/or the time it takes to generate these effectors from memory cells relative to parasite growth [40,92]. Ultimately, an effective vaccine against malaria may need to address the challenge of short-lived memory/protective responses.

Malaria vaccine development

Vaccines are recognised the world over as important epidemiological control tools for disease prevention. The natural acquisition of anti-disease immunity in adults living in malaria-endemic areas and the successful treatment of susceptible individuals with gamma globulins from semi-immune adults [55-57,93] suggests that the development of an antibody-mediated blood stage vaccine is feasible. A cost-effective vaccine would form a powerful additional component in current malaria control strategies. Malaria vaccine development has however proven very challenging because of the parasite's complex nature and the difficulty of correlating naturally acquired immune responses to protection against infection and/or disease. Current knowledge of the immune system suggests that an effective malaria vaccine would need to induce long-term CD4+ T cell responses irrespective of the parasite stage being targeted [17,33,35,94,95]. For vaccines targeting pre-erythrocytic stages of the parasite, complementary responses include CD8+ T cell as well as antibody responses, whilst robust antibody responses are mainly required against the erythrocytic stages [17,18,27]. An effective vaccine would be expected to achieve in infants, children first-time pregnant women and/or naïve adults at least the anti-disease immune status of semi-immune adults, though over a shorter period of time.

One strategy of malaria vaccine development involves immunisation with whole parasites (sporozoites) that have been attenuated chemically, genetically or through irradiation [96-100]. Attenuation ensures that sporozoites become non-replicating while maintaining a metabolically active state. This strategy has the advantage of not requiring adjuvants and exposes vaccinees to all parasite antigens in their native conformation, thus avoiding the selection of one or a few antigens since the exact targets of protective immunity are poorly defined. This strategy has demonstrated robust induction of both cell and antibody-mediated responses in rodent models as well as in humans [96-99]. This strategy, despite showing very promising results, has a number of inherent challenges including parasite (sporozoite) culturing, isolation, storage and vaccine delivery

conditions, which would inflate the cost of production as well as final vaccine safety. Despite these challenges, optimization of these conditions and clinical development of such vaccination strategies are still progressing.

Vaccination with merozoites, either emulsified with adjuvant or a low dose of live parasites followed by drug cure, represent another whole parasite strategy. The effectiveness of this strategy has been demonstrated with challenge studies in mice, monkeys and humans [101-107]. Administration of low doses of live *P. falciparum* parasites in human volunteers with subsequent drug cure induced sterile T cell-mediated protection that was characterised by production of high levels of interferon-gamma (IFN-γ) and increased activity of nitric oxide synthase but no detectable levels of interleukin4 (IL-4), IL-10 or malaria specific antibodies [107]. As with whole sporozoite vaccines, there are practical challenges to this approach, to which solutions are currently being sought.

Subunit vaccine development, the second general strategy, aims to focus the immune response on one or a few parasite antigens that have shown promise as vaccine candidates. This strategy has the advantage of using defined and highly purified recombinant antigens that allow a practical elucidation of the immune mechanisms underlying vaccine effectiveness. It however requires the selection of very essential targets that are highly antigenic/immunogenic and vaccination may be carried out with adjuvant-formulated antigen, or may be delivered as the gene preferably incorporated in a viral vector. Antigens may be specific to the pre-erythrocytic, erythrocytic or sexual stages of the developing parasite, or may be found in different stages of the parasite's life cycle. While erythrocytic and pre-erythrocytic candidates would prevent disease by targeting parasites before or during the asexual blood stage and therefore of benefit to the individual, sexual stage candidates are typically transmission-blocking and only have community-related benefits.

Though the main targets of protective immunity to malaria are not clearly defined, a limited number of accessible parasite antigens expressed at various stages of the parasite's complex life cycle have been linked with protective responses in *in vitro* studies as well as in murine and simian malaria models [108-114]. These antigens were identified, and subsequently characterised, through their binding to antibodies purified from hyperimmune sera. A handful of these antigens are currently undergoing pre-clinical and clinical testing. Prominent amongst these are the variant surface antigens (VSAs) that are expressed on the surface of infected red cells. Other prominent antigens are either expressed/localized to the parasite surface or associated with the parasitophorous vacuole and include circumsporozoite protein (CSP), thrombospondin-related anonymous protein (TRAP), the liver stage antigens (LSAs), apical membrane antigen 1 (AMA1), the merozoite surface proteins (MSPs), Glutamate-rich protein (GLURP), erythrocyte binding antigens (EBA), the parasite protein anchor molecule glycosylphosphatidylinositol (GPI) as well as the gametocyte/ookinate-specific antigens Pfs25, Pfs28, Pfs48/45 and Pfs230.

While some of these antigens are developmental stage-specific (eg. LSA, TRAP), others like AMA1, GLURP and EBA-175 are found in multiple developmental stages [115-118]. Most of these antigens are believed to be essential for the parasite's survival and/or effective transition from one developmental stage to another. Targeting these antigens would therefore kill parasites directly or interrupt their life cycle.

The RTS,S subunit vaccine, comprising the antigenic C-terminal repeat regions of *P. falciparum* CSP fused to the N terminal of the surface antigen of Hepatitis B virus is the most clinically advanced malaria vaccine. Development of this candidate started over two decades ago, and different adjuvant formulations of this candidate have over the years demonstrated a generally good safety and tolerability profile. Earlier challenge studies of this candidate formulated with SBAS2 (currently known as AS02) in malaria-naïve adult volunteers showed protection against sporozoite challenge in 6 out of 7 volunteers [119]. A phase IIb trial with AS02A as adjuvant in children 1-4 years showed a 37% reduction in prevalence of *P. falciparum* infection compared to Hepatitis B-vaccinated controls, and a vaccine efficacy (time to first clinical episode) of 57.7% over a 6-month follow-up period [120]. Later analysis of data for all randomized subjects showed a 30.5% vaccine efficacy against first time infection and 34% lower *P. falciparum* prevalence in the vaccine group compared to controls over the 45-month surveillance period [121]. A trial of RTS,S formulated with AS01E in 894 children aged 5 – 17 months showed vaccine efficacy (against first time infection) of 39.2% 12 months after the final vaccination and efficacy of 45.8% in a cohort followed-up for 15 months [122]. In the same study vaccine efficacy after the first 8 months of follow-up was 53% [123]. Another trial with AS02D formulations in 340 newborns/infants demonstrated 65.2% efficacy against first infection with *P. falciparum* 6 months after administration of the last vaccine dose [124]. In a phase I/IIb trial of RTS,S formulated with AS02D in 214 newborns/infants, vaccine efficacy against clinical malaria was estimated at 33% over a 4-month follow-up period [125]. Based on these varied yet encouraging trial results, RTS,S has been described as a "leaky" vaccine that confers partial protection to most or all vaccinees [126,127]. The vaccine's protective effects are believed to be mediated mainly by antibodies and CD4+ T cells to the CSP repeat regions, with very little evidence for CD8+ T cell activity [128-130]. The RTS,S vaccine formulated in AS01 is currently undergoing phase III multi-site trials in Africa, involving about 16,000 infants and children in 11 centres with varying transmission intensities.

Antigenic polymorphism and immune evasion

Most subunit vaccine candidate antigens currently under investigation are polymorphic in nature, and this presents a formidable challenge to their development as vaccines. Allelic polymorphism is believed to be a host immune evasion strategy that renders a functional host response to one parasite variant

ineffective against other variants [131-133]. This view is supported by the fact that polymorphism usually occurs in portions of the parasite's conformationally intact surface antigens that are likely to be accessible to antibodies *in vivo* [133,134]. Parasites have also been shown to modulate the host's immune system by dampening anti-parasite effector responses and inducing regulatory responses in various immune cell types in the skin and other immune cell compartments [44,81,135,136], thereby inducing immunosuppression to their survival benefit.
The discovery of potent novel candidate antigens is crucial to the development of a vaccine against malaria. The advent of new technologies and the current advancement in -*omics* research makes this ever more feasible. Nevertheless, there is the need to better understand and improve on immune responses to already known candidates that exhibit antigenic variation and allelic polymorphism by tweaking vaccine formulation and delivery systems since most of these known candidates naturally elicit functional responses. Two general approaches can be employed to ensure that such candidates yield desired immune responses. The first is to develop subunit vaccines based on conserved and immunogenic parts of candidates such as MSP1, MSP3, GLURP and CSP. The second approach involves tackling allelic polymorphism by incorporating a number of allelic forms either as fusion proteins or as antigen cocktails in a single vaccine. The vaccine candidate AMA1-C1 [137] being developed by NIAID, and the DiCo strategy [138] being developed by BPRC Rijswijk are examples of multi-allele vaccines for dealing with extensive polymorphism in AMA1.

AMA1, a polymorphic vaccine target

AMA1 is a type 1 trans-membrane protein that is expressed as an 83 kDa precursor protein and initially located in micronemes of the merozoite [139-142]. This precursor is further processed to a 66 kDa protein by the removal of the N-terminal prosequence [141,143,144]. The precise role of AMA1 has not been fully elucidated, but it is believed to be involved in the formation of the contact junction between parasite membrane and the red cell membrane following apical re-orientation and prior to red cell invasion [145,146]. Around the time of merozoite release from schizonts, the 66 kDa AMA1 is translocated to the merozoite surface where it interacts with other invasion-associated antigens like the RON proteins at the point of attachment to the red cell surface [140,145,147-152]. Its release from micronemes has been shown to be triggered by differences in the ionic environment between the erythrocyte cytosol and the intravascular compartment, and is sequentially followed by the release of rhoptry antigens [153,154]. The ectodomain of AMA1, which is the vaccine target, is shed as 44 and 48 kDa alternate proteins from the merozoite surface upon red cell invasion [141,155]. The amino acid sequence of the AMA1 ectodomain has 16 cysteine residues that are conserved in all known AMA1 sequences and these form disulphide bonds that result in a structure with three

distinguishable but interactive domains (Figure 3). The vaccine potential of AMA1 is informed by the fact that antibodies against the ectodomain can interfere with AMA1 processing and prevent red cell invasion *in vitro* [71,72,156,157], and this effect requires immunisation with correctly folded AMA1 [112,158]. Despite the short exposure time for merozoites in between red cell invasions and the low abundance of AMA1 relative to other merozoite antigens, anti-AMA1 antibody levels in naturally exposed populations are usually high compared to antibodies against other blood stage antigens [159-161]. Additionally, a significant proportion of new-borns and infants show relatively higher levels of placentally transferred anti-AMA1 antibodies [162]. Antibodies to domain I of the molecule are more prevalent than those to domains II and III, even though dominant antibody epitopes seem to be dependent on more than one domain [163-165].

The vaccine potential of AMA1 is limited by allelic polymorphism resulting from single amino acid substitutions in an estimated 17% of the 622 amino acid positions within the AMA1 molecule (Remarque, unpublished data). A significant number of amino acids at polymorphic positions however occur at very low frequency. Polymorphism results from non-synonymous single nucleotide substitutions within the single *ama1* gene locus [131,166-168], a phenomenon that has been linked with selective pressure from the host immune system.

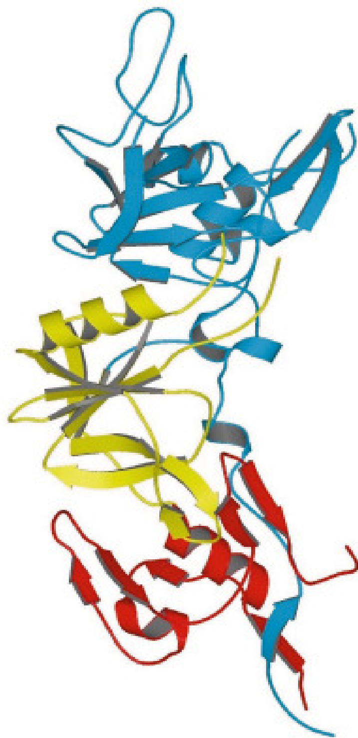

Figure 3. Ribbon structure of the ectodomain of AMA1.
The three antigen domains are shown in blue (domain I), yellow (domain II) and red (domain III).
Source: ESRF website (http://www.esrf.eu/news/spotlight/spotlight19malaria/)

The majority of amino acid substitutions occur within domain I of the ectodomain and are di- or trimorphic, with a single heptamorphic site at amino acid position 197 [133,169,170]. Polymorphic residues have been shown to occur predominantly on one surface of the AMA1 molecule, and this suggests that this face at the parasite surface is more accessible to antibodies [169,171], and/or that antigenic variation on the more conserved surface is subject to strong functional constraints. Polymorphism in AMA1 renders a fully functional immune response against one parasite strain less effective against strains expressing other allelic variants. This has been extensively demonstrated in murine models [109,110,172] and this may also be the case in humans [173,174]. Thus the immune response to AMA1, believed to be mainly antibody-mediated, is to both strain-specific and conserved epitopes [112,163,165]. A vaccine strategy based on AMA1 would therefore have to circumvent the inherent strain specificity of immune responses to the molecule in order to have broad functional benefits. This may be achieved through vaccine formulations that incorporate a number of polymorphic alleles, and the AMA1-C1 vaccine candidate, developed by the US NIAID and comprising an equimolar mixture of AMA1 alleles from the FVO and 3D7 parasite strains [137,175,176] in an example. This strategy of vaccine formulation was also exploited in the *in silico* design of three Diversity-Covering (DiCo) proteins based on 355 published unique sequences of AMA1 that were available at the time of design [138]. The three antigens expressed in the yeast *Pichia pastoris* and purified from culture supernatants. When formulated as a cocktail with adjuvant, they induce antibody responses that show broad AMA1 allele recognition and *in vitro* inhibition of diverse parasite strains.

Aims and outline of thesis

An effective malaria vaccine will be a valuable and cost-effective addition to currently available malaria control tools, and a lot of effort and resources are being devoted to finding an effective vaccine. All approaches to malaria vaccine development face peculiar challenges, and in addition to discovering novel vaccine targets, there is the need to fine-tune immune responses against already known and available targets. One such vaccine target is the ectodomain of *P. falciparum* AMA1 (*Pf*AMA1), an essential parasite protein that exhibits extensive polymorphism. There are currently 1778 valid published *Pf*AMA1 protein sequences (GenBank), from which 649 unique alleles have been identified (Remarque, personal communication). This huge diversity begs the question whether there are common antigenic determinants to which functional antibodies can be induced, since a broad-acting vaccine would need to be effective against most or all parasite variants.

This thesis focuses on strategies for *Pf*AMA1 vaccine formulation that can broaden the antibody response and ultimately achieve near universal functionality. It addresses issues relating to the fine dissection of antibody

responses to *Pf*AMA1 as well as to existing approaches for assessing vaccine effectiveness.

In chapter 2 of this thesis, a competition ELISA protocol with high sensitivity and reproducibility for analyzing antibody specificities to allelic variants of *Pf*AMA1 was developed. This assay, together with *in vitro* parasite growth inhibition assays, was used to show that rabbits immunised with a three-antigen *Pf*AMA1 cocktail vaccine elicit antibodies that exhibit greater cross-reactivity and broader *in vitro* inhibitory activity than antibodies induced against a single *Pf*AMA1 allele. The induction of functional anti-AMA1 antibodies to epitopes shared by a number of different *Pf*AMA1 alleles was also established.

In chapter 3, the antibody responses induced in rabbits against cocktail vaccines incorporating three and seven antigens were compared. Data obtained showed that a cocktail of three DiCo AMA1 antigens (DiCo mix) induced antibodies with similar specificity and functionality as antibodies induced with a seven-antigen (DiCo mix + AMA1 of FVO, HB3, 3D7 and CAMP strains of *P. falciparum*) vaccine cocktail. It was also established here that DiCo mix vaccines in three different adjuvants induced very similar proportions of antibodies against shared AMA1 epitopes, irrespective of the adjuvant used for formulation. The absolute levels of induced antibodies were however dependent on the choice of adjuvant. This is important for the further development of *Pf*AMA1-based vaccines, as adjuvant selection for subsequent clinical development will be determined mainly by the antibody potentiation (quantity) properties of the adjuvant.

Data showing that immunisation of rabbits with three different *Pf*AMA1 alleles (from the FVO, HB3 and 3D7 parasite strains) either in sequence or as a vaccine cocktail, yields antibodies with comparable specificity and functionality is presented in chapter 4. Since clinical immunity to malaria develops after (sequential) exposure to a diversity of parasites, a vaccine cocktail would be expected to induce immunity similar to that acquired through natural infection, but over a shorter period of time. The data therefore suggests that acquisition of natural immunity involves accumulation of antibodies to shared epitopes with time and exposure to variant parasite strains.

The induction of antibodies with broadened allele recognition and parasite inhibition capacity after immunisation with a multi-allele *Pf*AMA1 vaccine was confirmed in rhesus monkeys as a more representative model for immunity to malaria in humans, and the data is presented in chapter 5. Different groups of monkeys were immunised with DiCo mix formulated in two different adjuvants (CoVaccine HT and Montanide ISA51), as well as with a *Pf*AMA1-MSP1_{19} fusion protein in Montanide ISA51, with all vaccine formulations demonstrating a generally good tolerability profile.

In chapter 6, the effect of antigenic diversity on the laboratory measurement of anti-*Pf*AMA1 antibodies in naturally exposed individuals was investigated. Antibody levels in the plasma of infants and children exposed naturally to *P. falciparum* were measured against 4 *Pf*AMA1 allelic variants as well as 3 antigen

cocktails and compared. The data showed that humans, especially infants and younger children with very few parasite encounters, discriminate between different *Pf*AMA1 alleles, just as is the case for other mammalian species. The implication of this for the measurement of antibody responses against *Pf*AMA1 as a polymorphic antigen in vaccine studies and especially in the field has been highlighted.

A general discussion of the key findings in this thesis, the current status of malaria vaccine development as well as the vaccine-related implications of antigenic diversity in AMA1 that is found naturally in *P. falciparum* isolates from malaria-endemic areas are finally presented in chapter 7.

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