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

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To Stella, the love of my life

and to Cheryl, the shining star in my life

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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.

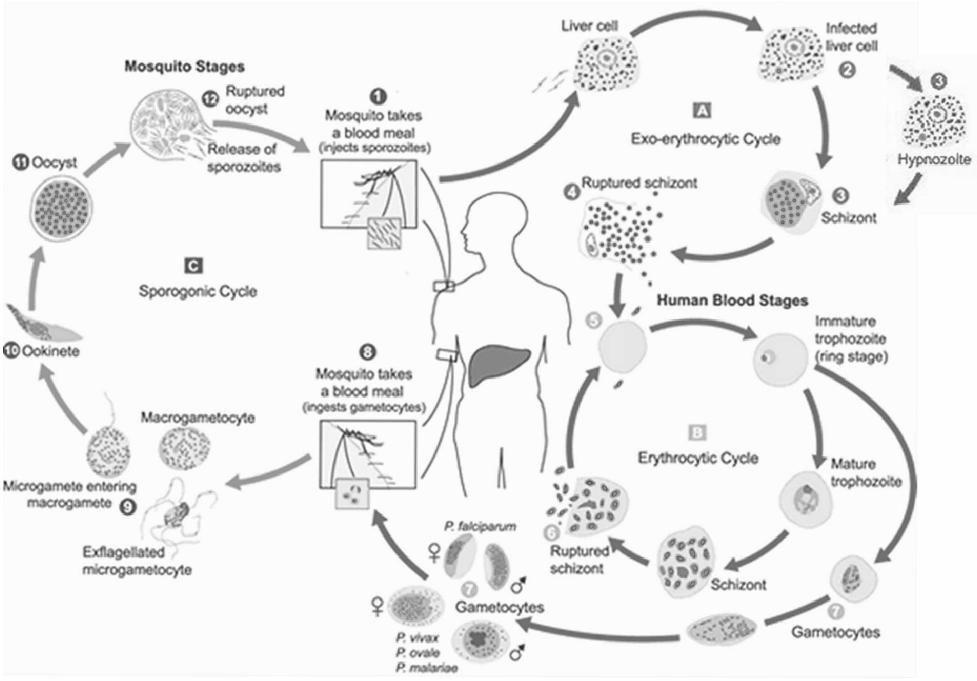

Figure 1. Life cycle of the human *Plasmodium* species. The asexual liver (A) and blood (B) stages occur in the primary host (humans), while the sexual stages (C) occur in the secondary host (mosquito). Source: http://www.uni-tuebingen.de/modeling/Mod_Malaria_Cycle_en.html, with slight modification.

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].

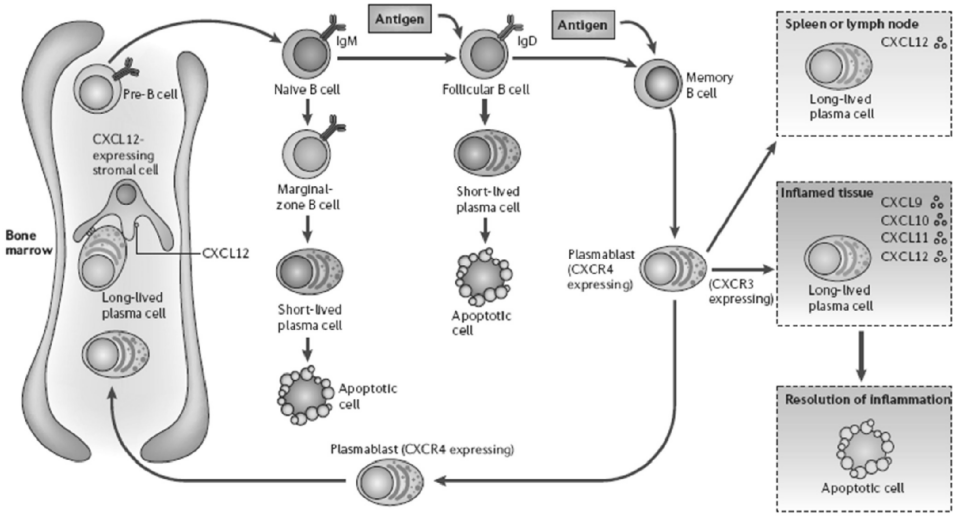

Figure 2. Maturation, activation, differentiation and survival of B-lymphocytes.
Source: Radbruch et al. [46].

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.

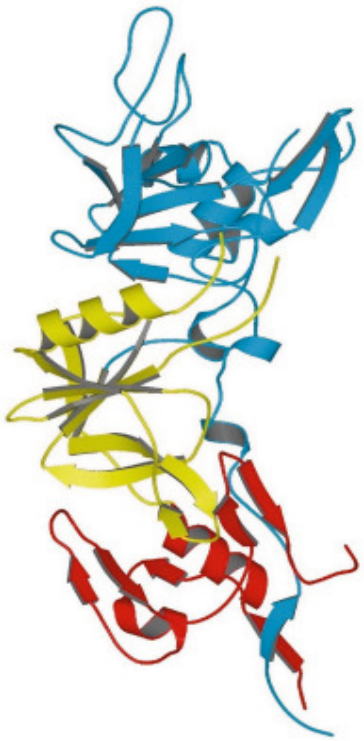

Figure 3. Ribbon structure of the ectodomain of AMA1.
The three antigen domains are shown in blue (domain I), yellow (domain II) and red (domain III).
Source: ESRF website (http://www.esrf.eu/news/spotlight/spotlight19malaria/)

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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Chapter 2

Humoral immune response to mixed *Pf*AMA1 alleles; multivalent *Pf*AMA1 vaccines induce broad specificity

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ABSTRACT

Apical Membrane Antigen 1 (AMA1), a merozoite protein essential for red cell invasion, is a candidate malaria vaccine component. Immune responses to AMA1 can protect in experimental animal models and antibodies isolated from AMA1-vaccinated or malaria-exposed humans can inhibit parasite multiplication *in vitro*. The parasite is haploid in the vertebrate host and the genome contains a single copy of AMA1, yet on a population basis a number of AMA1 molecular surface residues are polymorphic, a property thought to be primarily as a result of selective immune pressure. After immunisation with AMA1, antibodies more effectively inhibit strains carrying homologous *AMA1* genes, suggesting that polymorphism may compromise vaccine efficacy. Here, we analyse induction of broad strain inhibitory antibodies with a multi-allele *Plasmodium falciparum* AMA1 *(Pf*AMA1) vaccine, and determine the relative importance of cross-reactive and strain-specific IgG fractions by competition ELISA and *in vitro* parasite growth inhibition assays.

Immunisation of rabbits with a *Pf*AMA1 allele mixture yielded an increased proportion of antibodies to epitopes common to all vaccine alleles, compared to single allele immunisation. Competition ELISA with the anti-*Pf*AMA1 antibody fraction that is cross-reactive between FVO and 3D7 AMA1 alleles showed that over 80% of these common antibodies were shared with other *Pf*AMA1 alleles. Furthermore, growth inhibition assays revealed that for any *Pf*AMA1 allele (FVO or 3D7), the cross-reactive fraction alone, on basis of weight, had the same functional capacity on homologous parasites as the total affinity-purified IgGs (cross-reactive + strain-specific). By contrast, the strain-specific IgG fraction of either *Pf*AMA1 allele showed slightly less inhibition of red cell invasion by homologous strains.

Thus multi-allele immunisation relatively increases the levels of antibodies to common allele epitopes. This explains the broadened cross inhibition of diverse malaria parasites, and suggests multi-allele approaches warrant further clinical investigation.

INTRODUCTION

Malaria continues to be one of the most important human parasitic diseases, with a global estimate of about 247 million clinical cases and almost 1 million deaths annually [1]. The greater burden of the disease is caused by *Plasmodium falciparum* in sub-Saharan Africa, where children under 5 years old, pregnant women (mostly primigravid) and their foetuses are at the greatest risk. A cost-effective vaccine would form a powerful additional component in control strategies for malaria and a number of *Plasmodium* antigens expressed at different stages of the parasite's complex life cycle are currently undergoing clinical evaluation [2].

Among the candidates in clinical testing is *Plasmodium falciparum* Apical Membrane Antigen 1 (*Pf*AMA1), a protein expressed in sporozoites and in merozoites of both liver and asexual erythrocytic development stages, the vaccine-related properties of which has recently been reviewed [3]. In brief, AMA1 is a merozoite membrane protein initially located in micronemes. Around the time of merozoite release from schizonts AMA1 is translocated to the merozoite surface, where it is involved in merozoite/red cell interactions preceding invasion [4–6]. Anti-AMA1 antibodies can interfere with AMA1 function and prevent invasion *in vitro* [7–10], this effect requiring immunisation with correctly folded AMA1 [11,12]. The ectodomain of AMA1, which is the vaccine target, is shed as 44 and 48 kDa alternate proteins from the merozoite surface upon RBC invasion [13]. The amino acid sequence of the ectodomain has 16 cysteine residues that are conserved in all AMA1 sequences and these form disulphide bonds that result in a structure with three distinguishable but interactive domains (reviewed in [3]).

Polymorphism in AMA1 has long been evident [14], thought to be an effect of selection exerted by host immune responses [15,16]. Immunisation with one allele of AMA1 ectodomain induces antibodies that inhibit homologous parasite growth *in vitro* to a greater extent as compared to heterologous parasites [12,17,18]. The induction of functional antibodies has been demonstrated in a number of ways, including rodent and primate challenge/passive immunisation studies [15,19–21]. In some cases, and particularly with the rodent parasite *P. chabaudi*, antibodies are protective against parasites expressing homologous AMA1 but not those expressing heterologous AMA1 alleles [15,22]. Antibodies to both conserved and strain-specific *Pf*AMA1 antibody epitopes have been observed in malaria-exposed humans [23,24].

About 10% of amino acid residues of AMA1 are polymorphic, and even when appearing distant in the primary structure, may cluster within the tertiary structure [25–27]. These polymorphic clusters occur predominantly on one surface of the AMA1 molecule, which suggests that this face is accessible to antibody at the parasite surface [28]. This points to the significance of strain-specific epitopes in eliciting protective antibodies [12,17,29], although conserved AMA1 epitopes are also targets for inhibitory antibodies [30,31].

Antibodies induced by immunisation with a combination of two allelic forms of *Pf*AMA1 (FVO and 3D7) inhibit the *in vitro* growth of both parasite strains to the same extent as antibodies raised against the single respective antigens [17], although there was no significant gain in growth inhibition against unrelated parasite strains. Similar observations from antigen recognition in ELISA have been reported in human trials with vaccine candidates incorporating *Pf*AMA1 proteins from the FVO and 3D7 parasite strains [32–34], and multi-allele immunisation has been proposed as one way of overcoming strain-specificity in *Pf*AMA1 responses. The question remains whether antibodies elicited by a multi-allele vaccine would be effective against parasites expressing a relatively distant natural *Pf*AMA1 allele from those of the vaccine components.

An effective *Pf*AMA1 vaccine will be required to overcome allelic diversity. We have therefore obtained sera from rabbits immunised separately with the full ectodomain *Pf*AMA1 from FVO, 3D7 and HB3 strains, as well as sera from rabbits immunised with a mixture of these 3 *Pf*AMA1 alleles, to assess the feasibility and mechanism of broadening the antibody response by immunisation with a mixture of *Pf*AMA1 alleles. Strain-specific and cross-reactive anti-*Pf*AMA1 antibody fractions were also used to assess the relative importance and contribution of these two IgG fractions to the overall *in vitro* functional capacity of anti-*Pf*AMA1 antibodies. The study was also used to validate the competition ELISA methodology for assessing antibody responses to naturally-occurring *Pf*AMA1 antigens and establish it as an analytical tool for dissecting humoral immune responses to polymorphic antigens. Our data shows the feasibility of broadening the functional antibody response to *Pf*AMA1 by immunisation with a mixture of three *Pf*AMA1 alleles. Such a vaccine preferentially induces the expression of antibodies to epitopes that are common to the vaccine component alleles by diluting out responses to the strain-specific epitopes. Common or cross-reactive antibodies, in the absence of strain-specific antibodies, are capable of inhibiting the *in vitro* growth of parasites represented by the vaccine *Pf*AMA1 alleles as well as parasites expressing *Pf*AMA1 alleles not included in the vaccine. The data indicates that *Pf*AMA1 multi-allele vaccine strategies should be pursued and provide a justification for further clinical investigation.

METHODS

Protein production and rabbit immunisations

The full ectodomain of AMA1 allelic forms from the *P. falciparum* strains FVO, HB3, 3D7 and CAMP were expressed in *Pichia pastoris* by a similar methodology as described elsewhere [35,36]. Potential N-glycosylation sites were removed from the *Pf*AMA1 gene sequences by mutagenesis before expression.

All animals were handled in strict accordance with good animal practice as defined by the Belgian national animal welfare regulations, and all animal work

was approved by the ethics committee of the Centre d'Economie Rurale (CER Groupe, Marloie, Belgium). Rabbit immunisations were done intramuscularly (Eurogentec SA, Seraing, Belgium) with 4 doses of *Pf*AMA1 formulated in Montanide ISA720 (Seppic, Paris, France) as adjuvant, according to the manufacturer's instructions. New Zealand white rabbits were immunised on days 0, 28, 56 and 82, and the final bleed sera collected on day 95 were used in this study. Four groups of 2 rabbits each were immunised as follows: the first 3 groups received 30 µg/dose of FVO AMA1, 3D7 AMA1 or HB3 AMA1 respectively. The 4th group received 30 µg/dose of a mixture (10 ug each) of the 3 *Pf*AMA1 alleles, also formulated in the same adjuvant.

Antibody purification

Serum antibodies were purified on Protein A sepharose (GE Healthcare, Etten-Leur, The Netherlands) columns. Binding and elution buffers (Pierce, Rockford, IL) were used according to manufacturer's protocols. After elution, antibodies were concentrated and exchanged into RPMI 1640 using AmicronUltra-15 tubes (30-kDa cutoff; Millipore, Ireland). Antibodies were subsequently sterile-filtered with 0.22 µm Ultrafree MC centrifugal filter units (Millipore), the concentration determined with a Nanodrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and stored at -20°C until use.

3D7-specific AMA1 antibodies were purified from the total serum IgGs (protein A purified) of a 3D7-immunised rabbit using a 3D7 AMA1-coupled sepharose matrix. The eluted 3D7-specific IgG fraction was further separated into strain-specific (flow-through) and cross-reactive (eluate) IgGs by passage over an FVO AMA-coupled sepharose matrix. Protein A purified anti-FVO AMA1 antibodies were also affinity fractionated as has been described for anti-3D7 AMA1, first over an FVO AMA1-coupled sepharose matrix, then the eluate over a 3D7 AMA1-coupled matrix (Figure S1, page 155 of this thesis). The constituent IgG in all fractions were confirmed by ELISA and all fractions were concentrated, sterile filtered and stored at –20°C until use.

ELISA

Competition ELISA assays were carried out with AMA1 from 4 different *P. falciparum* strains (FVO, HB3, 3D7 and CAMP) to define specificities of antibodies raised against the 3 *Pf*AMA1 vaccine antigens (FVO, 3D7, HB3). An initial titration was performed to determine the optimal dilution of purified IgG required for the competition assay with each of the 3 vaccine antigens as coating antigen. Briefly, 96-well flat bottom Microlon titre plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 100 µl/well of 1 µg/ml FVO, 3D7 or HB3 AMA1 ectodomain at 4°C overnight. Plates were washed 5 times with PBS, 0.05% Tween 20 (PBS-T) using an automated plate washer (Bio-TEK Instruments, Inc, VT) and blocked with 200 µl/well of 3% BSA in PBS-T for at

least 1.5 h. IgG samples were titrated 3-fold from 1:10,000 and incubated for 2 h. A pool of sera from rabbits immunised with a mixture of the 3 *Pf*AMA1 alleles, titrated 2-fold from 1:100,000, was used as standard calibrator on all plates. Protein A purified IgG from rabbit pre-immunisation sera, titrated 2-fold from 1:10,000, was used as negative control on all plates. All samples, standards and controls were diluted with 0.5% BSA in PBS-T and added in duplicate (100 µl/well). After sample incubation, plates were washed and 100 µl/well of 1:1250-diluted goat anti-rabbit IgG/alkaline phosphatase conjugate (Pierce, Rockford, IL) added for 1 h. Plates were then washed, incubated with 100 µl/well p-nitrophenyl phosphate (pNPP; Fluka, Poole, UK) as substrate for 30 min. and the optical density (OD) at 405 nm read with a 96-well ELISA plate reader (BioRad, Japan). ODs were converted to arbitrary units (AUs) by the standard curve included on each plate using an excel-based four-parameter logistic function, which after correcting for variation approximates the IgG dilution that gives an OD of 1.0 to one arbitrary unit (1AU).

Dilutions that resulted in an AU of 2 were extrapolated for each rabbit serum/purified IgG and used for the subsequent antigen competition assay. The assay involved co-incubation of different allelic forms of *Pf*AMA1 with the same dilution of test IgGs in plates coated entirely with one of the vaccine *Pf*AMA1 alleles, such that there was competition between the added (competitor) antigens and the coated antigen for binding to test IgGs. The procedure was similar to that described for the pre-titration, except for the addition of 50 µl/well rabbit IgG at 2 times the desired dilution (equivalent to an AU of 4) to 50 µl/well of titrated soluble antigens in coated and blocked plates. Antibodies from all rabbits were co-incubated with the 4 *Pf*AMA1 alleles (FVO, HB3, 3D7, CAMP) separately, on plates coated with either FVO, HB3 or 3D7 AMA1. The competitor/soluble antigens were titrated 3-fold from 30 – 0.005 µg/ml over 9 duplicate wells, and the 10th sample wells were left without soluble antigen. The appropriately diluted IgG sample was then added to all 10 duplicate wells for each soluble antigen and after incubation for 2 h, plates were developed as described above.

To further dissect the nature and underlying mechanism of humoral responses to the different *Pf*AMA1 alleles, IgG pools made from the single allele immunisations, were also compared with IgGs from the single and multi-allele immunisations by competition ELISA.

Duplicate OD values (from residual antibody binding to the coated antigen after competition) for wells that had soluble antigens were converted to arbitrary units and expressed as a percentage of AU values from wells without soluble antigen. The percent residual binding values were then plotted (points) alongside the predicted percent values (curves) based on a least squares approximation from the following four-parameter logistic function;

$$Y = \frac{(100 - Y\min)}{1 + e^{(Xmid - X)sc}} + Y\min$$

where *Y* is the predicted % residual binding, $\boldsymbol{Y_{min}}$ is the maximal depletion at infinite soluble antigen concentration (minimum value), ***X*** is the soluble antigen concentration (log scale), $\boldsymbol{X_{mid}}$ is the soluble antigen concentration (log scale) at which 50% antibody depletion is achieved (midpoint between the maximum and minimum depletion values), and ***sc*** is the slope of the curve. Percent antibody depletion for any competitor/soluble antigen is therefore the difference between 100% (binding in the absence of soluble antigen) and the residual binding.

The competition assay was initially validated by testing anti-FVO AMA1 IgG or serum at dilutions equivalent to 0.2, 0.5, 1, 2, 4 and 8 times the titre (1AU) on FVO-coated plates (100 ng/well) with the same soluble antigen concentrations (3-fold titration from 30 µg/ml over 9 duplicate wells). The assay was shown to be reproducible and independent of the antibody source (serum or purified IgG) and the dilution provided the OD values in wells with no competitor antigen were within the linear portion (ODs of 0.3 – 2.5 over blank) of the standard curve.

Antibody avidity measurements

The binding capacity of antibodies raised by single and mixed allele immunisations were determined by avidity ELISA with sodium isothiocyanate (NaSCN) elution. Briefly, 96-well flat bottom Microlon titre plates were coated with AMA1 allelic antigens as described above, and after blocking, incubated with a pre-determined titre (1AU) of sera from immunised rabbits for 1 h. Plates were then washed and incubated with an increasing concentration of NaSCN (0, 0.25, 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5 and 3.0M) in different duplcate wells for 15 min. Plates were again washed and subsequently developed with goat anti-rabbit IgG/alkaline phophatase conjugate and pNPP substrate as already described. Avidity index, the concentration of NaSCN required for 50% dissociation of bound antibodies (relative to duplicate wells without NaSCN) was the extrapolated in Microsoft excel for each rabbit serum sample.

Parasite cultures and Growth Inhibition assays

Protein A and affinity-purified IgG fractions were tested for *in vitro* activity in parasite growth inhibition assays (GIAs) as described elsewhere [18]. All IgGs and IgG pools were tested in triplicate on FCR3, NF54, HB3 or CAMP parasite strains at a 3-fold serial dilution from 6 mg/ml (protein A purified IgG) or 1 mg/ml (affinity-purified IgG) in 96-well culture plates. Parasites were cultured under standard conditions (an atmosphere of 5% CO_2, 5% O_2, and 90% N_2, 37°C), and the *Pf*AMA1 antigen expressed by all parasite strains were verified by PCR and restriction fragment length analysis. Parasite cultures were mycoplasma-free and synchronized with 0.3 M alanine, 10 mM Hepes pH 7.5 before use in an

assay. Late trophozoite/early schizont stages at a parasitaemia of 0.3 ± 0.1% and 2% final haematocrit were used in all assays. The final culture volume was 50 µl/well and parasites were incubated for 40-45 hrs. Parasite growth was assessed by measuring parasite lactate dehydrogenase levels with the lactate/diaphorase/APAD substrate system, and plates were read at 655 nm after 30 min of development. Parasite growth inhibition was expressed as;

$$\%\ \ inhibition = 100 - \frac{(A_{655}Sample - A_{655}RBC)}{(A_{655}SZ - A_{655}RBC)} \times 100$$

where $A_{655}Sample$ is the OD_{655} for any test sample well, $A_{655}SZ$ is the average OD_{655} of schizont control wells included on each plate and $A_{655}RBC$ is the average OD_{655} of RBC control wells. The data was presented as the arithmetic mean % inhibition from each sample triplicate.

Statistical analyses

Residual binding (or minimum) values in competition ELISA, and the corresponding confidence intervals were generated by a 4-parameter logistic fit with least squares approximation using the *R* statistical package (R Development Core Team, 2008, version 2.8.1). Comparisons between minimum values estimated in the non-linear regression were done with Student's *t* test; *p* values < 0.05 were considered statistically significant. All plots were prepared with the *R* statistical package. Since antibody depletion patterns in ELISA were similar for IgG samples from the two rabbits in each immunisation group, data presented (ELISA and GIA) are for only one rabbit per group and is therefore of a qualitative nature.

RESULTS

Competition ELISA validation

The competition ELISA assay used was based on coating plates with one allele of *Pf*AMA1 and mixing test antibody samples with the same or other *Pf*AMA1 alleles to determine the degree to which antibody binding to the coating material was inhibited. The assay was reproducible within and between runs and at various initial serum/IgG concentrations (CV was generally below 2%, and maximally 15% for very low OD values). Antibody depletion patterns were similar irrespective of whether serum or protein A-purified IgG samples were used for the assay (data not shown). Depletion patterns, plotted as percentages, were similar irrespective of the final serum /IgG dilution used provided the OD value was within the linear portion of the standard curve. An 8-fold active dilution range (0.5 – 4 AU) gave optimal results (Figure S2, page 156 of this thesis).

Dilutions above this range (8AU or higher) shifted the curves to the left (suggesting less antigen required for depletion compared to dilutions within the linear range), and dilutions below the range (0.2AU or lower) shifted curves to the right (suggesting more antigen required for depletion compared to dilutions within the linear range) (Figure S2). Antibody depletion patterns were also very similar for each pair of rabbits immunised with the same antigen, irrespective of the original antibody response/titre (data not shown).

A 4-parameter logistic plot was used to assess the reproducibility and robustness of the assay by comparing data from three different assays and at three different antibody dilutions using final ODs within the linear portion. A statistical comparison of the minimum values, the most informative parameter for comparing antibody specificities by this assay, is presented in Table 1 for an assay involving co-incubation of 4 different competitor antigens with total IgG from an FVO AMA1 immunisation in FVO AMA1-coated plates. For any competitor antigen, the minimum value represents the proportion of antibodies that do not bind to the competitor antigen, but do bind to the coating antigen. This fraction of the test antibodies represents the strain-specific component with respect to the competitor antigen, while the fraction depleted by the competitor

Table 1. Residual IgG binding estimates from competition ELISA validation

	Competitor antigen (*Pf*AMA1)			
	FVO	**HB3**	**3D7**	**CAMP**
Expt.1 (1AU)	4.2 (-1.8 - 10.2)	14.2 (-3.8 - 32.2)	50.1 (43.7 - 56.5)	33.1 (29.3 - 36.8)
Expt.1 (2AU)	3.3 (-1.9 - 8.5)	8.4 (-18.3 - 35.0)	50.2 (45.0 - 55.3)	34.1 (29.6 - 38.5)
Expt.2 (2AU)	3.7 (-1.3 - 8.6)	22.4 (14.8 - 29.9)	49.9 (42.0 - 57.9)	31.6 (25.5 - 37.6)
Expt.3 (4AU)	6.6 (1.2 – 12.0)	5.4 (0.1 - 10.6)	51.6 (42.6 - 60.7)	36.2 (28.0 - 44.4)
Mean of all Expts.	4.4 (0.3 - 8.6)	13.5 (6.1 - 20.9)	50.4 (44.9 - 55.9)	33.7 (29.8 - 37.5)

The assay was validated by repeated assessment of anti-FVO AMA1 antibody depletion by FVO, HB3, 3D7 and CAMP AMA1 competitor/soluble antigens, on FVO AMA1-coated plates. Values are the estimated minimum residual binding and reported as minimum value (95% CI) for each of the competitor/soluble antigens. Assays were performed on three different days with three different antibody dilutions (1AU, 2AU, 4AU). 1AU is equivalent to the antibody titre.

antigen represents antibodies that are capable of binding to both the coated and competitor antigens. With the exception of HB3 AMA1, competitor antigens had highly consistent minimum values between assays and with different starting antibody dilutions. Minimum values for 3D7 and CAMP AMA1 competitor antigens were consistently and significantly different from the homologous FVO AMA1 competitor antigen. The shape of the HB3 AMA1 competitor antigen curve suggested that at higher concentrations, further antibody depletion was possible. This was not the case for the other competitor antigens as the minimum values had almost reached a plateau at the highest antigen concentration used. As a result, relatively wide confidence intervals were obtained for minimum values when HB3 AMA1 competed for anti-FVO AMA1 antibodies (Table 1).
The slope describes an inverse relationship between antibodies not depleted by the competitor antigen and the log-concentration of competitor antigen, with a higher absolute value representing a steeper curve. At different antibody dilutions for FVO, 3D7 and CAMP AMA1 competitor antigens, the slope was fairly consistent between assays (-0.26 to -0.52), with most values around -0.40. *Xmid*, like IC_{50}, is the concentration of competitor antigen that results in 50% antibody depletion. However, interpretation of this parameter is confounded by the different minimum values for the different competitor antigens (Figure S2).

Antibodies from multi-allele immunisation have an increased cross-reactivity

Once validated, the competition ELISA assay was used to compare the relative proportions of cross-reactive and strain-specific antibody fractions induced by single and mixed *Pf*AMA1 allele immunisations. Antibody depletion patterns revealed significantly increased cross recognition of all heterologous competitor antigens by antibodies from the mixed allele immunisation (anti-Combi) compared to those from the single allele immunisations (Figure 1, Table 2). This implies that there is a higher proportion of antibodies to common epitopes in the mixed allele immunisation compared to the proportion in any single allele immunisation. For example, recognition and depletion of antibodies by the FVO AMA1 competitor antigen increased significantly from 60% (40.2% residual binding) for IgGs from the 3D7 AMA1 single allele immunisation to over 80% (17.8% residual binding) for anti-Combi antibodies ($p<0.0001$) in competition assays with 3D7 AMA1 as coating antigen (Figure 1A). Similarly, depletion by HB3 AMA1 in the same assays was almost 70% (29.9% residual binding) with anti-3D7 AMA1 antibodies and up to 95% (5.73% residual binding) with anti-Combi antibodies ($p=0.0006$). Depletion by CAMP AMA1, which was not a component antigen of the mixed allele vaccine, also increased significantly from 60% (39.9% residual binding) with anti-3D7 AMA1 antibodies to almost 75% (25.7% residual binding) with anti-Combi antibodies ($p=0.0008$). Similar trends of increasing recognition and depletion were observed by comparing antibodies

from single allele immunisation with HB3 AMA1 and FVO AMA1 with the mixed allele immunisation when assays were performed with the respective *Pf*AMA1 alleles as coating antigens (Figures 1B and 1C). Depletion of anti-Combi antibodies was consistently highest for all competitor antigens when FVO AMA1 was used as the coating antigen. CAMP AMA1 highly recognized and depleted anti-FVO AMA1 antibodies (31.6% residual binding) and least recognized anti-HB3 antibodies (55.0% residual binding), but relatively similar degrees of anti-Combi antibodies were recognized/depleted by CAMP AMA1 irrespective of the coating antigen used (Table 2).

To further assess the nature of antibodies raised in a mixed allele immunisation, protein A-purified antibody pools containing anti-FVO/HB3, anti-FVO/3D7 and anti-HB3/3D7, as well as a pool of antibodies against all three *Pf*AMA1 alleles (anti-FVO/HB3/3D7) were made from antibodies raised in the single allele-immunised rabbits. These antibody pools were compared to antibodies from single and mixed allele immunisations in competition ELISA with FVO, HB3 or 3D7 AMA1 as coating antigens. Depending on the coating antigen/IgG pool combination, there were small changes in depletion by the four native *Pf*AMA1 alleles compared to the IgGs from single allele immunisations, with mixed significance (data not shown). The antibody pools with anti-HB3 IgGs were predictably less recognized by CAMP AMA1. Recognition/depletion patterns of the pool of three (anti-FVO/HB3/3D7) were intermediate between the observed patterns for antibodies from single and mixed allele immunisations (Figure 1). Generally, there was the tendency for greater depletion (lower residual binding) of anti-Combi antibodies by all competitor antigens compared to the depletion of antibodies from the anti-FVO/HB3/3D7 IgG pool (Table 2). This suggests that although IgG pooling would result in a decreased proportion of strain-specific antibodies, immunisation with the mixed antigens yields even lower levels of antibodies to epitopes that are specific to the component vaccine antigens. In order to assess the functional capacity of these antibodies in relation to the observations made by competition ELISA, growth inhibition assays were performed with the FCR3 (one pro-sequence amino acid difference from FVO AMA1), HB3, NF54 (with identical AMA1 to clone 3D7) and CAMP strains of *P. falciparum*. In assays with NF54, HB3 and FCR3 parasite strains, growth inhibition levels decreased more rapidly with decreasing concentration of antibodies against heterologous *Pf*AMA1 alleles (Figure 2). Thus the extent of *in vitro* growth inhibition of any parasite strain was dependent on the antibody source (homologous versus heterologous), and generally for heterologous parasites, also on the number of amino acid variants between the vaccine and parasite AMA1 alleles (see Table 3). For example, the growth of NF54 parasites was best inhibited by anti-3D7 AMA1 antibodies and least by anti-FVO AMA1 antibodies over the 4 dilutions tested, with anti-HB3 AMA1 antibodies yielding intermediate inhibition (Figure 2).

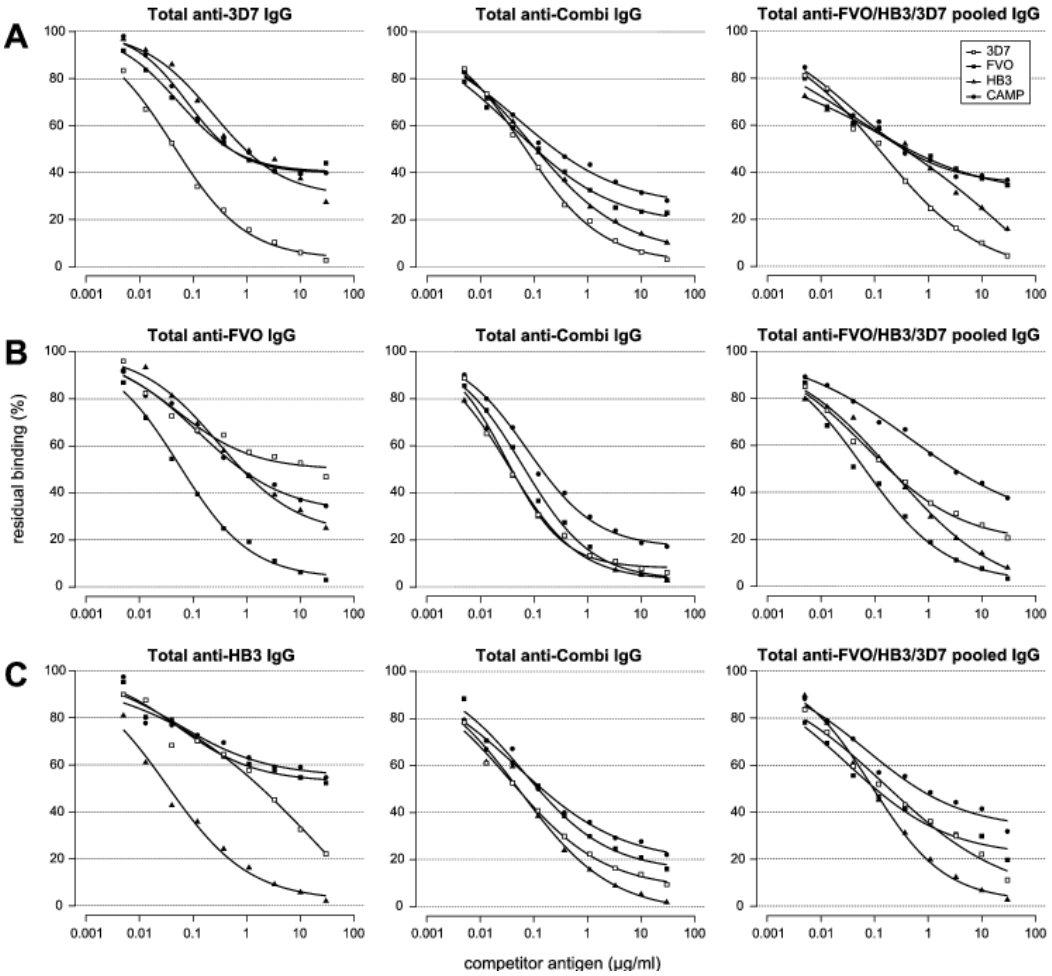

Figure 1. Competition ELISA with protein A-purified antibodies and antibody pools made from single allele immunisations. A) Assay on 3D7 AMA1-coated plates with anti-3D7 AMA1, anti-Combi (mixed allele immunisation) and anti-FVO/HB3/3D7 antibody pool. B) Assay on FVO AMA1-coated plates with anti- FVO AMA1, anti-Combi and anti-FVO/HB3/3D7 antibody pool. C) Assay on HB3 AMA1-coated plates with anti- HB3 AMA1, anti-Combi and anti-FVO/HB3/3D7 antibody pool. IgG pools were made from antibodies raised in single allele immunisations with FVO, HB3 and 3D7 AMA1. All assays were performed with FVO, HB3, 3D7 and CAMP AMA1 proteins as competitor antigens. All IgGs were used at 2 times the pre-determined antibody titre. Plots are representative of at least 2 assay repeats using IgGs from one rabbit per group since the depletion patterns were similar for both rabbits in each immunisation group.

Most importantly, however, anti-Combi antibodies resulted in growth inhibitions that were comparable with that of antibodies from single allele immunisations on the respective homologous parasite strains in all cases. Furthermore, the anti-Combi antibodies yielded the best growth inhibition of CAMP parasites compared to antibodies from the 3 single allele immunisations, even though CAMP AMA1 was not included in the mixed allele vaccine.

Table 2. Residual IgG binding estimates for antibodies raised in single and multi-allele immunisations.

Coating antigen	IgG Sample	Competitor antigen (*Pf*AMA1)			
		FVO	**HB3**	**3D7**	**CAMP**
FVO AMA1	anti-FVO	3.7 (-1.3 - 7.2)	22.4 (14.8 - 29.9)	49.9 (42.0 - 57.9)	31.6 (25.9 - 37.2)
	anti-Combi	3.0 (-1.3 - 7.2)	2.9 (0.1 - 5.8)	8.0 (3.1 - 12.9)	16.8 (12.6 - 20.9)
	IgG pool§	1.9 (-7.1 - 10.9)	-3.2* (-15.9 - 9.4)	18.6 (12.5 - 24.7)	25.5 (14.1 - 36.8)
		FVO	**HB3**	**3D7**	**CAMP**
3D7 AMA1	anti-3D7	40.2 (36.8 - 43.6)	29.9 (19.6 - 40.2)	3.3 (-0.8 - 7.4)	40.0 (36.0 - 43.8)
	anti-Combi	17.8 (13.4 - 22.2)	5.7 (2.4 - 9.0)	1.9 (-1.5 - 5.4)	25.7 (19.2 - 32.2)
	IgG pool§	29.9 (18.8 - 40.9)	-150* (-546 - 246)	-3.0* (-11.1 - 5.0)	34.6 (28.9 - 40.3)
		FVO	**HB3**	**3D7**	**CAMP**
HB3 AMA1	anti-HB3	52.7 (46.2 - 59.1)	2.3 (-6.0 - 10.5)	-57.7* (-299 - 184)	55.0 (43.0 - 67.0)
	anti-Combi	15.1 (7.6 - 22.5)	-2.2* (-11.7 - 7.4)	7.5 (1.5 - 13.5)	18.8 (8.2 - 29.5)
	IgG pool§	21.2 (12.6 - 29.8)	2.1 (-2.3 - 6.5)	3.3 (-16.8 - 23.4)	32.9 (22.1 - 43.7)

Residual binding values are the predicted minimum values based on the measured values for each competitor antigen, and were generated with a four-parameter logistic fit with least squares approximation. Values are estimated with the R statistical package and reported as % residual binding or minimum value (95%CI).
§A pool of IgGs from the single allele immunisations with FVO, HB3 and 3D7 AMA1.
*Negative estimate of residual binding (minimum values have not reached a plateau yet). Minimum values cannot be accurately estimated.

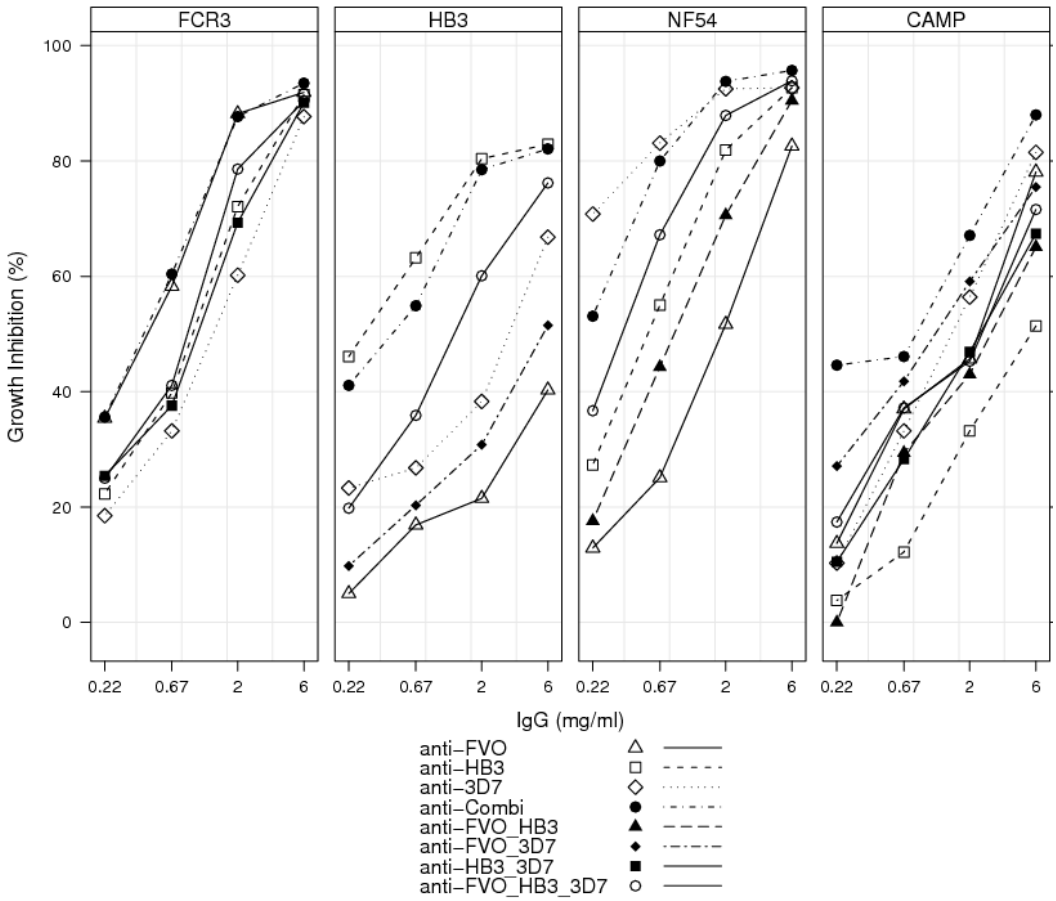

Figure 2. Growth inhibition levels exhibited by protein A-purified IgGs from single/mixed *Pf*AMA1 immunisations and IgG pools. All IgG fractions were tested in a single growth cycle assay with FCR3, HB3, NF54 and CAMP strains of *P. falciparum*. For all strains, assays were performed with 0.3 ± 0.1% parasitaemia and a final haematocrit of 2%. IgG samples were tested at 4 dilutions (3-fold titration from 6 mg/ml). IgG pools were made from antibodies raised in single allele immunisations with FVO, HB3 and 3D7 AMA1. The data presented is representative of at least two assay repeats using IgGs from one of the two rabbits per group.

The relatively similar ELISA titres of mono-specific antibodies (950,000 for anti-FVO, 1,150,000 for anti-HB3 and 1,280,000 for anti-3D7 AMA1) on the respective homologous *Pf*AMA1 alleles compared to anti-Combi antibodies (1,230,000 on FVO, 1,140,000 on HB3 and 1,120,000 on 3D7 AMA1) as measured in protein G-purified fractions eliminates the possibility of this observation being due to higher titres of anti-Combi antibodies. The observed GIA activity cannot also be attributed to a better quality of the anti-Combi antibodies since these were shown to have antigen-binding capacities in the same order as mono-specific antibodies when titres were normalized (Table 4). The current observation may therefore be mainly attributed to the induction of a more cross-reactive antibody profile and represents a broadened inhibitory capacity of anti-Combi antibodies compared to antibodies induced in single allele immunisations.

GIAs with the antibody pools were also performed to assess the trends in growth inhibition with respect to the varying antibody specificities. Results showed that differences in growth inhibition between the pools were dependent on the parasite strain/antibody pool combination.

Table 3. Number of amino acid variants between vaccine and *Pf* parasite AMA1 alleles.

Vaccine antigen	Parasite strain			
	NF54	HB3	FCR3	CAMP
3D7	***6** (2,3,1)	**29** (15,8,6)	**30** (19,8,3)	**26** (14,9,3)
HB3	**30** (16,8,6)	***5** (1,3,1)	**24** (13,7,4)	**31** (17,8,6)
FVO	**30** (19,8,3)	**23** (12,7,4)	***6** (2,3,1)	**20** (11,6,3)

Values represent only the differences in domains I, II and III of *Pf*AMA1 ectodomain. Differences per domain have been presented in brackets as (domain I, domain II, domain III).
*The 6 variant amino acids between "homologous" AMA1 alleles (5 for HB3) are due to amino acid substitutions introduced to prevent protein glycosylation and cleavage. Substitutions occur at positions 162 and 288 in domain I (position 288 only for HB3), positions 373, 422 and 423 in domain II, and position 499 in domain III.

Table 4. Measured avidity Indices for mono-specific and anti-Combi antibodies against vaccine *Pf*AMA1 alleles.

	Avidity Index by capture antigen		
Rb ID (Immunising Ag)	**FVO**	**HB3**	**3D7**
1455 (FVO)	1.23	0.91	1.01
1456 (HB3)	0.89	1.10	0.81
1459 (3D7)	1.15	1.03	1.38
1461 (Combi)	1.04	1.06	1.31

Avidity index of AMA1-specific antibodies was estimated as the concentration of NaSCN required to dissociate 50% of AMA1-bound antibodies. The avidity indices of mono-specific antibodies were determined against both the immunising ("homologous") allele and the other two "heterologous" alleles. That for anti-Combi antibodies was determined against all vaccine component alleles.

The anti-FVO/3D7 antibody pool, for example, inhibited the growth of CAMP parasites better (Figure 2) than the other double pools (anti-FVO/HB3, anti-HB3/3D7), even though these pools had similar antibody titres as measured by ELISA (data not shown). This is likely due to the fact that HB3 AMA1, being the most distant allele from CAMP AMA1 in terms of amino acid residues, shares fewer functional epitopes with CAMP AMA1 such that the pools with anti-HB3 AMA1 antibodies cross-reacted least with CAMP AMA1, resulting in relatively lower parasite growth inhibitions.

Inhibition of all strains by anti-Combi antibodies was higher compared with the anti-FVO/HB3/3D7 IgG pool, confirming the induction of higher levels of antibodies to common epitopes by multi-allele immunisation. Antibodies to common allele epitopes are predominantly induced since strain-specific epitopes on each of the vaccine alleles have been diluted out in the vaccine antigen mixture, and this translates to a broadened scope of *Pf*AMA1 recognition. The functional assay therefore confirms observations made by competition ELISA, and shows that mixed allele immunisation predominantly yields antibodies to common allele epitopes and low levels of antibodies to strain-specific epitopes. High levels of antibodies to the common vaccine allele epitopes are invariably required for broad strain inhibition.

Most cross-reactive antibody epitopes are shared by all alleles

To assess the relative contributions of strain-specific and cross-reactive antibodies to overall antigen recognition and parasite inhibition, strain-specific and cross-reactive antibody fractions were affinity-purified from total IgGs of rabbits immunised with 3D7 AMA1 alone and FVO AMA1 alone. Cross-reactive and strain-specific IgG fractions for each of the two *Pf*AMA1 alleles were prepared with respect to the other allele (procedure presented schematically in Figure S1). Up to 90% of recovered IgGs from anti-3D7 AMA1 antibodies were cross-reactive with FVO AMA1, while over 95% of recovered IgGs from anti-FVO AMA1 antibodies were cross-reactive with 3D7 AMA1. These IgG fractions were compared with the respective un-fractionated affinity-purified anti-3D7 or anti-FVO IgGs by competition ELISA. The strain-specific fraction of anti-3D7 AMA1 IgGs had very little reactivity with HB3 and CAMP AMA1 alleles (Figure 3A). There was however, an improved recognition and depletion of IgGs in the cross-reactive fraction by all the heterologous *Pf*AMA1 alleles used (FVO, HB3, CAMP). Similar observations were made with anti-FVO AMA1 strain-specific and cross-reactive IgG fractions, except that the strain-specific fraction of anti-FVO AMA1 IgG was still highly reactive with HB3 AMA1, and to a lesser extent with CAMP AMA1 (Figure 3B). Based on these observations, it is likely that FVO AMA1 may induce the production of antibodies that are more cross-reactive in comparison with 3D7 AMA1. The affinity-purified antibody fractions were also tested for

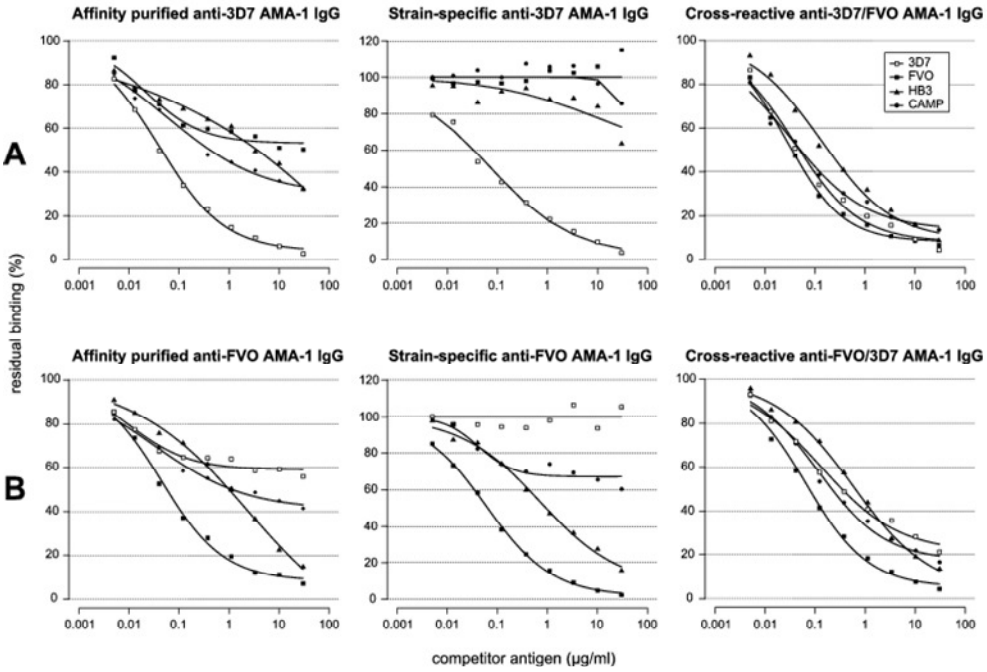

Figure 3. Competition ELISA with *Pf*AMA1-specific IgG fractions. Anti-3D7 AMA1 IgG was affinity-purified from total (Protein A) IgG of one of the 3D7 AMA1-immunised rabbits. A portion of this IgG fraction was afterwards fractionated into 3D7 AMA1 strain-specific IgG (flow through) and 3D7/FVO cross-reactive IgG (eluate) by passage over an FVO AMA1 affinity matrix. Similar specific fractions were made from total IgGs from one of the FVO AMA1-immunised rabbits, first over an FVO AMA1 matrix, and then over a 3D7 AMA1 matrix. All IgG fractions were used for competition assays at 2 times the pre-determined antibody titre. AMA1 antigens from the 3D7, HB3, FVO and CAMP parasite strains were used as competitor antigens in all assays. Assays were done using plates coated with 3D7 AMA1 (A) and FVO AMA1 (B), and plots are representative of data from at least 2 repeat assays.

functional capacity by *in vitro* growth inhibition assays on FCR3 (FVO), CAMP and NF54 (3D7) parasite strains (Figure 4). The cross-reactive fractions alone had the same functional capacity on homologous parasites as the respective total affinity-purified IgGs when both were tested at the same concentrations. The anti-3D7 cross-reactive fraction showed slightly less inhibition on FCR3 heterologous parasites over the four antibody concentrations tested as compared to the anti-FVO cross-reactive fraction, and the reverse was true for the inhibition of NF54 parasites. Furthermore, both cross-reactive fractions yielded slightly lower inhibition of CAMP parasites compared to the inhibitions observed for same fractions on their respective homologous parasites. By contrast, the strain-specific fractions showed slightly less inhibition of red cell invasion by homologous parasites compared to the cross-reactive and total fractions. Both strain-specific fractions had negligible inhibitory effect on heterologous parasites, including the CAMP heterologous strains. These observations confirm the need to induce cross-reactive antibodies in overcoming allelic diversity to *Pf*AMA1, but also show that the cross-reactive antibody

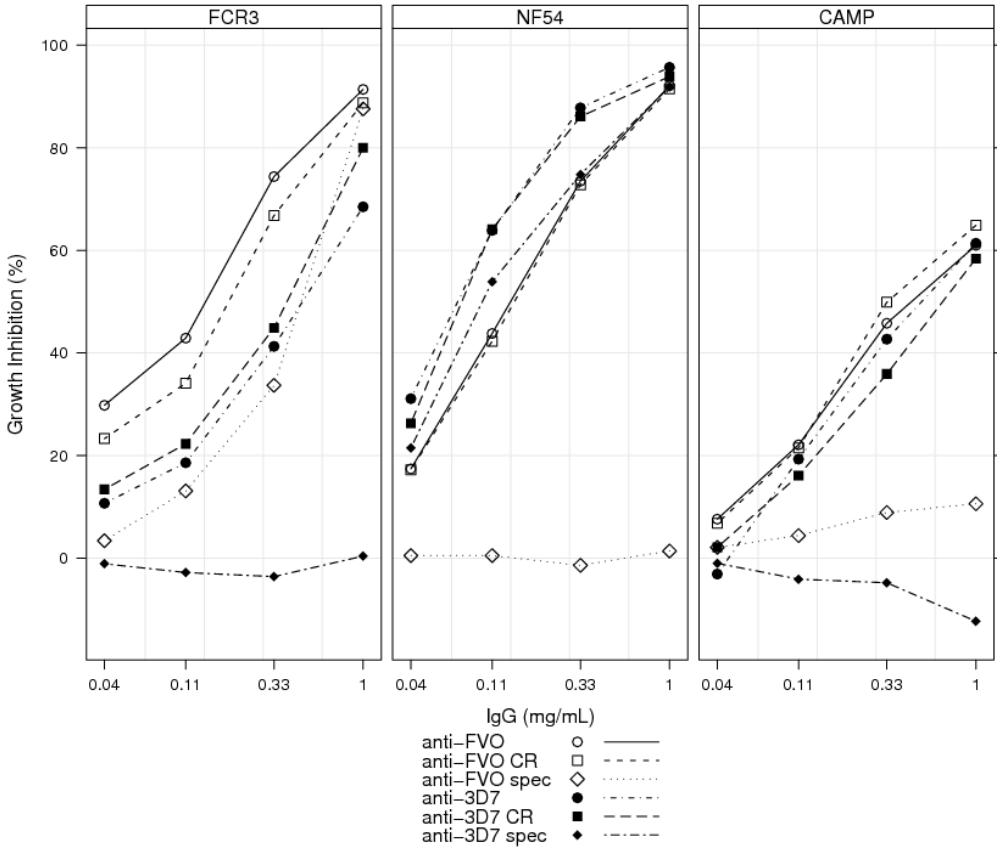

Figure 4. Representative data showing levels of parasite growth inhibition exhibited by affinity-purified AMA1-specific IgG fractions. All fractions were tested for functional activity on FCR3, NF54 and CAMP strains of *P. falciparum*. For all strains, assays were done with 0.3 ± 0.1% parasitaemia and a final haematocrit of 2%. IgG samples were tested at 4 dilutions (3-fold titration from 1mg/ml). *Anti-FVO* and *anti-3D7* are the respective total affinity-purified IgGs, *anti-FVO CR* and *anti-3D7 CR* designate the cross-reactive fractions, while *anti-FVO spec* and *anti-3D7 spec* designate the respective strain-specific fractions.

fraction from a single *Pf*AMA1 allele immunisation may not be as efficient for achieving significant parasite inhibition.

DISCUSSION

Allelic polymorphism in AMA1 is due to single amino acid substitutions and has been linked with host immune pressure on the parasite [15,25]. Although this makes AMA1 a possible target for natural as well as vaccine induced responses, polymorphism presents the practical challenge of developing a broadly effective vaccine since immunisation with one AMA1 haplotype appears not to protect against parasites expressing relatively distant haplotypes [12]. Preliminary analysis of about 745 *Pf*AMA1 amino acid sequences pulled from PubMed through GeneBank shows 236 unique AMA1 haplotypes, with an estimated 189 occurring in domain I alone (unpublished data). An effective vaccine is expected to protect against this diversity of parasites globally or at least within a

particular endemic region. It is worth noting that while about 10% of amino acid residues in the AMA1 ectodomain are polymorphic, most of these residues are dimorphic, a few are tri- or tetramorphic, and a single position in domain I (197) is heptamorphic [25,28]. Polymorphic residue linkages present within the molecule also tend to limit the choice of amino acids in certain polymorphic positions [18], providing some level of polymorphic stability. Overcoming *Pf*AMA1 polymorphism is therefore a key step in vaccine development, and immunisation with a mixture of *Pf*AMA1 alleles has been proposed as one possible solution to this challenge [3,17,18,37].

The aim of the present study was to determine the relative functional importance of cross-reactive and strain-specific antibody fractions elicited upon immunisation with a particular *Pf*AMA1 allele, and also to assess the feasibility of achieving broad strain recognition by antibodies to a multi-allele *Pf*AMA1 vaccine. We validated a competition ELISA assay for assessing antibody specificities providing a highly reproducible and robust methodology for dissecting specific antibody responses to immunisation with a *Pf*AMA1-based vaccine. The assay was independent of the specific antibody source (serum or purified IgG) and the antibody dilution factor when the working OD values fell within the linear portion of the standard/calibration curve. The latter observation may be explained by the fact that the antigen-antibody complex reaction, being reversible, would always have very similar percentage proportions of reaction components when it attains a dynamic equilibrium state. It is also worthwhile noting that the anti-*Pf*AMA1 antibody depletion patterns observed for the various competitor *Pf*AMA1 alleles in competition assays were generally predictive of the extent of growth inhibition of the different parasite strains by the anti-*Pf*AMA1 antibodies *in vitro*.

The major findings of this study are that i) immunisation with a mixture of allelic *Pf*AMA1 forms predominantly induces the production of cross-reactive anti-*Pf*AMA1 antibodies, and ii) the cross-reactive fraction of antibodies to any *Pf*AMA1 allele has the same functional capacity (GIA) as the total anti-*Pf*AMA1 antibodies (cross-reactive + strain-specific) at the same concentration, hence induction of antibodies to epitopes that are common to a number of *Pf*AMA1 alleles will not reduce the inhibitory capacity against parasites expressing any of the vaccinating alleles.

The increased recognition and depletion of anti-Combi antibodies by all competitor antigens, including the out-group antigen CAMP, compared to that of mono-specific antibodies implies a broadened antibody response (Figure 1). This is most likely due to the induction of antibodies predominantly to epitopes that are common to the component alleles of the vaccine, and similar observations have been made in earlier studies [17,27,37]. Since strain-specific epitopes on each vaccine allele are expected to be present at relatively low quantities in the mixture, they will have a decreased probability of presentation to the relevant immune system effectors compared to common epitopes. Indeed, the levels of

antibodies to common epitopes in the mixed allele immunisation, as assessed by competition ELISA assays, were constantly higher relative to the levels in antibody pools made from the three single immunisations with the same antigens (Figure 1). This was confirmed by the growth inhibition patterns observed when all antibody fractions were tested against the FCR3, HB3, NF54 and CAMP parasite strains (Figure 2).

Recognition and depletion of affinity-purified IgG fractions from FVO and 3D7 AMA1 mono-specific immunisations provide further evidence for the induction of antibodies against common epitopes. Most (≥ 80%) of the cross-reactive epitopes between these two *Pf*AMA1 alleles are shared with CAMP and HB3 AMA1 alleles (Figure 3). Additionally, for the anti-3D7 IgG fraction, affinity depletion of anti-FVO cross-reactive antibodies removed up to 80% of antibodies reactive to the HB3 and CAMP AMA1 alleles. These observations may possibly extend to the many other *Pf*AMA1 alleles not tested in this study. The patterns of recognition and depletion of the affinity-purified, cross-reactive fractions closely resemble those of antibodies induced by the mixed allele immunisation in ELISA.

Observations from competition ELISA were confirmed by functional parasite growth inhibition assays with the FCR3, NF54, HB3 and CAMP strains of *P. falciparum.* The extent of antigen recognition by these antibody fractions was predictive of the degree of functional parasite inhibition observed *in vitro.* Furthermore, assays with affinity-purified anti-*Pf*AMA1 antibodies on FCR3 and NF54 parasite strains showed that at the same concentration, the cross-reactive fractions had the same growth inhibitory effects as the total anti-*Pf*AMA1 IgGs on homologous parasites, while the strain-specific fractions had slightly lower inhibitions over the IgG concentrations tested (Figure 4). Thus in the absence of the strain-specific fraction of antibodies against any *Pf*AMA1 allele, the cross-reactive fraction alone is still highly inhibitory against homologous parasites. The limited cross inhibition of heterologous parasites by antibodies from single allele immunisations may therefore be attributed to the proportions of cross-reactive and strain-specific antibodies; the cross-reactive antibody fraction may not be enough to effectively inhibit heterologous parasite invasion of RBCs to the same extent as homologous strain inhibition, which would involve both cross-reactive and strain-specific antibody activity.

Cross-reactive antibody fractions of both anti-FVO and anti-3D7 AMA1 IgGs, initially derived from mono-specific sera, inhibited the respective heterologous strains less effectively *in vitro* (Figure 4). Since the same concentration of both antibody fractions resulted in greater growth inhibition of the respective homologous parasites, the current observation may be a potential consequence of the affinity purification process, or logically due to avidity differences in binding to homologous and heterologous parasite *Pf*AMA1 alleles. This latter observation, if confirmed, would imply that the cross-reactive fraction of antibodies generated by single allele immunisation may not be as equally good as

cross-reactive antibodies induced by multi-allele immunisation in terms of functional capacity against heterologous parasites.

The measured avidity indices for anti-Combi and mono-specific antibodies on the immunising antigen(s) as well as for mono-specific antibodies on "heterologous" *Pf*AMA1 alleles were comparable (Table 4). Due to antibody titre normalization however, the quantity of each mono-specific serum that was used for the avidity determination on heterologous alleles was up to 2-fold higher than the quantity of mono-specific and anti-Combi antibodies used on the respective homologous alleles. Thus cross-reactive antibodies, irrespective of the source, bound the allelic *Pf*AMA1antigens to very similar degrees, and the only factor that influences the extent of *in vitro* parasite inhibition was the absolute levels of these functional antibodies. These observations, taken together with the fact that the functional activity of antibodies is generally linked with their antigen binding strength [38,39], support the conclusion that anti-Combi antibodies are most likely high-avidity in nature. Additionally, most low-avidity antibodies are likely to be lost during affinity purification of *Pf*AMA1-specific antibodies. Depletion patterns for the affinity-purified cross-reactive fractions from both anti-3D7 and anti-FVO AMA1 antibodies (Figure 3) were however, similar to those of anti-Combi antibodies (Figure 1) which were Protein A-purified and should therefore include any low-avidity AMA1-specific antibodies. The absence of such low-avidity, cross-reactive antibodies after affinity purification would be expected to result in antibody depletion patterns that are rather similar to those of mono-specific antibodies (Figure 1).

Considering the data presented, it may be hypothesized that apart from antibody specificity, an optimal concentration of antibodies is also necessary in order to achieve a good degree of parasite inhibition. These findings are important for two main reasons, that i) there is no loss in *in vitro* inhibitory capacity by mainly inducing antibodies to common epitopes, and ii) the antibodies thus induced will also cross-react with epitopes on other *Pf*AMA1 alleles that are similar to those on the vaccine's component alleles to which they were raised. High titres of antibodies to such common epitopes imply broadened recognition and inhibition of a wide range of parasite strains. Taken together, these results provide evidence for the induction of high levels of inhibitory antibodies to common epitopes by immunisation with a mixture of *Pf*AMA1 alleles. An added advantage of this strategy over a typical multi-antigen or multistage vaccine is the possible limitation on the number of different antigens (with very different epitopes) that can be practically included in a multistage vaccine formulation without compromising effectiveness. Such a multistage vaccine may induce low antibody titres to each of a wide variety of antigens/epitopes, some of which may not be anti-parasitic enough, such that the overall response will be affected by the reduced effective antibody concentration [27,40,41]. A multi-allele strategy with a promising antigen like *Pf*AMA1, by comparison, will focus the humoral response on relevant epitopes that are common to all constituent alleles. In

theory, increasing the number of constituent alleles will reduce the number of common/overlapping epitopes and these repeated epitopes will form the bulk of all epitopes present in the mixture. This would be expected to translate to relatively higher antibody titres against these common epitopes, with the result being broad antibody specificity. Duan and others [42] have recently proposed such an approach, with a recommended minimum of six *Pf*AMA1 alleles as components of a universal vaccine. The number of alleles that can be practically included in such a vaccine may however be limited by high costs (of producing six different proteins) and/or practical developmental difficulties (of expressing fusion proteins with six component antigens), especially if AMA1 is to be combined with other highly immunogenic antigen(s) in a multi-antigen vaccine. On this basis, the diversity covering approach [18], comprising three synthetic and highly divergent *Pf*AMA1-based proteins, appears to be a more practical approach to *Pf*AMA1 vaccine development. Being highly divergent sequences, the three DiCo antigens are expected to have fewer common/overlapping antibody epitopes. These would nevertheless represent the greater proportion of antibody epitopes in the DiCo mixture, and would induce high antibody titres upon immunisation. It is therefore practically possible and more cost-effective to induce a significantly broad humoral response to *P. falciparum* strains using the DiCo proteins, at least as components of a multi-antigen vaccine.

In summary, the present study has shown that broad functional specificity of anti-*Pf*AMA1 antibodies to diverse *P. falciparum* strains can be achieved by multi-allele immunisation. The humoral response is most likely focused on epitopes that are common to the constituent alleles, which would form the bulk of all epitopes present, and leads to induction of antibodies to these common epitopes. The results also show that majority of B cell epitopes are shared by the *Pf*AMA1 alleles used in this study, and possibly by many other *Pf*AMA1 alleles. Thus antibodies induced against a multi-allele vaccine are also highly likely to be effective against parasites expressing diverse *Pf*AMA1 alleles. Of central importance to this immunisation strategy is the demonstration of good levels of homologous parasite inhibition by cross-reactive anti-*Pf*AMA1 antibodies, compared to the total antibody fraction at the same concentration *in vitro*. This is necessary to ensure that in aiming to broaden the antibody response, functionality against parasites expressing the vaccine *Pf*AMA1 alleles is not compromised.

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COMPETING INTERESTS

Three of the authors are in the process of obtaining a patent for a set of three synthetic Diversity-Covering (DiCo) AMA1 proteins.

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Chapter 3

Generation of humoral immune responses to multi-allele *Pf*AMA1 allele vaccines; effect of adjuvant and the number of component alleles on the breadth of response.

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ABSTRACT

There is increasing interest in multi-allele vaccines to overcome strain-specificity against polymorphic vaccine targets such as Apical Membrane Antigen 1 (AMA1). These have been shown to induce broad inhibitory antibodies *in vitro* and formed the basis for the design of three *Di*versity-*Co*vering (DiCo) proteins with similar immunological effects. The antibodies produced are to epitopes that are shared between vaccine alleles and theoretically, increasing the number of component AMA1 alleles is expected to broaden the antibody response. A plateau effect could however impose a limit on the number of alleles needed to achieve the broadest specificity. Moreover, production cost and the vaccine formulation process would limit the number of component alleles.

In this paper, we compare rabbit antibody responses elicited with multi-allele vaccines incorporating seven (three DiCos and four natural AMA1 alleles) and three (DiCo mix) antigens for gains in broadened specificity. We also investigate the effect of three adjuvant platforms on antigen specificity and antibody functionality.

Our data confirms a broadened response after immunisation with DiCo mix in all three adjuvants. Higher antibody titres were elicited with either CoVaccine HT™ or Montanide ISA 51, resulting in similar *in vitro* inhibition (65 – 82%) of five out of six culture-adapted *P. falciparum* strains. The antigen binding specificities of elicited antibodies were also similar and independent of the adjuvant used or the number of vaccine component alleles. Thus neither the four extra antigens nor adjuvant had any observable benefits with respect to specificity broadening, although adjuvant choice influenced the absolute antibody levels and thus the extent of parasite inhibition. Our data confirms the feasibility and potential of multi-allele *Pf*AMA1 formulations, and highlights the need for adjuvants with improved antibody potentiation properties for AMA1-based vaccines.

INTRODUCTION

Malaria is a disease caused by the apicomplexan parasite *Plasmodium* and remains a global scourge despite recent coordinated efforts at control and prevention in endemic areas. The development of an effective malaria vaccine has become ever more urgent in the face of increasing parasite/vector resistance to currently available drugs/insecticides for disease treatment and vector control [1–3]. Subunit vaccine development requires the identification of immunogenic targets from the wide array of antigens expressed by the parasite. A number of antigens expressed by *Plasmodium falciparum*, the parasite mostly responsible for severe forms of malaria, are currently at various stages of pre-clinical and clinical testing [4–6] with apical membrane antigen 1 (AMA1) being an important candidate. The vaccine properties and potential of AMA1 have recently been reviewed [7].

Despite the current global interest in AMA1 as a vaccine component, its potential is limited by allelic polymorphism resulting from single amino acid substitutions in an estimated 16% of amino acids (100 out of 622 positions in 1294 sequences accessed from GenBank) within the AMA1 molecule (Remarque, unpublished data). Polymorphism is generally an immune evasion strategy of the parasite that renders a fully functional immune response against one parasite strain less effective against strains expressing other allele variants, and this has been extensively demonstrated for AMA1 in murine models [8–10] as well as in clinical trials in malaria-naive populations [11]. This suggests that the immune response to AMA1, believed to be mainly antibody-mediated, is to both strain-specific and conserved epitopes [12–14]. We have previously shown that for any *Pf*AMA1 allele, the cross-strain anti-*Pf*AMA1 antibody fraction inhibits the homologous parasite strain *in vitro* to a similar extent as the total antibody fraction (strain-specific + cross-reactive) when both are tested at the same concentration [15]. On this premise, a universally effective *Pf*AMA1 vaccine should have two basic features; i) be able to induce functional responses to antibody epitopes that are common to, or conserved in the widest diversity of parasite strains possible, and ii) be such that strain-specific epitopes on the component alleles are diluted out or eliminated from the epitope repertoire. Attempts at achieving such responses have been made through immunisation with recombinant vaccines incorporating a number of *Pf*AMA1 alleles [15–17], and have been the basis for the *in silico* design of three *Di*versity-*Co*vering (DiCo) *Pf*AMA1-based proteins with similar immunological effects when mixed in a vaccine formulation [18]. The three DiCo proteins, incorporating 80 - 97% of AMA1 amino acid diversity found naturally at the time of design, are distinctly different from one another and are together expected to elicit functional cross-strain antibodies. Initial functional assays with protein A-purified rabbit antibodies (6 mg/ml) against DiCo mix in Montanide ISA 51 showed an average

in vitro inhibition of 70% against the highly diverse FVO, HB3 and 3D7 parasite strains [18].

It seems reasonable to assume that increasing the number of different *Pf*AMA1 alleles included in such a multi-allele vaccine would further broaden the specificity of the expected anti-*Pf*AMA1 antibody response. This idea raises the question of how many alleles would be necessary to achieve the broadest possible functional antibody response. It should also be noted that the number of alleles that can be included in such a vaccine would be limited by the cost of producing many different antigens prior to mixing, and the potential inherent difficulties with quality assurance/control required for vaccine formulation. Another level of complexity would be the appropriate adjuvant system for vaccine formulation. Adjuvants are known to modulate the specificity of antibodies elicited against some parasite antigens [19,20].

In the present study, we further examine the broadened antibody response after immunisation of rabbits with a mixture of the three DiCo proteins by competition ELISA and *in vitro* growth inhibition assays (GIAs) with six distinct *P. falciparum* parasite strains. We compare this response with that induced by a similar immunisation with a mixture of seven antigens (the three DiCo proteins and natural AMA1 alleles from FVO, HB3, 3D7 and CAMP strains of *P. falciparum*) for gains in broadened specificity. We also assess the effect of the choice of adjuvant on the antigen specificities of antibodies elicited against the DiCo mix vaccine.

Our data suggests a practical limit to the number of *Pf*AMA1 alleles that need to be incorporated in a multi-allele vaccine to achieve broad specificity, and shows no adjuvant effect on antigen binding specificity of the elicited antibodies. It also highlights a potentially important role for cross-reactive antibodies in naturally acquired immunity to malaria, and the need for new adjuvants with improved antibody potentiation properties and safety for use in *Pf*AMA1-based subunit vaccine development.

METHODS

Ethics Statement

All animals used in this study were handled in strict accordance with good animal practices within the respective jurisdictions (German and European Union guidelines for BioGenes GmbH, Berlin, and the Belgian national animal welfare regulations for Eurogentec SA, Seraing). Rabbit immunisation work at BioGenes was under approval from NIH/OLAW (ID number #A5755-01) and immunisations at Eurogentec had approval from the ethics committee of the Centre d'Economie Rurale (CER Groupe, Marloie, Belgium).

Protein production, adjuvants and rabbit immunisations.

The full ectodomain of the AMA1 allelic forms from *P. falciparum* strains FVO, HB3, 3D7 and CAMP, as well as the *in silico*-designed DiCo 1, DiCo 2 and DiCo 3 antigens were expressed as recombinant proteins in *Pichia pastoris* by a similar methodology as described elsewhere [21]. Groups of rabbits were immunised with *Pf*AMA1 multi-allele formulations in three different adjuvants. CoVaccine HT™, an enhancer of both humoral and cellular immune responses, is a novel proprietary vaccine adjuvant from Protherics Medicines Development Limited, a BTG Company (London, UK). It is a squalane-based oil-in-water adjuvant that has a sucrose fatty acid sulphate ester (SFASE) [22]. Montanide ISA 51 is a water-in-oil emulsion developed by SEPPIC (Paris, France), containing the naturally metabolizable oil Drakeol 6 VR and mannide mono-oleate as emulsifier, and the adjuvant promotes antibody formation and significant cytotoxic T-cell activity [23,24]. Montanide IMS 4112 VG PR, (one of the Montanide IMS group of adjuvants from SEPPIC), is a water-dispersible composition containing (undisclosed) immuno-stimulatory organic compounds and excipient [25].

The first group of rabbits (Gp 1, n=9) was immunised with a mixture of 7 antigens (DiCo 1, DiCo 2, DiCo 3, and *Pf*AMA1 allelic antigens from FVO, HB3, 3D7 and CAMP parasite strains, ~ 7 µg of each antigen, 50 µg total antigen per dose) in CoVaccine HT™. The second group (Gp 2, n=8) was immunised with a mixture of 3 antigens (~ 17 µg each of DiCo 1, DiCo 2 and DiCo 3, here referred to as DiCo mix; 50 µg in total) in CoVaccine HT™. The third group of rabbits (Gp 3, n=8) was immunised with DiCo mix (~ 17 µg of each DiCo protein, 50 µg in total) in Montanide IMS, and the fourth group (Gp 4, n=5) with DiCo mix (10 µg of each DiCo protein, 30 µg in total) in Montanide ISA 51. All immunisations were done intramuscularly with 3 full human doses (500 µl/dose) of vaccine on days 0, 28 and 56, and exsanguination for all groups was on day 70. Vaccine formulation in all cases was according to the adjuvant manufacturers' specifications. For CoVaccine HT™, formulation involved mixing 300 µl of antigen at 200 µg/ml with 300 µl of CoVaccine HT™ with an SFASE concentration of 40 mg/ml. One 500-µl dose of the resulting formulation therefore contained 50 µg antigen and 10 mg SFASE. Montanide IMS formulation also involved mixing 300 µl of antigen with 300 µl of adjuvant such that 500 µl of the resulting mixture contained 50 µg of antigen. Formulation with Montanide ISA 51 was in a 50/50 (w/w) ratio of antigen to adjuvant; 276 µl of antigen at 130 µg/ml was mixed with 324 µl of adjuvant and the mixture was emulsified by 20 passages through a 22-gauge syringe-coupling piece prior to injection with 500 µl.

Rabbits in the first three immunisation groups were housed at BioGenes GmbH (Berlin, Germany), while the last group (Gp 4) was housed at Eurogentec SA (Seraing, Belgium). The second and third group immunisations (Gp 2 and Gp 3) were done simultaneously in the same experiment at BioGenes, while the first

and fourth (Gp 1 and Gp 4) were done in separate experiments. Final bleed sera from the four different immunisation groups were used in the assays described here.

Antibody purification

Antibodies from all groups were purified on Protein G sepharose (GE Healthcare, Etten-Leur, The Netherlands) columns. Binding and elution buffers (Pierce, Rockford, IL) were used according to manufacturer's protocols. After elution, antibody eluates were filtered (0.22 µm), concentrated and exchanged into RPMI 1640 using pre-sterilized AmicronUltra-15 tubes (30-kDa cutoff; Millipore, Ireland). The concentration of each antibody fraction was subsequently determined with a Nanodrop ND1000 spectrophotometer using the IgG molar extinction coefficient (Nanodrop Technologies, Wilmington, DE) and stored at -20°C until use.

ELISA

Competition ELISA was performed as previously described [15]. Briefly, 96-well flat bottom Microlon titre plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 1 µg/ml (100 µl/well) of the relevant antigen (DiCo 1, DiCo 2, DiCo 3, FVO AMA1, HB3 AMA1, CAMP AMA1 or 3D7 AMA1) at 4°C overnight. After blocking with 200 µl/well of 3% BSA in PBS-T, 60 µg/ml of each of six competitor/soluble antigens (AMA1 from strains FVO, HB3, 3D7 or CAMP, a mixture of the 4 AMA1 alleles designated NM, and DiCo mix designated DM) were titrated 3-fold over 9 duplicate wells at a 50 µl/well final volume. Fifty microlitres/well of a fixed dilution (two arbitrary units or 2AU) of rabbit IgGs (titre pre-determined using the relevant capture antigen) was then added to the titrated competitor antigens and co-incubated for 2 h. A pool of sera from rabbits immunised with a mixture of 3 *Pf*AMA1 alleles (FVO, HB3, 3D7 strains), titrated 2-fold from 1:100,000, was used as standard calibrator on all plates. After sample incubation, plates were developed with 100 µl/well of 1:1250-diluted goat anti-rabbit IgG/alkaline phosphatase conjugate (Pierce, Rockford, IL). Colour development was with 100 µl/well of p-nitrophenyl phosphate (pNPP; Fluka, Poole, UK) for 30 min. and the optical density (OD) at 405 nm determined. Measured ODs were converted to arbitrary units (AUs) by the standard curve included on each plate and expressed as a percentage of AU values from wells without competitor antigen. Residual binding (%) at the highest competitor antigen concentration was estimated from AU values based on a least squares approximation from the following four-parameter logistic function;

$$Y = \frac{(100 - Y\min)}{1 + e^{(Xmid - X)sc}} + Y\min$$

where *Y* is the predicted % residual binding, $\boldsymbol{Y_{min}}$ is the maximal depletion at infinite soluble antigen concentration (minimum value), ***X*** is the soluble antigen concentration (log scale), $\boldsymbol{X_{mid}}$ is the soluble antigen concentration (log scale) at which 50% antibody depletion is achieved (midpoint between the maximum and minimum depletion values), and ***sc*** is the slope of the curve. Percent antibody depletion for any competitor/soluble antigen is therefore the difference between 100% (binding in the absence of soluble antigen) and residual binding at the highest competitor antigen concentration of 30 µg/ml.

Parasite cultures and growth inhibition assays

Protein G-purified IgG fractions were tested for *in vitro* activity in parasite growth inhibition assays (GIAs). All IgGs were tested in triplicate on FCR3 (one amino acid difference in the pro-domain from the FVO strain, with *ama1* GenBank accession no. M34553), NF54 (parent strain of the 3D7 clone with *ama1* GenBank accession no. U65407), HB3 (accession no. U33277), L32 (accession no. EF221749), 7G8 (accession no. M34555) and CAMP (accession no. M34552) parasite strains at a 2-fold serial dilution from 6 mg/ml in 96-well half area cell culture plates (Greiner, Alphen a/d Rijn, The Netherlands). Parasites were cultured under standard conditions (an atmosphere of 5% CO_2, 5% O_2, and 90% N_2, 37°C), and the *Pf*AMA1 antigens expressed by all parasite strains were verified by PCR and restriction fragment length analysis. Parasite cultures were mycoplasma-free and synchronized with 0.3 M Alanine, 10 mM Hepes pH 7.5 before use in assays. Late trophozoite/early schizont stages at a parasitaemia of 0.3 ± 0.1% and 2% final haematocrit were used in all assays. The final culture volume was 50 µl/well and parasites were incubated for 42-46 h. Parasite growth was assessed by measuring parasite lactate dehydrogenase levels and plates were read at 655 nm after 30 min of development. Parasite growth inhibition was expressed as;

$$\%\ \ inhibition = 100 - \frac{(A_{655}Sample - A_{655}RBC)}{(A_{655}SZ - A_{655}RBC)} \times 100$$

where $A_{655}Sample$ is the OD_{655} for any test sample well, $A_{655}SZ$ is the average OD_{655} of schizont control wells included on each plate and $A_{655}RBC$ is the average OD_{655} of RBC control wells. The data is presented as the arithmetic mean % inhibition from each sample triplicate.

Statistical analyses

Residual antibody binding ($\boldsymbol{Y_{min}}$) for each competitor antigen in competition ELISA was estimated by a 4-parameter logistic fit with least squares approximation using the *R* statistical package (R Development Core Team, 2009, version 2.10.1). The mean % depletion (100-$\boldsymbol{Y_{min}}$) and 95% confidence intervals

of a competitor antigen on one capture antigen were calculated and compared with mean values of the same competitor antigen on other capture antigens within the same immunisation group, or with mean values obtained for the same competitor antigen in other immunisation groups.

ELISA antibody titres on seven different capture antigens are presented as dotplots superposed with boxplots indicating the median as well as the lower and upper quartiles for each immunisation group. GIA data is presented as the mean % growth inhibition ± standard error of mean per immunisation group against each of the six parasite strains tested. Associations between antibody titre and GIA activity for four parasite strains with matching ELISA data were estimated with a four-parameter logistic fit.

The original Gp 1 study involved 98 rabbits immunised with the seven-antigen mixture in CoVaccine HT™. ELISA was performed on a random sample of 9 out of the 98 available. Since small volumes (~100 µL) of the individual rabbit sera were available, only a single antibody sample, representing a pool of IgGs from the 98 rabbits, was available for *in vitro* growth inhibition assay. The growth inhibitory capacity of this single sample against three parasite strains (FCR3, HB3, NF54) was therefore compared directly with that of IgGs purified from a pool of sera from all 8 rabbits immunised with DiCo mix in CoVaccine HT™ (Gp 2). All plots were prepared with the *R* statistical package.

RESULTS

Three-antigen and seven-antigen immunisations induce antibodies with similar specificity profiles.

Specificity profiles of antibodies from rabbit immunisations with the three-antigen (DiCo mix, Gp 2) and seven-antigen (DiCo mix + AMA1 alleles from FVO, HB3, 3D7 and CAMP strains of *P. falciparum*, Gp 1) vaccines, both formulated in CoVaccine HT™, were determined by a standardized competition ELISA with the three DiCo proteins used separately as capture antigens. Initial titrations with the separate DiCos as capture antigens showed that antibody titres were higher (1.4 – 1.7 times) in the three-antigen immunisation group (Gp 2) compared to the seven-antigen immunisation group (Gp 1), and the differences were statistically significant when DiCo1 and DiCo 2 were used as capture antigens (Figure 1).

In competition ELISA, antibodies from the three and seven-antigen immunisation regimens were co-incubated with four natural *Pf*AMA1 alleles (from 3D7, HB3, FVO and CAMP parasite strains) and two different antigen mixtures (DiCo mix or DM, and a mixture of the four natural *Pf*AMA1 alleles, designated NM) in coated and blocked plates. Antibody depletion by all 6 competitor antigens/mixtures was fairly consistent for all rabbits within the same immunisation group when assays were done on DiCo 1, DiCo 2 and DiCo 3-coated plates, respectively.

Statistical comparison of all rabbit antibodies from the three-antigen (Gp 2, n=8) and seven-antigen (Gp 1, n=9) immunisation groups, both formulated in Co-Vaccine HT, showed no significant difference in mean % antibody depletion by all six competitor antigens/mixtures (Table 1, Gps 1 & 2). For both vaccines, antibody depletion by the competitor antigen mixtures (DM and NM) was between 91% and 99% on all three DiCo capture antigens as was expected (Table 1). Of the four natural *Pf*AMA1 competitor antigens,
HB3 AMA1 depleted the most antibodies (87– 95%) on all the DiCo capture antigens, whilst 3D7 AMA1 depleted the least (67 – 82%) (Table 1, Gps 1 & 2). The mean % antibody depletion amongst competitor antigens varied most on DiCo 2-coated plates compared to DiCo 1- and DiCo 3-coated plates. Mean % antibody depletion was least for 3D7 AMA1 (~ 68%) on DiCo 2-coated plates, irrespective of whether the antibodies were raised with the three- or seven-antigen vaccines.

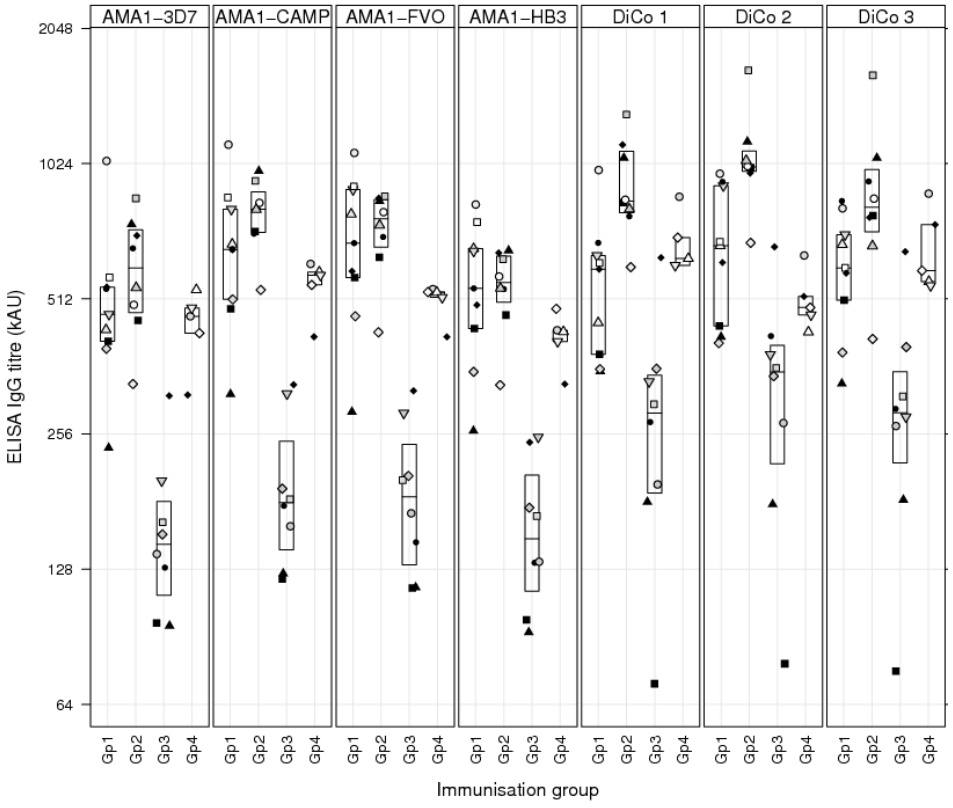

Figure 1. Levels of anti-AMA1 antibody elicited with the four multi-allele vaccine formulations in rabbits. Gp 1 rabbits were immunised with seven AMA1 antigens (DiCo mix and AMA1 from FVO, HB3, 3D7 and CAMP parasite strains) in CoVaccine HT™, and the other groups were immunised with DiCo mix in CoVaccine HT™ (Gp 2), Montanide IMS (Gp 3) and Montanide ISA 51 (Gp 4) respectively. Antibody titres were determined by a standardized ELISA with DiCo 1, DiCo 2, DiCo 3, FVO, HB3, 3D7 and CAMP AMA1-coated plates. Data is presented on a Log2 scale as dotplots with a boxplot superpose indicating the lower and upper quartiles as well as the median per immunisation group. Within the same immunisation group, plotting symbols represent the antibody titre of individual rabbits on all coating/capture antigens.

Table 1. Mean % antibody depletion from DiCo 1, 2 and 3-coated plates.

Capture antigen	Competitor antigen	Gp 1 (7Ag/HT) n=9	Gp 2 (3Ag/HT) n=8	Gp 3 (3Ag/IMS) n=8	Gp 4 (3Ag/ISA 51) n=5
DiCo 1	FVO	84.7 (81.6 – 87.8)	83.0 (79.5 – 86.6)	77.0 (71.9 – 82.0)	77.4 (73.0 – 81.8)
	HB3	93.9 (91.7 – 96.1)	90.1 (88.5 – 91.6)	90.7 (87.9 – 93.6)	83.4 (76.7 – 90.1)
	DM	96.1 (93.9 – 98.3)	98.6 (98.2 – 99.1)	97.2 (95.9 – 98.4)	91.9 (88.3 – 95.5)
	CAMP	87.6 (84.2 – 90.9)	86.3 (84.5 – 88.0)	78.3 (74.5 – 82.1)	77.4 (71.4 – 83.5)
	3D7	75.2 (72.2 – 78.2)	76.9 (73.1 – 80.8)	69.3 (65.0 – 73.6)	61.4 (54.9 – 68.0)
	NM	97.6 (96.1 – 99.0)	92.4 (91.3 – 93.4)	92.1 (90.8 – 93.5)	88.2 (85.8 – 90.6)
DiCo 2	FVO	90.0 (87.2 – 92.8)	86.4 (84.2 – 88.6)	84.8 (79.4 – 90.2)	83.6 (81.2 – 86.1)
	HB3	94.0 (91.8 – 96.2)	94.4 (91.1 – 97.8)	97.1 (94.8 – 99.4)	83.7 (71.6 – 95.7)
	DM	96.1 (93.9 – 98.4)	97.6 (96.4 – 98.9)	98.1 (96.6 – 99.6)	92.9 (90.4 – 95.5)
	CAMP	83.0 (79.6 – 86.4)	83.5 (80.1 – 87.0)	77.9 (75.3 – 80.5)	77.3 (76.1 – 78.6)
	3D7	67.6 (63.3 – 71.9)	68.3 (65.9 – 70.6)	63.6 (58.7 – 68.5)	56.6 (48.3 – 64.9)
	NM	95.0 (93.7 – 96.4)	92.9 (91.2 – 94.7)	95.0 (92.2 – 97.8)	88.9 (85.5 – 92.3)
DiCo 3	FVO	81.2 (77.7 – 84.6)	81.9 (78.2 – 85.6)	72.8 (66.9 – 78.7)	74.1 (66.8 – 81.4)
	HB3	91.9 (89.6 – 94.3)	87.1 (85.2 – 88.9)	85.1 (82.5 – 87.7)	80.9 (74.8 – 87.0)
	DM	97.3 (92.2 – 102.2)	97.8 (96.4 – 99.3)	96.8 (95.4 – 98.2)	91.0 (89.1 – 92.9)
	CAMP	80.2 (77.0 – 83.3)	80.8 (76.7 – 84.9)	66.3 (59.7 – 73.0)	67.8 (62.5 – 73.1)
	3D7	82.1 (78.6 – 85.6)	80.6 (77.9 – 83.2)	66.5 (61.4 – 71.7)	57.8 (47.9 – 67.6)
	NM	94.2 (93.1 – 95.4)	91.4 (89.0 – 93.7)	86.5 (83.6 – 89.4)	84.0 (77.6 – 90.4)

Antibody depletion after competition ELISA, values reported as mean % depletion (95%CI). 7Ag – vaccine containing DiCo mix + four AMA1 alleles from FVO, HB3, 3D7 and CAMP parasite strains; 3Ag – vaccine containing DiCo mix; NM &DM – competitor antigen mixtures comprising natural AMA1 alleles (FVO, HB3, 3D7, CAMP) and DiCo mix, respectively.

By contrast, mean % antibody depletion was greatest for FVO AMA1 competitor antigen (86.4% and 90.0% respectively for antibodies raised in the three- and seven-antigen immunisations) and HB3 AMA1 (94.4% and 94.0%, respectively) on DiCo 2-coated plates. 3D7 AMA1 as a competitor antigen depleted most antibodies from both immunisation groups (80.6% and 82.1% respectively for antibodies raised in the three- and seven-antigen immunisations) on DiCo 3-coated plates (Table 1, Gps 1 & 2). The 9 antibody samples from the seven-antigen immunisation in CoVaccine HT™ were randomly selected from a total of 98 such rabbits, and a pool of antibodies from all 98 rabbits was subsequently tested in GIA. The ELISA antibody depletion pattern of this IgG pool was shown to be similar to that of the individual antibody samples (data not shown).
Differences in % antibody depletion amongst the four allelic *Pf*AMA1 competitor antigens may be related to the DiCo protein coverage of polymorphism found in natural alleles. Alignments of DiCo protein amino acid sequences with those of the four natural alleles (Figure 2) reveal that apart from position K376 which was mutated in the three DiCo proteins to avoid protein cleavage there are five other positions in 3D7 AMA1 that are not present in any of the DiCo proteins. CAMP AMA1 sequences have two uncovered positions, HB3 AMA1 has three and FVO AMA1 has all amino acid residues represented at least once in the three DiCo proteins.

The majority of antibodies raised in three-antigen and seven-antigen immunisations recognise shared epitopes in natural PfAMA1 alleles.

In order to further test the hypothesis that multi-allele vaccines induce high proportions of antibodies to epitopes that are shared by the vaccine component alleles, the specificities of antibodies elicited by all four vaccine formulations to four different *Pf*AMA1 alleles were assessed by competition ELISA. Antibody titres were initially determined by a standard ELISA on plates coated with *Pf*AMA1 from FVO, HB3, 3D7 and CAMP parasite strains respectively, and titres were comparable to those obtained when the same antibodies were titrated on the immunising DiCo antigens (Figure 1). In our hands, rabbit immunisations with antigen from concentrations of 30 - 100 µg/ml have usually yielded similar results, hence the differences in the antigen dose between the first three groups and the DiCo mix/Montanide ISA 51 group (Gp 4) may not result in different titres. The four *Pf*AMA1 alleles (from FVO, HB3, 3D7 and CAMP parasite strains) were used as competitor antigens in subsequent competition ELISA on plates coated with FVO and 3D7 AMA1 antigens respectively.

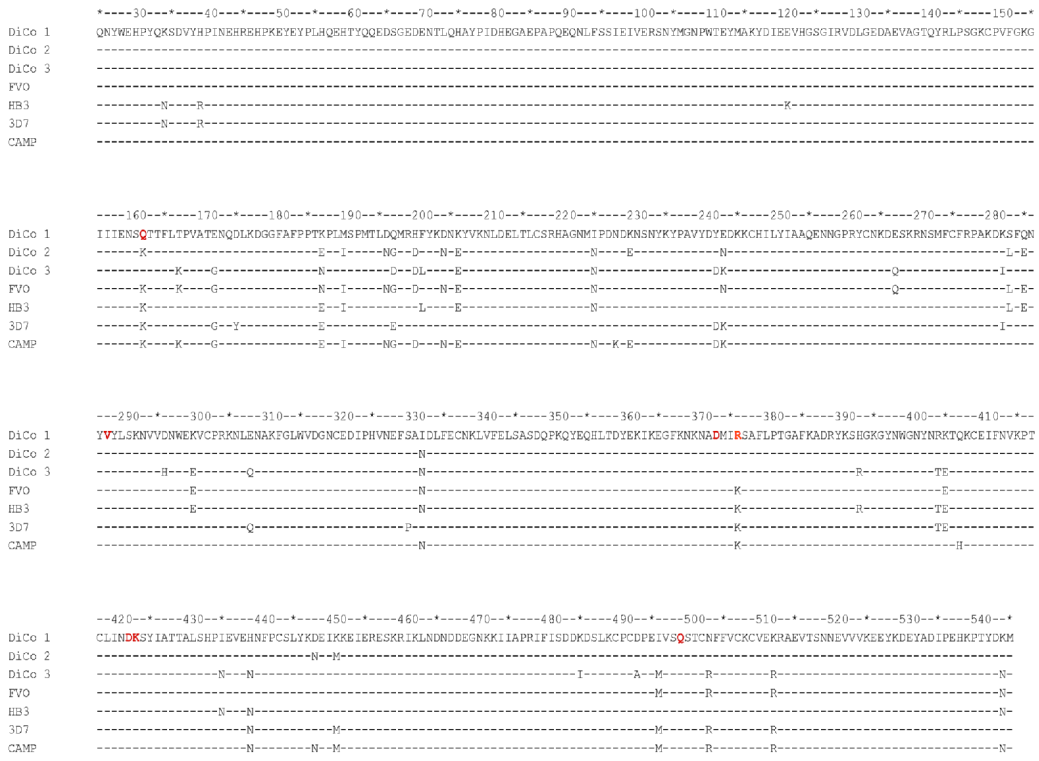

Figure 2. Alignment of the protein sequences (aa25-545) of *Pf*AMA1 antigens used in this study. DiCo proteins were used to immunise rabbits and as capture antigens in ELISA. Natural allele AMA1 proteins were used to immunise rabbits, and as capture and competitor antigens in (competition) ELISAs. All proteins were produced in *Pichia pastoris* and are devoid of N-glycosylation sites. These have been replaced with amino acid residues that occur in AMA1 sequences from other malarial species (N162Q, T288V, S373D, N422D, S423K, N499Q). Residue 162 is unique as it is also a polymorphic residue. Additionally, all DiCo sequences contain a point mutation at position 376 (K to R). This was necessary to prevent protein cleavage by *P. pastoris* proteases.

In assays performed with FVO AMA1-coated plates, antibodies from all four immunisation groups showed the greatest depletion with the homologous FVO AMA1 (94 – 98%) and HB3 AMA1 (94 – 97%) as expected (Table 2). CAMP AMA1 and 3D7 AMA1 depleted 78 – 84% and 67 – 82%, respectively. Likewise, assays performed with 3D7 AMA1-coated plates using the same rabbit antibodies resulted in 92 – 98% depletion by 3D7 AMA1, 85 – 86.5% depletion by CAMP AMA1, 88 – 90.5% depletion by HB3 AMA1 and 81 – 84% depletion by FVO AMA1 (Table 2). Between groups, antibody depletion by an individual competitor antigen was similar on the same capture antigen, and irrespective of the number of immunising antigens (Table 2, Gps 1 & 2) or the adjuvant used in vaccine formulation (Table 2, Gps 2, 3 & 4). On average, for all immunisation groups, the extent of recognition of FVO-binding antibodies by 3D7 AMA1 competitor antigen was about 8.8% less than the recognition of 3D7 AMA1-binding antibodies by FVO AMA1 competitor antigen. These observations

indicate that though the majority of antibodies recognise shared epitopes in the four natural *Pf*AMA1 alleles, a fraction of antibodies are still strain-specific. This may be explained by assuming that some antigen-specific epitopes on any of the three DiCo proteins is/are not present on the *Pf*AMA1 alleles of interest. Antibodies elicited against such epitopes only recognize the *Pf*AMA1 allele(s) that have these epitopes and will not bind to alleles without these strain-specific epitopes. Additionally, local distortions of the protein structure resulting from changes in amino acids that are in close proximity to, or within these epitopes may mean antibodies will have lower binding avidity/affinity for these epitopes. This may result in a continuum of binding specificities, such that some antibodies will have high avidity for epitopes on some *Pf*AMA1 alleles and lower avidity for corresponding, albeit altered epitopes on other alleles. With the exception of FVO AMA1, the three other natural *Pf*AMA1 alleles have amino acids that are not present in any of the three DiCo proteins (Figure 2).

Table 2. Mean % antibody depletion from FVO and 3D7 AMA1-coated plates.

Capture antigen	Competitor antigen	Gp 1 (7Ag/HT) n=9	Gp 2 (3Ag/HT) n=8	Gp 3 (3Ag/IMS) n=8	Gp 4 (3Ag/ISA 51) n=5
3D7 AMA1	FVO	83.9 (81.6 - 86.2)	85.4 (81.8 –89.0)	81.4 (78.1 – 84.7)	82.8 (79.1 – 86.5)
	HB3	90.5 (89.2 – 91.8)	89.8 (88.4 – 91.1)	90.5 (88.0 – 93.0)	88.1 (85.3 – 91.0)
	CAMP	86.4 (84.1 – 88.7)	86.5 (81.9 – 91.2)	85.6 (81.7 – 89.4)	85.3 (82.6 – 88.0)
	3D7	96.8 (95.7 – 97.9)	98.1 (96.6 – 99.6)	98.0 (95.3 – 100.7)	92.5 (90.3 – 94.7)
FVO AMA1	FVO	96.1 (95.3 – 96.8)	97.5 (96.3 – 98.7)	97.5 (96.4 – 98.6)	94.3 (93.0 – 95.6)
	HB3	95.0 (93.9 – 96.0)	95.7 (94.5 – 96.8)	96.3 (95.5 – 97.1)	94.1 (92.7 – 95.6)
	CAMP	82.9 (80.6 – 85.2)	83.6 (80.5 – 86.6)	78.3 (74.9 – 81.7)	83.6 (79.5 – 87.7)
	3D7	73.4 (70.5 – 76.3)	81.8 (79.7 – 83.9)	75.5 (70.2 – 80.8)	67.7 (61.8 – 73.7)

Antibody depletion after competition ELISA, values reported as mean % depletion (95%CI).
7Ag – vaccine containing DiCo mix + four AMA1 alleles from FVO, HB3, 3D7 and CAMP parasite strains; 3Ag – vaccine containing DiCo mix; NM &DM – competitor antigen mixtures comprising natural AMA1 alleles (FVO, HB3, 3D7, CAMP) and DiCo mix, respectively.

Epitopes that include these polymorphic amino acids are therefore prime candidates that may explain the remnant strain-specificity observed.

The choice of adjuvant determines the quantity but not the antigen specificity of elicited antibodies.

In order to assess the effect of adjuvant on the specificity of elicited responses, rabbit antibodies raised against DiCo mix in Co-Vaccine HT (Gp 2, n=8) were compared with those raised in separate immunisations with DiCo mix in Montanide IMS (Gp 3, n=8) and Montanide ISA 51 (Gp 4, n=5) as adjuvants. Antibody titres were dependent on the vaccine adjuvant when titrated in DiCo 1-, DiCo 2- or DiCo 3-coated plates. Antibody titres were generally comparable in the immunisation groups with Co-Vaccine HT and Montanide ISA 51, whilst titres were comparatively lower in the Montanide IMS group (Figure 1, Gps 2, 3 & 4)). Additionally, depletion patterns in competition ELISA showed a general, albeit insignificant trend of higher % depletions of antibodies against DiCo mix in CoVaccine HT™ (Gp 2) by all competitor antigens/mixtures, with 3D7 and CAMP AMA1 competitor antigens showing only borderline significance in some instances (Table 1, Gps 2, 3 and 4). Depletion of antibodies from the two Montanide immunisation groups was also generally comparable except for the two competitor antigen mixtures DM and NM. Thus though the choice of an adjuvant for immunisation affected the physiological levels of anti-DiCo mix antibodies in rabbits, the current data suggests that the adjuvant effect on epitope presentation to B cells, and by extension on the antibody specificity, may only be marginal. This may however be applicable only to the three adjuvants used in this study.

In vitro functional assays with anti-DiCo mix antibodies show similar inhibition of multiple P. falciparum strains.

The functional activity of anti-DiCo mix antibodies from the four immunisation groups was determined *in vitro* on a broad panel of culture-adapted *P. falciparum* strains (FCR3, NF54, HB3, L32, 7G8 and CAMP). Antibodies were tested at a 2-fold dilution from 6 – 0.75 mg/ml against all parasite strains. At the highest concentration tested, antibodies from the seven-antigen immunisation in CoVaccine HT™ (Gp 1, n=1, representing a pool with n=98) showed % growth inhibition of 75.1%, 81.9%, 87.2%, 88.3%, 89.1% and 93.9% against the L32, HB3, NF54, 7G8, FCR3 and CAMP parasite strains, respectively. The single sample available for testing in this group however meant these values could not be directly compared with the mean % inhibition of the 5 or 8 different rabbit IgGs in the other immunisation groups. The functional activity of this sample against three of the six parasite strains (FCR3, HB3, NF54) was therefore compared with that of IgGs purified from a serum pool from all 8 rabbits immunised with DiCo mix in CoVaccine HT™ (Gp 2) in separate experiments. The % inhibition of pooled antibodies from the seven-antigen immunisation were the

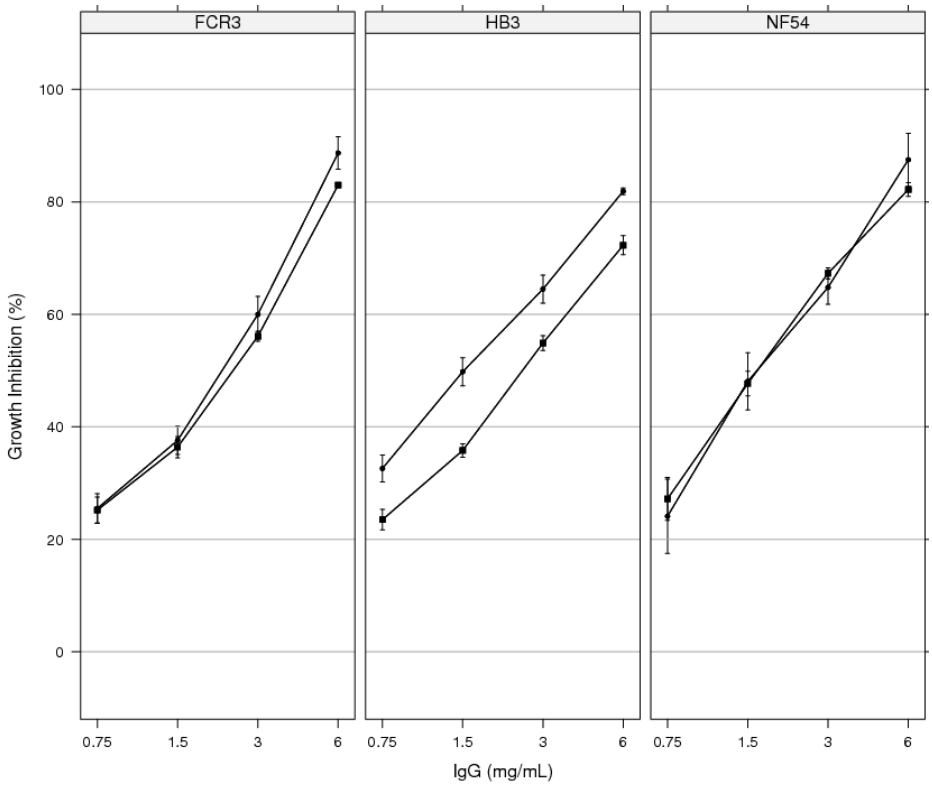

Figure 3. Growth inhibition of *Plasmodium* parasites by antibody pools from the 3-antigen and 7-antigen immunisation groups. The 7-antigen immunisation group (Gp 1) had only a single sample (antibodies purified from a pool of 98 rabbit sera), hence a direct comparison of the functional activities of antibodies from the 3 and 7-antigen immunisation groups could not be made. The growth inhibitory activity of IgGs purified from a pool of all rabbits immunised with DiCo mix (3-antigen, Gp 2) in the same adjuvant (CoVaccine HT™) was therefore compared with that of the single Gp 1 sample against the FCR3, HB3 and NF54 parasite strains. Plots represent the mean % inhibition ± SEM for replicate measurements for each sample. Filled circles (●) represent the Gp 1 pooled sample and the filled squares (■) represent the Gp 2 pooled sample.

same as that of the DiCo mix pooled antibodies against NF54 and FCR3 parasite strains, but slightly higher than that of DiCo mix pooled antibodies against HB3 parasites (Figure 3). Moreover, the % inhibition of these three parasite strains by the DiCo mix pooled antibodies compared favourably with the corresponding mean % inhibition estimated from the measured inhibitions of the individual rabbit IgGs to DiCo mix in CoVaccine HT™ (Figures 3 and 4).

The level of *in vitro* inhibition was the same for all the three DiCo mix immunisation groups (Gp 2, Gp 3 and Gp 4) against five of the six parasite strains, with the L32 strain being the exception (Figure 4). At the highest concentration tested (6 mg/ml), mean % growth inhibition of antibodies induced against DiCo mix in CoVaccine HT™ (Gp 2, n=8) was between 65 and 82% whilst that of antibodies against DiCo mix in Montanide ISA 51 (Gp 4, n=5) was between 65 and 81% against the five strains (Figure 4). Antibodies against DiCo mix in Montanide IMS (Gp 3, n=8) had the lowest mean % growth inhibitions (between 37% and 58%) against all five strains (Figure 4). Although inhibition of the L32

strain was generally lower in all immunisation groups (47.3% for Gp 2, 39.5% for Gp 3 and up to 50% for Gp 4 at 6 mg/ml total IgG), the trend across immunisation groups was similar to that for the other parasite strains. The observed lower inhibition of L32 parasites may be explained by the many Polymorphic amino acids (R197, D207, I224, N244, P330, R395 and T498) within the L32 AMA1 sequence that do not occur in any of the three DiCo proteins, aside those in the prodomain, and mutations at N-glycosylation and cleavage sites (Figure 5).

Since the proportions of strain-specific and cross-reactive antibodies as measured by competition ELISA were constant across immunisation groups, the lower GIA activity of the Montanide IMS group may be explained by the lower levels of the relevant cross-reactive antibodies measured in ELISA. Figure 6 shows that antibody levels against the relevant *Pf*AMA1 alleles correlate with the extent of *in vitro* inhibition of parasite strains that express those alleles. Thus the effect of adjuvant on the functionality of elicited antibodies is mainly on the quantity of cross-strain antibodies and not the quality of antibodies.

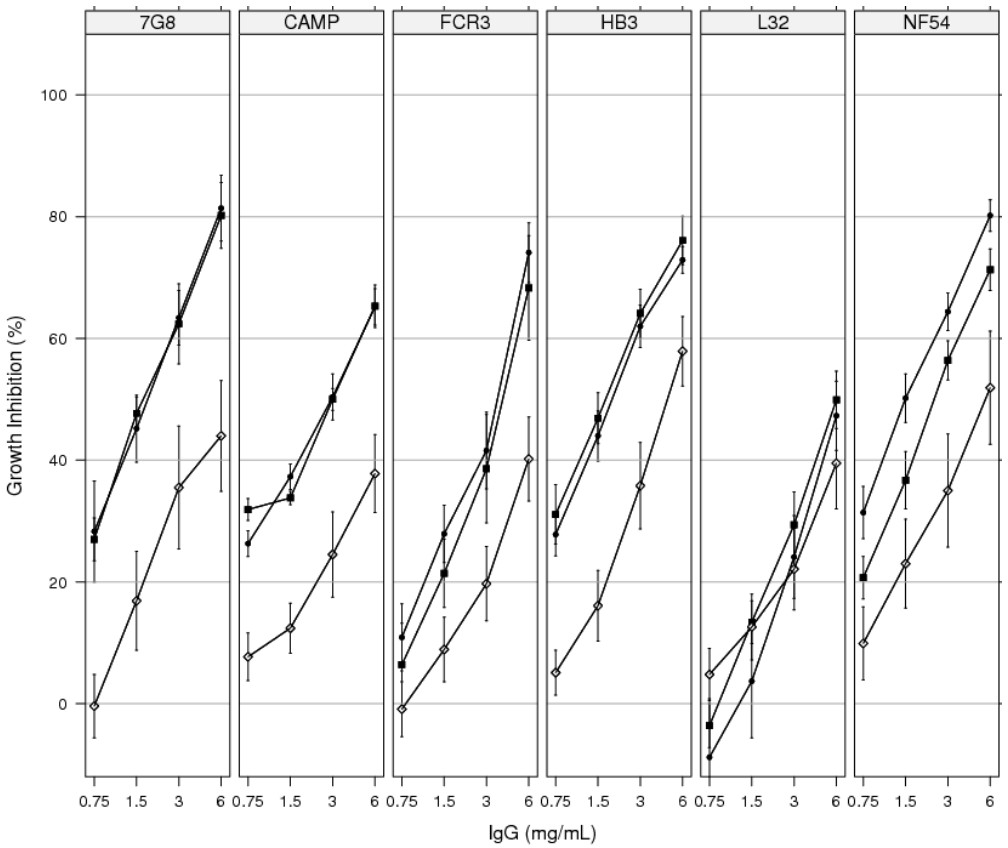

Figure 4. Growth inhibition of *Plasmodium* parasites by antibodies elicited with the three DiCo mix vaccines. Antibodies from all immunisation groups were tested on each of six culture-adapted strains (7G8, CAMP, FCR3, HB3, L32 and NF54) of *P. falciparum*. Plots represent the mean % inhibition ± SEM for all antibody samples within an immunisation group. Filled circles (●) represent Gp 2 (DiCo mix in CoVaccine HT™, n=8), open diamonds (◇) represent Gp 3 (DiCo mix in Montanide IMS, n=8) and filled squares (■) represent Gp 4 (DiCo mix in Montanide ISA 51, n=5).

DISCUSSION

Antibodies to epitopes that are shared between different *Pf*AMA1 alleles (cross-reactive/conserved) have been shown to be relevant for broad strain parasite inhibition *in vitro* [16,26,27], and may be potentially important in the field [28]. We have previously shown that immunisation with mixed *Pf*AMA1 alleles induces a high proportion of such antibodies [15], and this represents an important strategy for dealing with allelic polymorphism in key antigenic vaccine targets like AMA1. The number of allelic variants of the antigen that are needed for a universally functional vaccine however remains a subject of discussion. This number will be dependent on the underlying mechanism of antibody production upon immunisation with a mixture of slightly different immunogens. Our current working hypothesis is that such a formulation will have strain-specific epitopes diluted out and this will preferentially enhance the presentation of shared epitopes to the relevant antibody-producing effector systems *in vivo,* thereby increasing the breadth of antibody response.

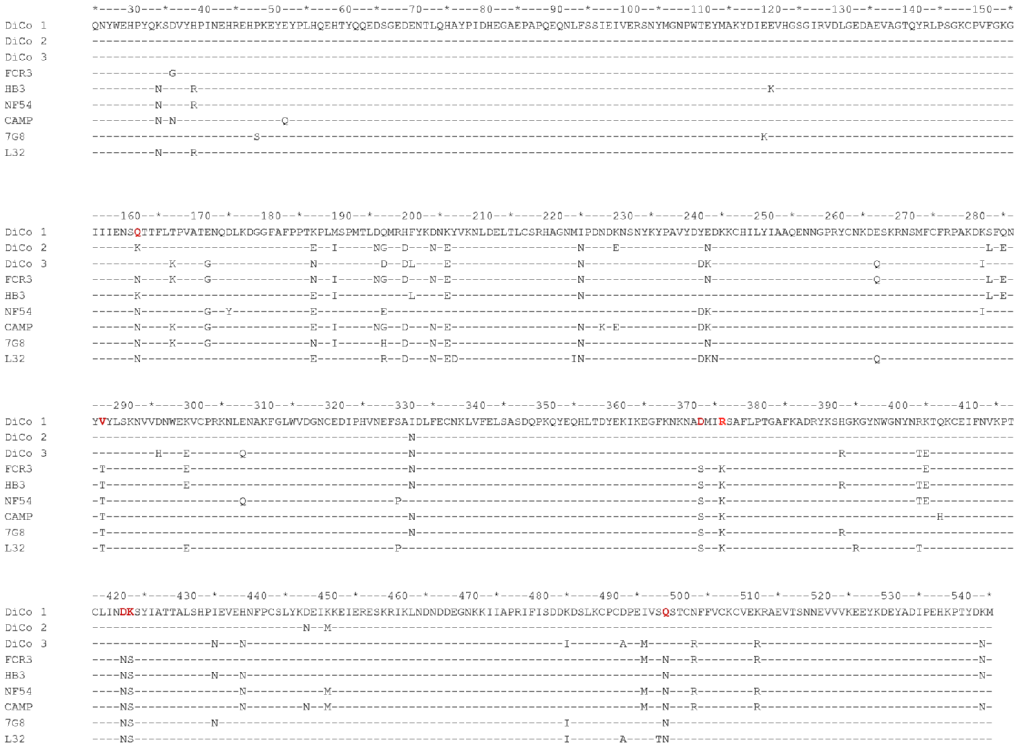

Figure 5. Alignment of DiCo protein sequences (aa25-545) with those of AMA1 present in GIA malaria parasites. AMA1 protein sequences of malaria parasites used for *in vitro* growth inhibition assays were accessed from the GenBank database. The DiCo sequences contain point mutations at the cleavage (K376R) and potential N-glycosylation (N162Q, T288V, S373D, N422D, S423K, N499Q) sites, and differ from parasite AMA1 sequences at these sites. Amino acids at 51 polymorphic sites (within aa25-525) also differ between sequences.

Our Diversity-Covering approach is premised on this hypothesis, and in designing the DiCo proteins, polymorphic amino acids of high frequency (> 10%) in naturally occurring *Pf*AMA1 sequences have been incorporated two or three times [18].

Thus the DiCo proteins intrinsically have another level of coverage of polymorphism, and are expected to further increase the breadth of antibody response. This study compared the proportions of cross-strain antibodies elicited in separate rabbit immunisations with three and seven antigens, and also assessed the adjuvant effect on antigen-binding specificity of elicited antibodies.

Data from competition ELISA on plates coated with any one of five AMA1 antigens (DiCo 1, DiCo 2, DiCo 3, FVO or 3D7) showed that the binding specificities of antibodies induced in the seven-antigen immunisation were not distinguishable from those induced in the three-antigen immunisation (Gps 1 & 2 of Table 1 and Table 2).

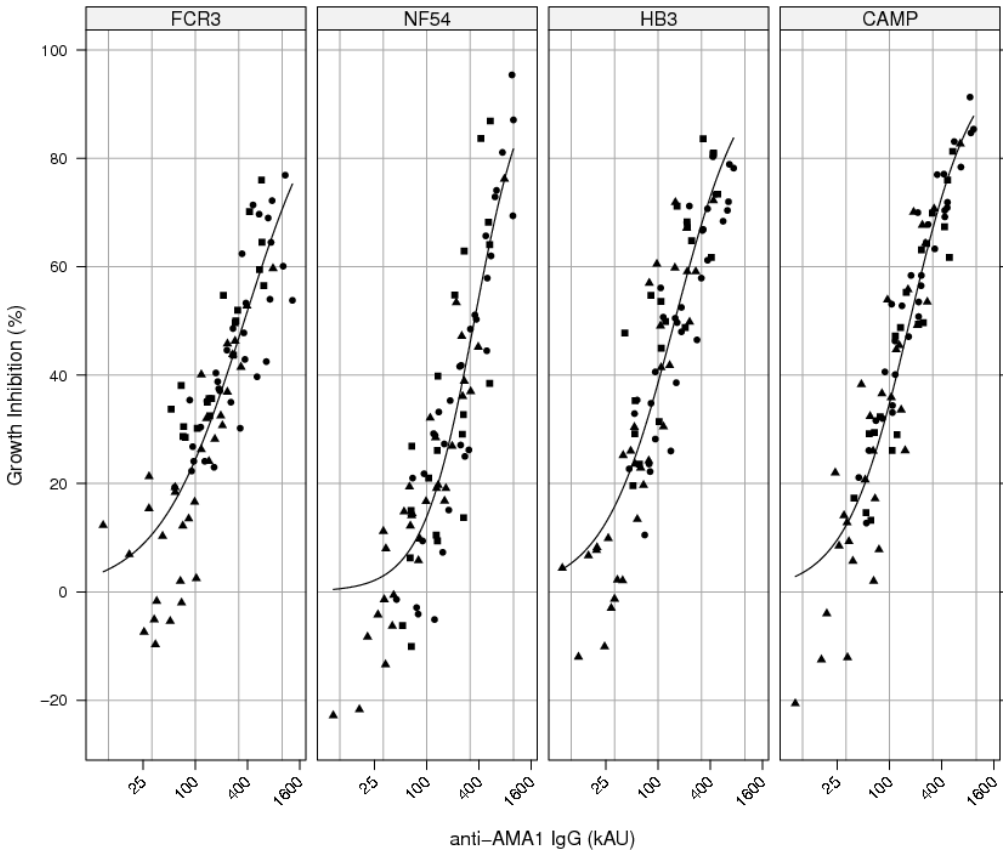

Figure 6. Relationship between ELISA antibody titre and *in vitro* parasite growth inhibition. Association of antibody levels with *in vitro* antibody functionality for three of the four immunisation groups (Gps 2, 3 and 4) is shown for parasite strains whose AMA1 allelic antigens were available for antibody measurement. In order to obtain an optimal estimate of the association, growth inhibition data at all four antibody concentrations tested (6.0, 3.0, 1.5 and 0.75 mg/ml) for each sample were included. Plots are based on a four-parameter logistic function, and each symbol represents individual rabbits in the same immunisation group.

Thus the addition of four extra antigens to the three in the DiCo mix vaccine did not lead to a detectable increase in the proportion of antibodies against cross-reactive epitopes. Antibodies induced against a mixture of three *Pf*AMA1 alleles have been previously shown to exhibit greater cross-reactivity than antibodies against a single allele [15].

The current data thus suggests a plateau effect and a practical limit on the number of alleles that are needed to elicit the broadest antibody response possible. Comparison of antibody depletion patterns of the four *Pf*AMA1 competitor antigens (FVO, HB3, 3D7 and CAMP) showed that the multi-allele vaccines least covered the 3D7 and CAMP AMA1 alleles, while HB3 and FVO AMA1 had reasonably better coverage (Tables 1 & 2). This was more so when assays were performed on DiCo 2 –coated (Table 1) or FVO AMA1-coated (Table 2) plates, and the observation was irrespective of the number of immunising alleles and the adjuvant used. Thus a fraction of antibodies elicited by multi-allele vaccine formulations is strain-specific. Alignment of the amino acid sequences of the three DiCo proteins alongside those of natural *Pf*AMA1 alleles (Figure 2) shows that apart from the cleavage site mutation (position K376R), there are five amino acids at polymorphic positions within the 3D7 AMA1 sequence (N34, R39, Y175, E197, P330) that are not present in any of the DiCo proteins, while all polymorphic amino acids in FVO AMA1 are present in at least one of the three DiCo proteins. Of the five unique amino acids in 3D7 AMA1, two in the prodomain (N34 and R39) do not have immunological significance since the *Pf*AMA1 prodomain is not present on the merozoite at the time of erythrocyte invasion [29,30]. The glutamic acid residue at the heptamorphic hot-spot position 197 [31] forms part of a cluster of inhibitory epitope residues within domain I of the protein [26], and occurs in only 8% of 1294 *Pf*AMA1 sequences available (Remarque, unpublished data). The two other residues (Y175 and P330) are located in other clusters that are also deemed important in antigenic escape [26]. Although the seven-antigen vaccine includes 3D7 AMA1, there is likely an over-representation of antibody epitopes that are not found within 3D7 AMA1. Lower levels of antibodies will thus be elicited against the 3D7 AMA1-type epitopes, and this fits our working hypothesis. CAMP and HB3 AMA1 sequences have two (K228 and H407) and three (N34, R39 and K121) polymorphic amino acids, respectively, that are not covered in the DiCo sequences (Figure 2). Residue K228 is likely to occur within an important domain I cluster [26], while residue H407 is in domain II and has not as yet been described as part of an important escape cluster. Antibody epitopes that include these polymorphic amino acids may therefore be relevant for antigenic escape. Residue K121 occurs within domain I and has not yet been described as part of an escape cluster. Residues N34 and R39 occur within the prodomain and are immunologically not relevant.

Amino acids at positions 175 and 228 are in close proximity to the conserved tyrosine residue at position 251 within the hydrophobic trough of AMA1 [32].

This tyrosine residue is believed to be important for interaction with other invasion-associated proteins [32]. Residues at positions 330 and 407 cluster away from the hydrophobic trough and residue 121 occurs on the more conserved face of the molecule. Some of these polymorphic residues have been predicted to belong to discontinuous B-cell epitopes within AMA1 (DiscoTope method, referenced in [33]).

Functional antibody assay data supports the observations made with competition ELISAs. Antibodies from DiCo mix immunisation groups (Gp 2, Gp 3 and Gp 4) showed consistent levels of inhibition of five out of six parasite strains tested, with the L32 strain being an exception (Figure 4). In addition, anti-DiCo mix antibodies elicited with two of the adjuvants (CoVaccine HT™ and Montanide ISA 51) consistently showed the greatest inhibition of all strains. These data collectively indicate an improved breadth of antibody response compared to antibodies from a single allele or two-allele immunisation [15,16]. The data shows that strain-specific antibodies, despite being present as determined by competition ELISA, did not have any significant functional effects on parasites *in vitro*. Since strain-specific anti-AMA1 antibodies are highly inhibitory against homologous strains [9,13,34], it can be concluded that the multi-allele vaccines induced only very low titres of these antibodies, confirming our working hypothesis.

The functional activity of the antibody pool from the seven-antigen immunisation group compared favourably with a similar pool made from all eight sera in the DiCo mix CoVaccine HT™ immunisation group (Figure 3). Thus the seven-antigen vaccine does not have significant functional benefits over the three-antigen DiCo mix vaccine. Though inhibition of one of the parasite strains tested (HB3) was significantly higher for the seven-antigen immunisation pooled antibodies compared to the three-antigen pooled immunisation antibodies, this singular observation may not have biological significance.

The lower *in vitro* inhibition of L32 parasites is likely due to the extent of coverage of polymorphism of the L32 AMA1 sequence by the DiCo mix vaccine. Apart from mutations at the cleavage (K376R) and N-glycosylation (N162K, T288V, S373D, N422D, S423K and N499Q) sites, there are seven polymorphic residues (R197, D207, I224, N244, P330, R395 and T498) that are not present in any of the three DiCo proteins (Figure 5). Residues at positions 197, 207, 224 and 244 have already been identified within or in proximity of important antigenic escape clusters [26], and it is imaginable that these, in addition to any functional epitope(s) that include residues P330, R395 and T498, will contribute to significant levels of strain-specific antibodies to L32 parasites.

Taken together, the data largely suggests parasite inhibition through recognition of shared *Pf*AMA1 allele epitopes in these diverse parasites. These functional epitopes are likely to occur in AMA1 from many other parasite strains, and antibodies to such epitopes may play a key role in naturally acquired immunity to malaria [28]. Though partial, clinical immunity is acquired over time after

repeated exposure to diverse parasite strains [35–38]. We therefore hypothesize that the antibody component of acquired immunity in semi-immune adults would be mainly to cross-strain epitopes, in contrast to the alternative model of a time-dependent acquisition of a diverse repertoire of strain-specific antibodies after repeated exposure to different parasite strains [28].

The choice of adjuvant for vaccine formulation has been shown to influence the quality and specificity of the elicited cellular and humoral responses [20,39,40]. In this study, adjuvant choice determined the levels of elicited antibodies and thus the extent of *in vitro* parasite inhibition observed (Figures 1 and 4). This however did not influence antigen specificity as antibodies induced in all four immunisation groups had near similar specificity profiles (Tables 1 and 2). This observation may be peculiar to DiCo mix as a vaccine candidate since the three DiCo proteins were designed to intrinsically cover polymorphism within natural *Pf*AMA1 sequences, and any specificity effects of the different adjuvants may have been masked by this property of the vaccine candidate. High levels of antibody of the right quality are required to counter parasite growth in both *in vitro* and *in vivo* systems [15,41]. Thus the choice of adjuvant for a DiCo mix vaccine, based on the current data, may have little or no effect on the induction of the relevant cross-strain antibodies. This is important for this vaccination strategy since adjuvant influence on antibody specificity would introduce another level of complexity in achieving the ultimate functional cross-strain antibodies.

In summary, we show that the four extra *Pf*AMA1 alleles, when added to DiCo mix in a seven-antigen formulation, do not broaden the antigen specificity of elicited antibodies further, and that the three DiCo antigens may on their own be sufficient to cover *Pf*AMA1 polymorphism. The DiCo mix vaccine elicits functional broad-strain antibody responses directed mostly to shared epitopes. Though up to three relevant polymorphic amino acids in AMA1 sequences from FCR3, HB3, CAMP, NF54 and 7G8 parasite strains were not covered by the DiCo antigens, the consistent level of inhibition of these strains by anti-DiCo mix antibodies suggest that 100% coverage of polymorphic residues may not be necessarily required for a universal vaccine. The lower inhibition of L32 parasites, which have as many as seven potentially important polymorphic residues missing from the DiCo sequences however imply that the DiCo approach would need to be constantly reviewed, just as is the case for seasonal influenza vaccines. This is necessary to account for increases in the frequency of polymorphic amino acids that were low (<10%) at the time of DiCo design, and indeed for new polymorphic positions that are likely to evolve in the field. We finally demonstrate that different adjuvants elicit anti-DiCo mix antibodies with similar antigen specificities, although the absolute levels of elicited antibodies differ. This enhances the feasibility of developing an effective multi-allele AMA1-based vaccine with reduced formulation complexity and warrants further development of multi-allele immunisation strategies. We also highlight a

potentially important role for cross-strain antibodies in naturally acquired immunity to malaria, and the need for novel adjuvants with improved antibody potentiation properties and safety for use in AMA1-based subunit vaccine development.

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COMPETING INTERESTS

Four of the authors are in the process of obtaining a patent for the three synthetic Diversity-Covering (DiCo) AMA1 proteins.

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Chapter 4

Immunisation with different *Pf*AMA1 alleles in sequence induces clonal imprint humoral responses that are similar to responses induced by the same alleles as a vaccine cocktail in rabbits

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ABSTRACT

Background

Antibodies to key *Plasmodium falciparum* surface antigens have been shown to be important effectors that mediate clinical immunity to malaria. The cross-strain fraction of anti-malarial antibodies may however be required to achieve strain-transcending immunity. Such antibody responses against *Plasmodium falciparum* apical membrane antigen 1 (*Pf*AMA1), a vaccine target molecule that is expressed in both liver and blood stages of the parasite, can be elicited through immunisation with a mixture of allelic variants of the parasite molecule. Cross-strain antibodies are most likely elicited against epitopes that are shared by the allelic antigens in the vaccine cocktail.

Methods

A standard competition ELISA was used to address whether the antibody response can be further focused on shared epitopes by exclusively boosting these common determinants through immunisation of rabbits with different *Pf*AMA1 alleles in sequence. The *in vitro* parasite growth inhibition assay was used to further evaluate the functional effects of the broadened antibody response that is characteristic of multi-allele vaccine strategies.

Results

A mixed antigen immunisation protocol elicited humoral responses that were functionally similar to those elicited by a sequential immunisation protocol ($p > 0.05$). Sequential exposure to the different *Pf*AMA1 allelic variants induced immunological recall of responses to previous alleles and yielded functional cross-strain antibodies that would be capable of optimal growth inhibition of variant parasites at high enough concentrations.

Conclusions

These findings may have implications for the current understanding of the natural acquisition of clinical immunity to malaria as well as for rational vaccine design.

BACKGROUND

Malaria caused by parasites of the *Plasmodium spp*. continues to be a major public health problem with half of the world's population at risk of infection [1]. The greatest risk of disease and fatality in *Plasmodium falciparum*-endemic areas occurs in children under 5 years and in first-time pregnant women. Natural immunity to clinical malaria is believed to develop in an age- and exposure-dependent manner, after repeated infection by a number of (different) parasite strains [2-4]. Even in adults who have had several parasite encounters, acquired clinical immunity is partial and is believed to be dependent on constant or periodic exposure to low-level parasitaemia [3,5].

The natural ability to acquire immunity to malaria, although partial, is a strong indication of the feasibility of developing at least an anti-disease vaccine directed against the blood stages of *Plasmodium*. Antigenic variation in immunogenic parasite targets however provides an immune escape route for parasites. Polymorphism in such well-known vaccine targets as the Merozoite Surface Proteins (MSPs) and Apical Membrane Antigen 1 (AMA1) have been associated with host immune pressure on parasites [6-10]. This presents malaria vaccine researchers with a formidable challenge since immunisation with one variant of these polymorphic antigens induces antibodies that show limited cross-inhibition/recognition of parasites expressing other allelic variants of the same antigen. This has been demonstrated extensively in animal models [11,12] and to some extent in human field studies [13-16].

There is growing interest in multi-allele/multi-antigen malaria vaccines and the potential of such vaccines for the induction of broad inhibitory antibody responses has been demonstrated [17-19]. The broadened response most likely results from diluting out strain-specific epitopes in the antigen mixture, with the bulk of remaining epitopes being those that are common to the vaccine component alleles [20].

The hypothesis that immunisation of rabbits with different *Pf*AMA1 alleles in sequence would result in boosting of only antibodies to epitopes that are common to all antigens was tested in this study. Antibodies to highly specific epitopes would not be boosted, and this is expected to further increase the proportion of induced cross-strain antibodies in comparison with antibodies induced by a multi-allele vaccine that incorporates the same allelic antigens. Such a mechanism of cross-strain antibody production would be based on the concept of original antigenic sin (clonal imprinting). Original antigenic sin results when prior exposure to one strain/antigen diverts the antibody response to shared epitopes following exposure to a second closely related strain/antigen such that the newly elicited antibodies still react strongly with the priming antigen [21-23]. A sequential immunisation protocol may mimic the development of natural clinical immunity and provide some insight into its acquisition in the field where over time an individual is exposed (sequentially) to

a number of variant parasite strains. The generated data shows that a sequential immunisation protocol may not be materially different from a mixed antigen protocol with respect to the proportions of elicited strain-specific and cross-strain antibodies. As expected, antibody production in the sequential immunisation groups was through associative immune recall of previous antigen encounter. This data is relevant to the current understanding of the acquisition of clinical immunity against malaria in endemic areas, as well as for rational vaccine design.

METHODS

Antigen production, rabbit immunisation and antibody purification

The full ectodomain of the AMA1 allelic forms from *P. falciparum* strains FVO, HB3, 3D7 and CAMP, as well as the *in silico*-designed *Di*versity *Co*vering antigens (DiCo 1, DiCo 2 and DiCo 3) [24], were expressed as recombinant proteins in *Pichia pastoris.* The three DiCo proteins were all expressed with the FVO AMA1 prodomain, and all antigens were mutagenized at up to six potential N-glycosylation sites within the *Pf*AMA1 ectodomain. The expression, purification and characterisation of all antigens were as described previously [25].

Rabbit housing and immunisation were at BioGenes GmbH (Berlin, Germany), and were in accordance with national and international animal welfare regulations. Rabbit immunisation at this facility was under approval from NIH/OLAW (ID number #A5755-01). Five groups of rabbits were immunised intramuscularly with three doses (30 µg per dose) of different *Pf*AMA1 vaccine formulations either in sequence or as an antigen cocktail on days 0, 28 and 56. All vaccines were formulated in a modified Freund's adjuvant (95 % paraffin oil, 2.4% Tween 40, 0.1% cholesterol and 0.01% lipo-polysaccharide from blue-green algae) provided by BioGenes, and formulation was according to the manufacturer's protocols. Three of the groups were immunised with *Pf*AMA1 alleles from the FVO, HB3 and 3D7 strains of *P. falciparum* in different orders (details in Table 1). A fourth group was immunised with three doses of an equimolar mixture of these three alleles (designated as NA mix, 10 µg of each allele, 30 µg dose), and the last group was immunised with three doses of an equimolar mixture of the three DiCo proteins (10 µg of each DiCo, 30 µg dose), referred to as DiCo mix (Table 1). Rabbits were exsanguinated on day 70 and sera from all five groups were analysed in ELISA while purified antibodies from these sera were used in growth inhibition assays described here. Antibodies from final bleed sera were purified on Protein G Sepharose (GE Healthcare, Etten-Leur, The Netherlands) columns. Binding and elution buffers (Pierce, Rockford, IL) were used according to manufacturer's protocols.

Table 1. Schedule indicating the order of *Pf*AMA1 antigen administration to rabbits.

Immunisation group	Antigens and immunisation			*Number of rabbits
	Day 0	Day 28	Day 56	
1 (f3h)	FVO AMA1	3D7 AMA1	HB3 AMA1	6
2 (hf3)	HB3 AMA1	FVO AMA1	3D7 AMA1	6
3 (h3f)	HB3 AMA1	3D7 AMA1	FVO AMA1	4
4 (NA mix)	NA mix	NA mix	NA mix	6
5 (DiCo mix)	DiCo mix	DiCo mix	DiCo mix	6

f (FVO), ***3*** (3D7) and ***h*** (HB3) in groups 1, 2 and 3 indicate the order of antigen administration.
NA mix – an equimolar mixture of AMA1 antigens from the FVO, HB3 and 3D7 strains of *P. falciparum*, administered at all three immunisation time points.
DiCo mix – an equimolar mixture of the three Diversity-covering (DiCo) proteins, administered at all three immunisation time points.

After elution, antibody eluates were filtered (0.22 μm), concentrated and exchanged into RPMI 1640 using pre-sterilized Amicon Ultra-15 tubes (30-kDa cutoff; Millipore, Ireland). The concentration of each antibody fraction was subsequently determined by a Nanodrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) using the IgG extinction coefficient, adjusted to 12 mg/ml and stored at -20°C until use.

ELISA and growth inhibition assays

Sera from all rabbits were titrated in a standardized ELISA on plates coated with recombinant AMA1 allelic antigens from FVO, HB3 or 3D7 parasite strains. Sera were also analysed with a harmonized competition ELISA protocol that has been described elsewhere [20]. FVO, HB3 and 3D7 AMA1 proteins were used as capture antigens and FVO, HB3, 3D7 and CAMP AMA1 antigens were used as competitor antigens in all assays.

Protein G-purified IgG fractions from final bleed sera were tested for *in vitro* activity in parasite growth inhibition assays (GIAs). All IgGs were tested in triplicate on FCR3 (one amino acid difference in the pro-domain from the FVO strain, with *ama1* GenBank accession no. M34553), NF54 (parent strain of the 3D7 clone with *ama1* GenBank accession no. U65407), HB3 (accession no. U33277), 7G8 (accession no. M34555) and CAMP (accession no. M34552)

parasite strains at a 2-fold serial dilution from 6 mg/ml in 96-well half area cell culture plates (Greiner, Alphen a/d Rijn, The Netherlands). Parasites were cultured under standard conditions (an atmosphere of 5% CO_2, 5% O_2, and 90% N_2, 37°C), and all parasite strains were verified by PCR and restriction fragment length analysis of the *Pf*AMA1 antigen they express. Parasite cultures were mycoplasma-free and synchronized with 0.3 M Alanine, 10 mM Hepes, pH 7.5 before use in assays. Late trophozoite/early schizont stages at a parasitaemia of 0.3 ± 0.1% and 2% final haematocrit were used in all assays. The final culture volume was 50 µl/well and parasites were incubated for 42-46 h. Parasite growth was assessed by measuring parasite lactate dehydrogenase levels and plates were read at 655 nm after 30 min of development. Parasite growth inhibition was expressed as 100 – (($A_{655}Sample$ - $A_{655}RBC$)/($A_{655}SZ$ - $A_{655}RBC$)) X 100, where $A_{655}Sample$ is the OD_{655} for any test sample well, $A_{655}SZ$ is the average OD_{655} of schizont control wells included on each plate and $A_{655}RBC$ is the average OD_{655} of RBC control wells. The data is presented as the arithmetic mean % inhibition from each sample triplicate.

Statistical analyses

All analyses and graphics were made using the *R* statistical package (R Development Core Team, 2009, version 2.10.1). ELISA antibody titres in day 70 sera were log-transformed and compared between groups by one-way analysis of variance (ANOVA) followed by the pair-wise Tukey Honest Significant Difference post hoc test which applies a correction for multiple comparisons. Titres are also presented as dotplots superposed with boxplots indicating the median, lower and upper quartiles. Residual antibody binding ($\boldsymbol{Y_{min}}$) for each competitor antigen in competition ELISA was estimated by a 4-parameter logistic fit with least squares approximation. The mean % depletion (100-$\boldsymbol{Y_{min}}$) and corresponding 95% confidence intervals (95% CI) for each competitor antigen are presented for all immunisation groups. GIA data is presented as the mean % growth inhibition ± standard error of mean per immunisation group against the five parasite strains. Associations between antibody titre and the corresponding *in vitro* parasite growth inhibition levels were estimated with a four-parameter logistic fit. Differences were considered statistically significant at $p < 0.05$, or when the 95% CI of groups being compared did not overlap.

One of the 6 rabbits in the first immunisation group (f3h) experienced pneumonia during the study and data from this rabbit was excluded from all analyses. Rabbits (n = 4) in one of the sequential groups (h3f, Table 1) were immunised in a different experiment.

RESULTS

Specificity of antibodies elicited with mixed allele and sequential allele immunisation protocols

Antibodies from rabbit sera drawn on day 70 from all five groups were titrated against *Pf*AMA1 alleles from FVO, HB3 and 3D7 parasite strains, and the data is presented in Figure 1. There were no statistically significant differences for comparisons of the log-transformed antibody titres either for any single immunisation group against all three capture antigens, or for the different immunisation groups against the same capture antigen ($P > 0.05$, one-way ANOVA).

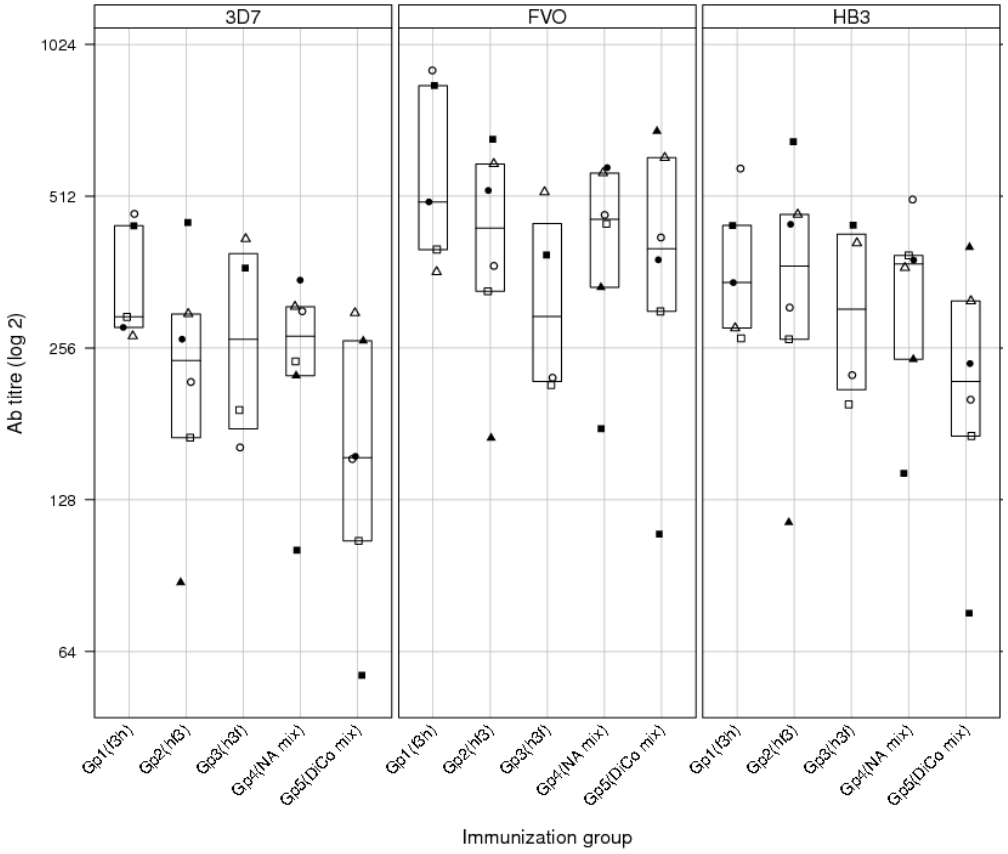

Figure 1. Absolute levels of anti-AMA1 antibodies elicited with mixed allele and sequential allele protocols in rabbits. Rabbits in groups 1 – 3 were immunised with three *Pf*AMA1 allelic antigens (from FVO, HB3 and 3D7 parasite strains) in three different sequences (refer to Table 1). The fourth group of rabbits was immunised with a cocktail of the three *Pf*AMA1 alleles, while the fifth group was immunised with a mixture of the three Diversity covering proteins (DiCo mix) at all immunisation time points. All vaccines were formulated with a modified Freund's adjuvant containing a lipo-polysaccharide from blue-green algae as adjuvant. Antibody titres of sera taken on day 70 were determined by a standardized ELISA with 3D7 (left panel), FVO (middle panel) and HB3 (right panel) AMA1-coated plates. Data is presented on a Log2 scale as dotplots with a boxplot superpose indicating the median, lower and upper quartiles per immunisation group. For each capture antigen, plotting symbols represent the antibody titre of individual rabbits within an immunisation group.

Pair-wise comparison of antibody titres for an immunisation group against any two capture antigens, or titres for any two groups against the same capture antigen also showed no significant differences (P > 0.05, Tukey HSD). Competition ELISA was performed to assess the relative proportions of cross-reactive and strain-specific antibodies induced against the individual vaccine antigens and a fourth *Pf*AMA1 allele (CAMP) that was not a component of any of the vaccines. The FVO, HB3 and 3D7 AMA1 allelic proteins were used as capture antigens and FVO, HB3, 3D7 and CAMP AMA1 as competitor antigens. Depletion (%) of antibodies with the different competitor antigens against each capture antigen is presented in Table 2. On each capture antigen, complete depletion of antibodies by the homologous competitor antigen was observed as expected. Heterologous antibody depletion was however dependent on the number of amino acid differences between the capture and competitor antigens (presented in Figure 2). For example, depletion was lowest for 3D7 AMA1 competitor antigen when FVO AMA1 was used as capture antigen, and lowest for CAMP AMA1 when HB3 AMA1 was used as capture antigen (Table 2).

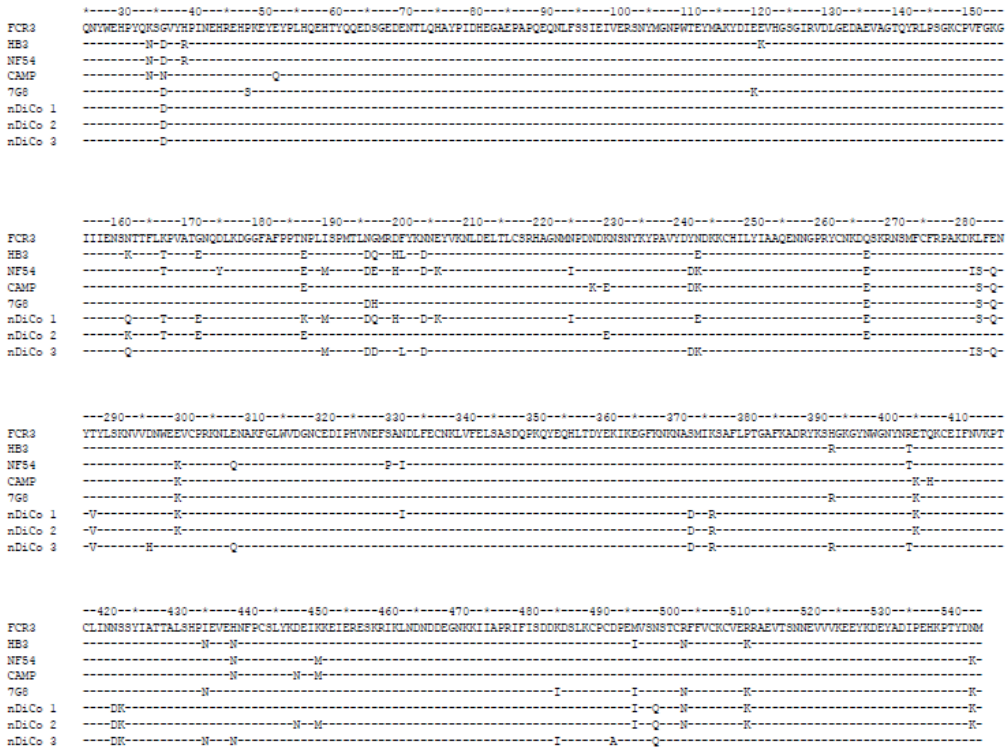

Figure 2. Protein sequence (aa 25 – 545) alignments for DiCo antigens and parasite AMA1 alleles. The recombinant AMA1 allelic antigens for FVO, HB3, 3D7 and CAMP used in ELISA differ at 6 positions (5 for HB3 AMA1) from the respective parasite sequences presented here. These differences (N162Q, T288V, S373D, N422D, S423K, N499Q) were introduced in the recombinant antigens to prevent N-glycosylation of the *Pichia pastoris*-expressed antigens.

Table 2. Mean % antibody depletion from FVO, HB3 and 3D7 AMA1-coated plates.

Coating antigen	Competitor antigen	Gp 1 (f3h) n=5	Gp 2 (hf3) n=6	Gp 3 (h3f) n=4	Gp 4 (NA mix) n=6	Gp5 (DiCo mix) n=6
FVO	FVO	98.5 (97.3 – 99.7)	95.9 (95.1 – 96.8)	98.2 (96.3 – 100.1)	96.8 (95.4 – 98.2)	97.6 (96.4 – 98.7)
AMA1	HB3	93.2 (88.1 – 98.3)	90.4 (87.0 – 93.8)	93.2 (86.0 – 100.4)	90.6 (87.4 – 93.9)	91.3 (89.7 – 92.9)
	3D7	84.5 (77.5 – 91.5)	75.5 (74.3 – 76.7)	76.6 (65.4 – 87.8)	70.4 (64.7 – 76.0)	77.0 (69.4 – 84.7)
	CAMP	78.7 (73.6 – 83.7)	70.3 (65.9 – 74.6)	74.2 (64.4 – 84.1)	70.3 (64.7 – 74.0)	84.0 (82.3 – 85.8)
HB3	FVO	87.5 (79.0 – 96.0)	86.0 (81.2 – 90.9)	91.4 (89.3 – 93.6)	86.6 (83.3 – 89.9)	84.1 (80.6 – 87.5)
AMA1	HB3	96.1 (93.6 – 98.5)	97.5 (95.4 – 99.6)	98.2 (98.1 – 98.4)	96.7 (95.0 – 98.4)	95.6 (93.7 – 97.4)
	3D7	86.7 (82.3 – 91.0)	84.3 (75.9 – 92.8)	77.9 (70.5 – 85.4)	73.7 (66.7 – 80.6)	74.7 (70.5 – 78.9)
	CAMP	76.8 (73.4 – 80.1)	68.5 (62.7 – 74.3)	74.4 (70.2 – 78.6)	66.8 (60.2 – 73.3)	81.2 (78.2 – 84.2)
3D7	FVO	83.0 (75.3 – 90.8)	91.8 (86.7 – 97.0)	91.3 (84.8 – 97.9)	73.2 (66.1 – 80.3)	86.9 (84.4 – 89.4)
AMA1	HB3	89.3 (85.2 – 93.4)	95.9 (92.7 – 99.1)	94.1 (89.2 – 99.0)	83.6 (76.1 – 91.1)	88.7 (86.2 – 91.2)
	3D7	100.2 (96.8 – 103.7)	98.2 (95.9 – 100.2)	96.8 (94.6 – 99.0)	95.8 (94.3 – 97.4)	97.1 (95.2 – 99.0)
	CAMP	79.5 (71.6 – 87.5)	82.0 (77.0 – 87.1)	85.7 (77.0 – 94.5)	71.0 (66.5 – 75.5)	89.2 (86.0 – 92.4)

Values reported as mean (95% CI) per immunisation group for the same competitor antigen on each coating antigen.

Comparison of the three sequential immunisation groups (f3h, hf3 and h3f) showed that though there were small differences (based on overlaps in 95% CI, Table 2) in the extent of heterologous antibody depletion, no clear trends emerged with respect to the order of antigen administration and the capture antigen used in assays. The three sequential immunisation groups also showed detectable quantities of antibodies that were specific to each of the three vaccine *Pf*AMA1 alleles in all polyclonal pools on day 70, despite the fact that each allelic antigen was administered at only one of the three time points.

Comparisons across all groups showed that antibody depletion by heterologous competitor antigens was generally lowest in the NA mix immunisation group compared to the three sequential immunisation groups (f3h, hf3 and h3f, Table2). The observed differences were however not always statistically significant since 95% CI sometimes overlapped. Antibody depletion by CAMP AMA1, an allele that was not in any of the vaccine formulations, was greatest for antibodies from the DiCo mix vaccine group compared to the other four groups in all assays (Table 2). CAMP AMA1 depletion of anti-DiCo mix antibodies was statistically significantly higher than that of anti-NA mix antibodies irrespective of the capture antigen, while differences between anti-DiCo mix antibodies and the sequential immunisation groups were not always statistically significant (Table 2).

Functional capacity of antibodies elicited with mixed allele and sequential allele immunisation protocols

Protein G-purified antibodies from day 70 bleeds were used for *in vitro* growth inhibition assays. Antibodies from the three sequential immunisation groups (f3h, hf3 and h3f) at 6 mg/ml showed similar mean levels of inhibition of two of the three parasites expressing the vaccine alleles (FCR3, $p = 0.60$; HB3, $p = 0.28$; one-way ANOVA) irrespective of the order of antigen administration (Figure 3). Mean inhibition levels against the NF54 strain were however higher for antibodies from the f3h group compared to those from the h3f group ($p = 0.02$, Tukey HSD). Pair-wise comparisons of mean growth inhibition levels in any sequential immunisation group with that of the NA mix group against any of the parasites showed no significant differences ($p > 0.05$, Tukey HSD) despite the weak trend of high antibody depletions from the sequential immunisation groups in competition assays (Table 2). Thus it did not matter whether the vaccine was administered as a mixture or in sequence the functional outcome *in vitro* was the same.

Antibodies from the sequential (f3h, hf3 and h3f) and NA mix immunisation groups generally showed a reduction in the extent of *in vitro* inhibition of "heterologous" parasite strains (CAMP, 7G8) compared to that of "homologous" strains (NF54, FCR3, HB3). Mean inhibition with antibodies from both the f3h (Gp 1) and NA mix (Gp 4) immunisations at 6 mg/ml were all higher against "homologous" strains (NF54, HB3, FCR3) compared to those against the CAMP and 7G8 strains ($p < 0.0001$, one-way ANOVA). Mean inhibition of antibodies from the hf3 group (Gp 2) were however only higher against HB3 strains when compared pair-wise with the "heterologous" strains ($p = 0.006$ for 7G8 and $p = 0.005$ for CAMP parasites, Tukey HSD). Antibodies from the h3f group (Gp 3) also showed higher mean inhibition against HB3 in comparison with the CAMP strain ($p = 0.024$, Tukey HSD). In contrast, antibodies from the DiCo mix group (Gp 5)

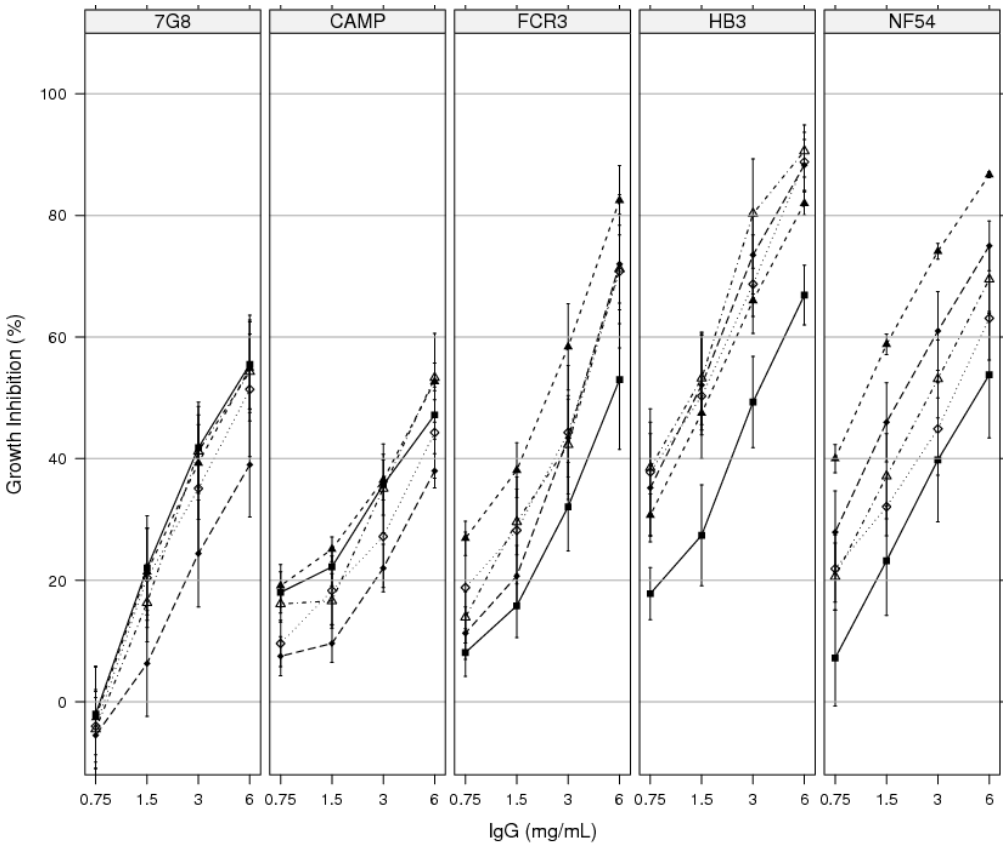

Figure 3. Growth inhibition of *P. falciparum* parasites by antibodies elicited with mixed and sequential allele protocols. Protein G-purified antibodies from all immunisation groups were tested on each of five culture-adapted strains (7G8, CAMP, FCR3, HB3 and NF54) of *P. falciparum*. Plots represent the mean % inhibition ± SEM for all antibody samples within an immunisation group. The filled triangle (▲) plot symbols represent data for group 1 (f3h, n = 5), open triangles (△) represent group 2 (hf3, n = 6), open diamonds (◇) represent group 3 (h3f, n = 4), filled diamonds (◆) represent group 4 (NA mix, n = 6) and filled squares (■) represent group 5 (DiCo mix, n = 6.

showed a generally consistent level of inhibition of all five parasite strains (Figure 3). Mean growth inhibitions ranged from 47.2% against the CAMP strain to 66.9% against the HB3 strain at 6 mg/ml total IgG, and these were not statistically significantly different (p = 0.55, one-way ANOVA).

The data generally suggests that effectiveness of the antibody response was dependent on the test parasite strain ("homologous" vs. "heterologous"), and the absolute levels of elicited antibodies. Higher antibody titres are expected to give greater *in vitro* parasite growth inhibition levels since antibody titres against specific alleles correlate well with the level of *in vitro* inhibition of parasites expressing those alleles (Figure 4).

DISCUSSION

An effective malaria vaccine is expected to confer similar or better immunity to malaria-susceptible individuals compared to that of adults who are resident in

endemic areas, but over a shorter period of time. In semi-immune adults, this level of anti-disease immunity is acquired after repeated infection with diverse parasite strains [2,3]. Antibodies are key components of anti-disease immunity [26,27], and the cross-strain fraction of anti-malarial antibodies may be important effectors against parasite strains that express diverse polymorphic antigens [7,14,28]. The main objectives of this study were to compare sequential and mixed *Pf*AMA1 immunisation protocols for the proportions of functional cross-strain antibodies they induce in rabbits, and to further demonstrate the specificity broadening effects of such vaccination strategies.

Statistically similar levels of antibodies were induced with all vaccine formulations, irrespective of the order of antigen administration (Figure 1). This suggests that the order of antigen exposure may not influence the levels of elicited antibodies. For sequential immunisation groups, this shows that

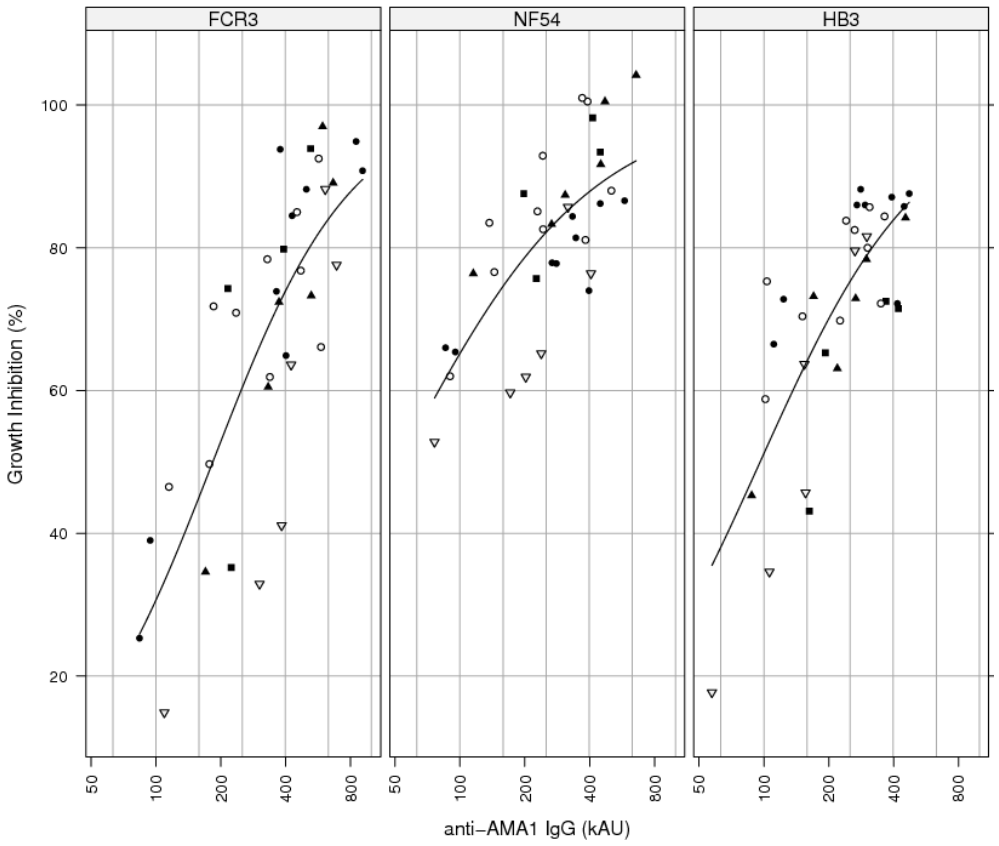

Figure 4. ELISA antibody titre correlates with *in vitro* parasite growth inhibition. Association of antibody levels with *in vitro* antibody functionality is shown for parasite strains FCR3 (FVO), HB3 and NF54 (3D7). *In vitro* inhibition of any parasite strain at 6 mg/ml of purified antibody has been plotted against antibody titres measured with the corresponding AMA1 allele. Plots are based on a four-parameter logistic function. Filled circles (●) represent animals in group 1 (f3h, n = 5), filled triangles (▲) represent group 2 (hf3, n = 6), filled squares (■) represent group 3 (h3f, n = 4), open circles (○) represent group 4 (Na mix, n = 6) and open triangles (△) represent group 5 (DiCo mix, n = 6).

"booster" responses were associative recall responses of previous allele vaccinations, and antibodies were mostly to epitopes that are shared by vaccine alleles. This is consistent with published data on both cellular and humoral immune responses to other polymorphic malaria antigens [7,29-31], and is a well-established phenomenon in immune responses to other parasitic and viral infections [21,22,32-34].

Antibody depletion data from competition assays showed marginally higher proportions of cross-strain antibodies in some sequential immunisation groups compared to the NA mix group (Table 2). Since significance was achieved only in some instances and there were no observable trends with respect to capture and competitor antigens, the order of antigen administration may only marginally influence the ultimate specificity of antibodies on day 70. The three-antigen DiCo mix vaccine generally yielded higher proportions of cross-strain antibodies compared to the three-antigen NA mix vaccine, especially against the out-group competitor antigen CAMP (Table 2). This suggests that the three DiCo antigens together present a greater proportion of epitopes that induce broad-reacting antibodies, and affirms the specificity broadening properties of the DiCo vaccine approach [9,35].

The determination of strain-specific antibodies against vaccine alleles in sequential immunisation groups on day 70 (Table 2) suggests that immunisation with a single allele does induce long-lived responses. This may reflect the persistence of strain-specific antibodies that were induced after antigen administration on day 0 (first antigen), 28 (second antigen) or 56 (third antigen). Alternatively, memory B cells to these specific antibody epitopes in previous vaccine alleles could be activated by the altered, corresponding low affinity epitopes on subsequently administered *Pf*AMA1 alleles, leading to high affinity secondary responses against the cognate epitopes. The latter phenomenon is in agreement with the mechanism underlying original antigenic sin, and supports the existence of a continuum of antibody specificities [7]. It must be noted that the specificity of an antibody for an antigen is directly related to the affinity of the antigen-antibody interaction hence an antibody that is "specific" to one *Pf*AMA1 allele may indeed have very low affinity for other alleles.

Data from *in vitro* growth inhibition assays was consistent with the observations in ELISA. The similar inhibition of FCR3, HB3 and NF54 parasite strains by purified antibodies from sequential immunisation (f3h, hf3 and h3f) and NA mix groups suggests that comparable levels of functional antibodies against all three parasite strains were present on day 70, irrespective of the order of antigen administration (Figure 3). This confirms the induction of antibodies mostly to shared epitopes based on the original antigenic sin phenomenon [7,22].

The observed higher inhibition of parasite strains expressing the vaccine alleles compared to the out-group strains (CAMP, 7G8) may be attributed to the generally low levels of antibodies induced in all immunisation groups. A similar observation was made in an earlier study where low titres of antibodies elicited

against DiCo mix in Montanide IMS resulted in lower *in vitro* parasite inhibition levels compared with the higher antibody levels against DiCo mix in two other adjuvants [35]. ELISA antibody titres and parasite inhibition levels in the present study are intermediate between those of anti-DiCo mix antibodies elicited in the previous study with Montanide IMS on the one hand and CoVaccine HT™/Montanide ISA 51 on the other hand. Similar proportions of cross-strain antibodies were measured in both studies despite the different absolute antibody titres. At such low antibody titres, high avidity strain-specific antibodies, which form a small proportion of elicited antibodies, most likely augment the functional effects of cross-strain antibodies against the respective homologous parasite strains. This fraction of strain-specific antibodies would however have very low avidities for AMA1 of the CAMP and 7G8 strains, resulting in lower levels of inhibition of these strains (Figure 3). Thus high titres of functional cross-strain antibodies are required to optimally inhibit "heterologous" strains. At high antibody titres, the additional inhibitory activity of any strain-specific antibodies against "homologous" parasites would put the overall inhibitory effect in the upper plateau region of the antibody binding-function curve (Figure 4). A possible limitation here however, is that some parasites, here the HB3 strain, may be inherently easier to inhibit than others, and this could mask the effect of vaccine responses described. Similar effect has been described for D10 parasites, which can be inhibited better with anti-3D7 AMA1 antibodies compared to inhibition of the homologous 3D7 strain [36,37].

Although anti-DiCo mix antibodies least inhibited the three "homologous" strains, these antibodies performed as well against the two out-group parasite strains as antibodies from all other vaccine formulations. Additionally, unlike the other vaccine formulations, anti-DiCo mix antibodies showed consistent inhibition of all parasite strains. This consistency, coupled with the significantly higher depletion of anti-DiCo antibodies by the out-group CAMP AMA1 competitor antigen when compared to anti-NA mix antibodies, suggests that DiCo mix may have a slight advantage as a vaccine candidate, since AMA1 from culture-adapted strains may not necessarily be encountered in the field.

The current *in vitro* growth inhibition data may seem inconsistent with our earlier published data [20], where rabbit antibodies elicited with a three-antigen (FVO, HB3, 3D7 AMA1) vaccine in Montanide ISA720 inhibited a heterologous parasite strain (CAMP) to a similar extent as the three "homologous" strains. This can however be explained by the fact that data from the earlier study was based on a single sample per immunisation group, with antibody titres that were 2-4 times higher than the average antibody titres in the study under discussion. The greater potency of the adjuvant used in the earlier study may partially account for the higher titres. Additionally, the earlier study used an immunisation protocol (4 vaccine doses on days 0, 28, 56 and 82, exsanguination on day 95) that is different from the one used in the current study (3 doses on days 0, 28 and 56, exsanguination on day 70). The extra booster dose, as well as the longer

study period, could account partially for the higher functional antibody titres, which were most likely at saturation levels. It must be noted that single allele immunisations with FVO, HB3 and 3D7 AMA1 alleles in the previous study also resulted in similar antibody titres to that of the three-antigen mixed allele vaccine but failed to achieve the same level of *in vitro* inhibition of heterologous parasites as the mixed allele vaccine [20]. Moreover, mixed allele vaccine antibodies from both studies showed similar binding specificities for the component antigens. Thus high titres of cross-strain antibodies are necessary for significant inhibition of variant parasites, and this is consistent with earlier published literature in both *in vitro* and *in vivo* settings [28,35].

This data may aid current understanding of the acquisition of clinical immunity to malaria in endemic areas. Induction of antibodies to polymorphic antigens in the field upon infection with different parasite strains may be through an original antigenic sin mechanism, and individuals will most likely accumulate a strain-transcending repertoire of antibodies over time. This will also explain why clinical testing of a mono-allelic vaccine based on a polymorphic antigen in an unexposed population yields antibodies that react better with homologous than heterologous antigenic alleles [13] while antibodies taken after a similar trial in a malaria-endemic population react equally well with both homologous and heterologous vaccine alleles [38]. A mixture of strain-specific and cross-strain antibodies are most likely induced in naïve individuals in the former instance while previous exposure in the latter results in a boost of responses to epitopes that are common to the vaccine and previously encountered alleles.

In summary, a mixed antigen immunisation protocol is expected to elicit humoral responses similar to those elicited by a sequential immunisation protocol, and by extension the response induced naturally in individuals in malaria-endemic populations. Thus the anti-AMA1 component of a natural immune response can be effectively mimicked by immunisation with a cocktail of AMA1 alleles. This finding may also apply to the many other polymorphic parasite antigens that are currently undergoing clinical evaluation. Additionally, sequential exposure to different AMA1 alleles induces immunological recall of responses to previous alleles and yields functional cross-strain antibodies that are capable of optimal parasite growth inhibitions at high enough concentrations. These findings may aid current understanding of the natural acquisition of clinical immunity to malaria as well as provide fresh insight into rational vaccine design.

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role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

COMPETING INTERESTS

Four of the authors are in the process of obtaining a patent for the three synthetic Diversity-Covering (DiCo) AMA1 proteins. This does not alter their adherence to any Malaria Journal policies on sharing data and materials.

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Chapter 5

Safety and immunogenicity of multi-antigen AMA1-based vaccines formulated with CoVaccine HT™ and Montanide ISA 51 in rhesus macaques

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ABSTRACT

Background

Increasing the breadth of the functional antibody response through immunisation with *Plasmodium falciparum* apical membrane antigen 1 (*Pf*AMA1) multi-allele vaccine formulations has been demonstrated in several rodent and rabbit studies. This study assesses the safety and immunogenicity of three *Pf*AMA1 Diversity-Covering (DiCo) vaccine candidates formulated as an equimolar mixture (DiCo mix) in CoVaccine HT™ or Montanide ISA 51, as well as that of a *Pf*AMA1-MSP1$_{19}$ fusion protein formulated in Montanide ISA 51.

Methods

Vaccine safety in rhesus macaques was monitored by animal behaviour observation and assessment of organ and systemic functions through clinical chemistry and haematology measurements. The immunogenicity of vaccine formulations was assessed by enzyme-linked immunosorbent assays and *in vitro* parasite growth inhibition assays with three culture-adapted *P. falciparum* strains.

Results

These data show that both adjuvants were well tolerated with only transient changes in a few of the chemical and haematological parameters measured. DiCo mix formulated in CoVaccine HT™ proved immunologically and functionally superior to the same candidate formulated in Montanide ISA 51. Immunological data from the fusion protein candidate was however difficult to interpret as four out of six immunised animals were non-responsive for unknown reasons.

Conclusions

The study highlights the safety and immunological benefits of DiCo mix as a potential human vaccine against blood stage malaria, especially when formulated in CoVaccine HT™, and adds to the accumulating data on the specificity broadening effects of DiCo mix.

INTRODUCTION

The development of an effective malaria vaccine remains an important public health objective for disease control in endemic areas. Vaccine strategies that control or prevent blood stage infection may be most desirable since blood stage parasites are responsible for clinical symptoms of the disease. Current knowledge of *Plasmodium falciparum*, the parasite responsible for the most severe form of disease suggests that a potentially effective vaccine would likely include multiple antigens, preferably expressed in different stages of the parasite's life cycle. Essential *P. falciparum* antigens that are currently being considered as subunit vaccine candidates include apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1). AMA1 is highly polymorphic and is found in both merozoite and sporozoite stages of the parasite [1-4] . It is initially expressed as an 83 kDa precursor protein in the micronemes and undergoes an N-terminal prosequence cleavage to form the 66 kDa antigen at the same site [5]. AMA1 translocates to the parasite membrane surface at the time of red cell invasion, and plays a key role in the invasion process [5-9]. The AMA1 ectodomain, which is the vaccine target, is shed as 44 and 48 kDa alternate antigens before the parasite enters the red cell [5,10]. The ectodomain has 16 cysteine residues that form disulphide bonds to divide the antigen's tertiary structure into three different but interactive domains [11].

MSP1, another important vaccine candidate, is the parasite major surface antigen that also plays a role in the red cell invasion process and is dimorphic [12-15]. MSP1 is expressed as a precursor protein of approximately 200 kDa on the surface of developing merozoites [16]. It is proteolytically processed into several fragments at the time of schizont rupture and red cell invasion. The 42 kDa fragment, which is a vaccine candidate, is subsequently processed into 33 kDa and 19 kDa fragments [17,18]. The 19 kDa fragment ($MSP1_{19}$), which is a major vaccine target, remains anchored to the merozoite surface and can be detected in early red cell stages of the parasite [19,20]. All other MSP1 fragments are shed as a peptide complex prior to red cell invasion.

These antigens have demonstrable vaccine properties in rodent and non-human primate models as well as in *in vitro* systems [21-27]. Their vaccine potential, which is exhibited mainly through antibody-mediated mechanisms [28,29], is however limited by allelic polymorphism [24,26,30-32]. Multi-allele vaccination studies, mostly in rabbits and rodents, have however shown promise in overcoming the strain-specific effects of polymorphism on immune responses to these antigens. This strategy informed the design, expression and purification of three Diversity-Covering (DiCo) *P. falciparum* AMA1 (*Pf*AMA1) antigens based on the sequences of 355 naturally occurring *Pf*AMA1 alleles [33]. The DiCo vaccine candidate is an equimolar mixture (DiCo mix) of the three DiCo antigens, hence apart from the design strategy which is to cover polymorphism, mixing of the three DiCo antigens cover polymorphism on a second level. DiCo mix formulated

with either Montanide ISA 51 or CoVaccine HT™ as adjuvant has been shown to induce rabbit humoral responses with similar high inhibitory capacities against multiple parasite strains *in vitro* [33,34]. Another strategy for dampening the effects of polymorphism on *Pf*AMA1 responses is to combine *Pf*AMA1 candidates with other highly immunogenic candidates that show limited polymorphism. This second strategy may involve mixing of the separately expressed and purified vaccine component antigens, or the expression and purification of component antigens as a single chimeric protein. In one such study, rabbits were immunised with *Pf*MSP1$_{19}$ (Welcome strain) and *Pf*AMA1 (domains I and II of the FVO strain) proteins formulated as a mixture or the expressed chimeric proteins in Montanide ISA720 and functionality of induced IgGs was tested against three parasite strains (FCR3, HB3, 3D7). IgG responses to *Pf*AMA1 alone or the *Pf*AMA1/*Pf*MSP1$_{19}$ vaccine products showed signs of strain specificity in functional assays, while responses to products containing *Pf*MSP1$_{19}$ alone showed limited strain-specificity [35]. Challenge studies in rodents immunised with a cocktail of *Plasmodium chabaudi* AMA1 and MSP1$_{42}$ antigens also showed a greater reduction in peak parasitaemia compared to separate immunisations with the single antigens [36]. Another study involving a combination vaccine that consists of *Pf*AMA1 and *Pf*MSP1$_{42}$ yielded responses that were directed against both antigens, though protection induced by the combination vaccine was not superior to that induced by the *Pf*AMA1 vaccine alone [27].

Most pre-clinical evaluations of these immunisation strategies are done in rodents and rabbits. Vaccine evaluation in a monkey model, though not legally mandatory, may be an important step as it could yield additional data on vaccine safety and immunogenicity ([37]). The *in vitro* parasite growth inhibition assay may have some limitations when used for assessment and down-selection of potentially important candidates, whose vaccine effects might be mediated by any of a number of different mechanisms. It nevertheless gives some idea of *in vivo* functionality, especially for antibody responses that are known to have a direct blocking effect on antigen processing and/or host cell invasion. In the current study the capacity of the DiCo mix and *Pf*AMA1-MSP1$_{19}$ candidates, formulated in either Montanide ISA 51 or CoVaccine HT™ as adjuvants, to induce functional broad-strain antibody responses in non-human primates was examined. The safety and tolerability of these adjuvant formulations were also assessed as a proxy to effects that are likely to be observed in clinical testing of these candidates.

METHODS

Antigens

All antigens were expressed as recombinant proteins in *Pichia pastoris* systems and details of gene expression and protein production have already been described elsewhere [33,35,38]. Natural *Pf*AMA1 alleles comprise the full length ectodomain (25-545) of the CAMP [GenBank:M58545], 3D7 [GenBank:U65407), HB3 [GenBank:U33277] and FVO (GenBank:AJ277646] strains of *P. falciparum.* DiCo proteins consist of amino acids 97 - 545 of the AMA1 ectodomain (domains I, II and III, without the signal sequence). The *Pf*AMA1-MSP1$_{19}$ fusion protein (designated AM) consists of amino acids 106 - 442 (domains I and II) of the FVO AMA1 ectodomain fused with a mutant form of *Pf*MSP1$_{19}$ (amino acids 1526 – 1621) of the Wellcome strain of *P. falciparum* [35]. All antigens were recognized by the reduction-sensitive rat monoclonal 4G2 antibody suggesting a correct folding of proteins. All antigens were devoid of N-glycosylation sites and the molecular size of the fusion protein product is comparable to that of any of the AMA1 proteins.

Animal welfare and ethical clearance

Animals used in this study were captive-bred for research purposes. Experimentation and housing were at the Biomedical Primate Research Centre (BPRC) animal facility in Rijswijk, the Netherlands, in accordance with Dutch laws and European Acts (directive 86/609/EEC) on animal experimentation. The BPRC is compliant with recommendations of the Weatherall report on the use of non-human primates in research [39]. The study was approved by an independent ethics committee at BPRC, constituted in accordance with Dutch law on animal experimentation. To minimize discomfort to animals, immunisation and blood sampling were all done under ketamine sedation. The study involved three experimental groups, each with six rhesus monkeys. Animals were assigned in a manner that ensured that age, weight and sex were similar amongst groups, and treatments were randomly assigned to groups.

Vaccine formulation, immunisation and bleeding

Two of the three groups of rhesus macaques were immunised with DiCo mix in either Montanide ISA 51 (Seppic, Paris, France) or CoVaccine HT™ (Protherics Medicines Development Limited, A BTG International Group Company, London, UK) as adjuvant, and the third group was immunised with the AM fusion protein formulated in Montanide ISA 51. Formulations were made under sterile conditions according to the respective adjuvant manufacturers' protocols. For Montanide ISA 51 formulations, 276 µl of antigen solution (130 µg/ml of DiCo mix or 217 µg/ml of AM) was added to 324 µl of adjuvant and the mixture emulsified by 20 passages through a Teflon-coated 22 gauge syringe-coupling piece. Five hundred microlitres (500 µl) of this formulation was administered

per animal. For CoVaccine HT™ formulation, 300 µl of DiCo mix (120 µg/ml in saline) was mixed with an equal volume of adjuvant to a sucrose fatty acid sulphate esters (SFASEs) concentration of 20 mg/ml. Five hundred microlitres (500 µl) of the resulting mixture with 10 mg SFASEs was administered after gentle mixing. Animals were immunised intramuscularly in alternating legs on days 0 (left), 28 (right) and 56 (left) with either 30 µg of DiCo mix (two groups, designated DiCo/ISA and DiCo/HT) or 50 µg AM fusion protein (1 group, designated AM/ISA). Small aliquots of blood (between 2 ml and 7 ml) were taken on days 0, 1, 7, 14, 28, 29, 35, 42, 56, 57, 63 and 70 for clinical chemistry, serology and haematology, and larger volumes (up to 27 ml) were taken on Day 70 for serology, IgG isolation and subsequent *in vitro* testing.

Safety monitoring

All treatment groups were monitored for safety by the assessment of local reactions (Draize scores), behaviour, appetite, stool and bodyweight as well as by clinical chemistry and haematology. Injection sites were inspected for local reactions on days 0, 1, 7 and 14 after each vaccination. Animals were also monitored on a daily basis by the caretakers and injection sites were frequently inspected. Clinical chemistry to assess organ and systemic functions was performed with a Cobas Integra 400 analyzer (Roche Diagnostics, Basel, Switzerland) according to standard methods and compared with normal values based on cumulative data from healthy animals within the same animal facility. Parameters measured include alkaline phosphatase, alanine transaminase, aspartate transaminase, bilirubin, lactate dehydrogenase, gamma glutamyl transpeptidase, cholesterol, glucose, iron, potassium, sodium, calcium, phosphate, chloride, bicarbonate, albumin, creatinine, total protein and urea. Haematology was performed with an automated analyser (Sysmex XT 2000iV platform; Goffin Meyvis, Etten-Leur, the Netherlands) and measurements were made for the red blood cell fraction (haemoglobin levels, erythrocyte count, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin) and for the white blood cell fraction (white blood cell, lymphocyte, neutrophil, monocyte, eosinophil and basophil counts) as well as for platelets and mean platelet volume.

Safety assessments were made before immunisation and one day, one week and two weeks following each immunisation. Since all parameters had returned to normal values by day 70, body weight was the only parameter monitored on days 99 and 126.

Immunological assessment of vaccine responses

Anti-AMA1 IgG levels in serum samples from blood drawn before immunisation on days 0, 28 and 56, as well as samples taken on days 14, 42, 70, 99 and 126 were determined using an indirect ELISA. IgGs were measured against seven different *Pf*AMA1 alleles (the three DiCo antigens and recombinant *Pf*AMA1

alleles from the FVO, HB3, 3D7 and CAMP parasite strains). Anti-$MSP1_{19}$ IgG levels in the AM vaccine group were measured with $MSP1_{19}$ as capture antigen. Briefly, plates were coated with 0.5 µg/ml of the relevant antigen in PBS and subsequently blocked with 3% BSA in PBS with 0.05% Tween 20. Serum samples were diluted in PBS containing 0.1% BSA and 0.05% Tween-20. A pool of hyperimmune sera with high AMA1 and $MSP1_{19}$-specific antibody titres was included on each plate as a standard calibrator. Antibodies were detected with an affinity-purified anti-human IgG coupled to alkaline phosphatase. Plates were developed with 1 mg/ml para-nitrophenyl phosphate, and the absorbance at 405 nm was measured. Antibody titres were subsequently expressed in arbitrary units (AU), with 1AU being equivalent to the reciprocal dilution at which an absorbance of 1 over background is achieved.

Antibody functionality was assessed by *in vitro* growth inhibition assays using the FCR3 (with one prodomain amino acid difference from AMA1 of the FVO strain [GenBank:M34553]), NF54 (parent clone of the 3D7 clone, [GenBank:U65407]) and HB3 [GenBank:U33277] culture-adapted parasite strains as has been previously described [33,38]. Antibodies were purified from day 70 sera using Protein A Sepharose (GE Healthcare, Etten-Leur, The Netherlands) columns and used at a final concentration of 10 mg/ml. Parasite strains were verified by restriction fragment length analysis and cultures were shown to be negative for mycoplasma.

Data analyses

Clinical chemistry and haematological data assessment was based on normal reference values calculated from cumulative data of similar measurements in healthy animals within the same facility. IgG titres determined by ELISA for the different immunisation groups were log-transformed to achieve normality and compared by one-way analysis of variance (ANOVA). Tukey HSD post hoc test with correction for multiple comparisons was used for pair-wise comparison of IgG titres in the same immunisation groups against different capture antigens. Student *t* tests were used for the pair-wise comparison of GIA data between immunisation groups against the same parasite strain, while the Tukey HSD post hoc test was used to compare data for the same immunisation group against different strains. ELISA antibody titres and GIA data from day 70 sera (or purified IgG) are also presented as dotplots superimposed with boxplots showing the median inhibition as well as the first and third quartiles per treatment group. Plot symbols represent individual animals within the same treatment group. All graphics and analyses were performed using the R statistical package (R Development Core Team, 2010, version 2.12.1).

RESULTS

Safety monitoring

Vaccine formulations with both adjuvants were generally well tolerated. All animals maintained body weights within reference ranges of normal values for the entire duration of the study. There were also no major changes in behaviour, appetite or stool over the period of observation. Apart from palpable inguinal lymph nodes there were no notable local reactions (oedema, erythema, indurations) in all 18 study animals. Levels of aspartate transaminase in the DiCo/ISA immunisation group as well as alanine transaminase and bilirubin in the AM/ISA group showed slight increases on the days following immunisation but returned to normal levels within a week. There was an increase in creatinine levels a day after immunisation in the DiCo/HT group but these also returned to normal levels within a week. Creatinine levels in the DiCo/ISA group were however above normal values at the start of the study and remained at similar high levels throughout the observation period. Levels of blood iron decreased on the days following immunisation in the DiCo/HT group but also returned to normal levels within a week. All other measured clinical chemistry parameters were within normal reference ranges throughout the 70-day observation period.

Of the parameters measured for haematology, increases in neutrophil count (and hence white blood cell count) were observed a day after each immunisation, but these again returned to normal values within a week. All other measured parameters were between normal ranges throughout the study.

Generally, local reactions were limited to mild reddening of the injection site area, and these resolved within a few days. One animal in the AM/ISA group however developed stiffness in the upper leg muscle to a degree that limited movement of the left leg. This adverse event was first observed 14 days after the last immunisation (day 70) and had not resolved on day 126. Given this observation, which lasted for more than 56 days, the amount of discomfort to this animal was rated moderate to serious.

ELISA antibody responses

IgG levels against seven AMA1 alleles and $MSP1_{19}$ at all sampling time points were determined using a harmonized ELISA. The three vaccine formulations induced appreciable levels of antibodies against the tested antigens and titres against all antigens increased in a similar manner and generally peaked on day 70, two weeks after the final vaccine injections were given. IgG titres against the FVO AMA1 allele at all time-points are presented in Figure 1 and day 70 titres against all antigens are presented in Figure 2. Beyond day 70, IgG titres in the two DiCo mix immunisation groups showed a decline that was statistically significantly lower on day 126 compared to day 70 levels ($p < 0.05$ for all AMA1 antigens, Student *t* test). Both the anti-AMA1 and anti-$MSP1_{19}$ levels for the

AM/ISA group were however not significantly different on days 70 and 126 ($p > 0.05$, Student *t* test).

Vaccine-induced IgG titres against all seven AMA1 capture antigens were generally highest in the DiCo/HT group by day 70. A comparison of the geometric mean titres, either for the same immunisation group against all AMA1 antigens, or for all groups against the same capture antigen, showed that there were no statistically significant differences ($P > 0.05$, one-way ANOVA). Anti-AMA1 IgG titres varied most amongst animals in the AM group, with four of the six animals having very low IgG titres against all antigens and the other two having exceptionally high titres (Figure 2).

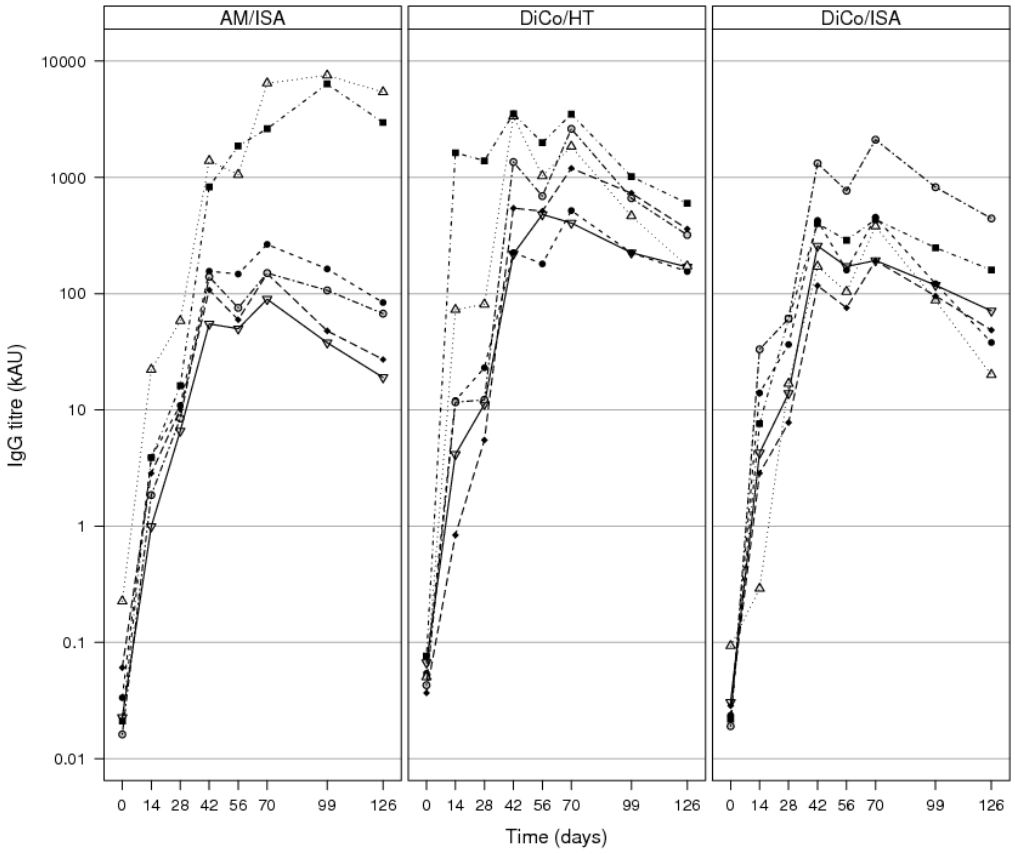

Figure 1. Anti-FVO AMA1 IgG titres in sera of animals immunised with AMA1-based vaccines. Three groups of six rhesus monkeys each were immunised with *Pf*AMA1-based formulations on days 0, 28 and 56. One group was immunised with DiCo mix formulated in CoVaccine HT™ (DiCo/HT), the second group with DiCo mix in Montanide ISA 51 (DiCo/ISA), and the third group with an AMA1-MSP1$_{19}$ fusion protein in Montanide ISA 51 (AM/ISA). IgG titres in samples taken prior to each immunisation, as well as in samples drawn on days 14, 42, 70, 99 and 126, were measured by an indirect ELISA. Similar patterns were observed for IgG measurements against six other *Pf*AMA1 alleles (vaccine antigens DiCo 1, DiCo 2 and DiCo 3 as well as *Pf*AMA1 from the HB3, 3D7 and CAMP parasite strains). For each panel, the different symbols represent individual animals.

Anti-MSP1$_{19}$ IgG titres were determined only for the AM group (Figure 2), and these were statistically comparable to anti-AMA1 IgG titres against all seven AMA1 capture antigens for the same group (p = 0.99, one-way ANOVA). The four animals with low anti-AMA1 IgG levels in this group also had the lowest anti-MSP1$_{19}$ IgG levels.

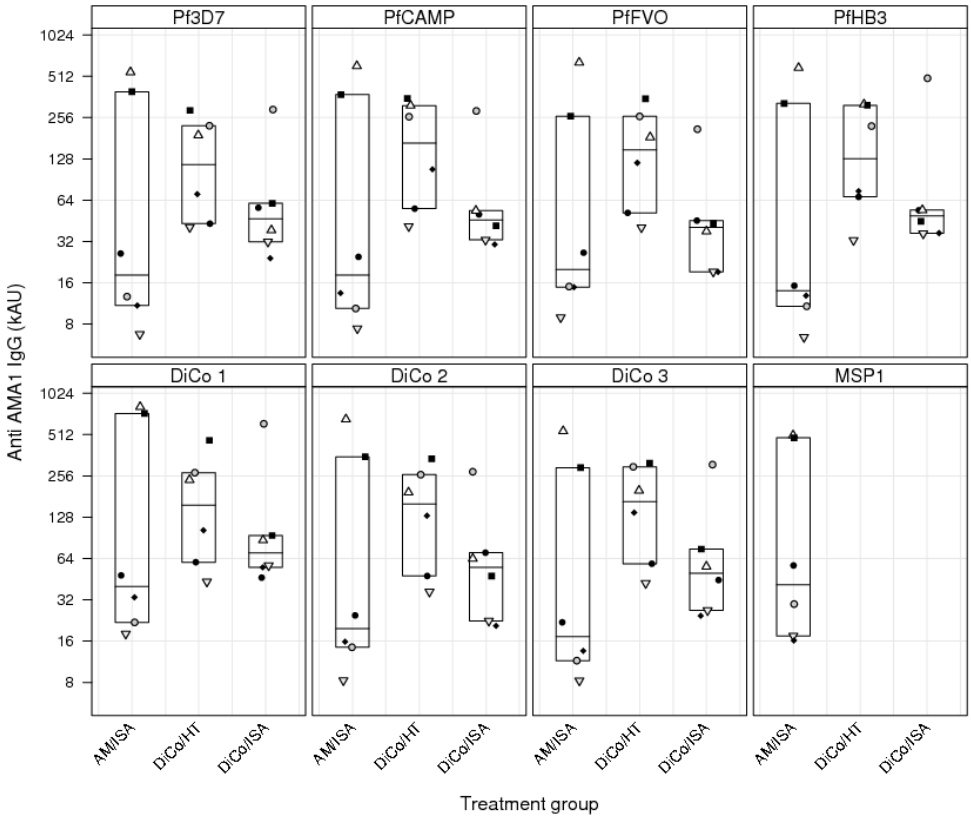

Figure 2. IgG titres in day 70 sera from all three immunisation groups. Anti-*Pf*AMA1 titres in all immunisation groups were measured against seven allelic vaccine antigens (DiCo 1, DiCo 2 and DiCo 3 as well as *Pf*AMA1 from the FVO, HB3, 3D7 and CAMP parasite strains) while anti-MSP1$_{19}$ IgG levels in the AM/ISA group were measured against *Pf*MSP1$_{19}$. For each immunisation group, plot symbols represent data from individual animals in all panels. Plot symbols are also the same as those used for the same animals in Figure 1.

In vitro growth inhibition data

In vitro growth inhibition assays were performed on the FCR3, HB3 and NF54 culture-adapted strains of *P. falciparum* using protein G-purified IgGs from day 70 sera. Purified IgGs were tested against each parasite strain at a final concentration of 10 mg/ml, and the data, presented in Figure 3, is the average of two independent assays per parasite strain. The amino acid sequence differences between the three DiCo antigens and the AMA1 alleles expressed by the *P. falciparum* strains used in assays is presented in Figure 4. All animals in the DiCo/HT group responded well to the vaccine by eliciting levels of IgG that substantially inhibited parasites, while IgGs elicited by one animal in the

DiCo/ISA group and four animals in the AM/ISA group had growth inhibition levels lower than 20% against all three strains. Growth inhibition levels for the AM/ISA group therefore had a very wide range.

At 10 mg/ml, IgGs from the DiCo/HT group had mean growth inhibition (56.3%, 57.8% and 83.7% for FVO, HB3 and NF54 strains, respectively) that was significantly higher than that of IgGs from the DiCo/ISA group (31.0%, 33.2% and 56.8% for FVO, HB3 and NF54 strains, respectively) against all three parasite strains ($p = 0.002$ for FCR3 strain, $p = 0.033$ for HB3 and $p = 0.006$ for NF54, student *t* test). Mean growth inhibition was significantly higher for the DiCo/HT group compared to the AM/ISA group (32.5%) against the NF54 strain alone ($p = 0.026$), while differences between the DiCo/ISA and AM/ISA groups against all three strains were not statistically significant ($p > 0.05$ in all cases). It must be noted that IgGs from one animal in the DiCo/HT group completely inhibited the NF54 strain, with the average of the two measurements being above 100%, an obvious artefact.

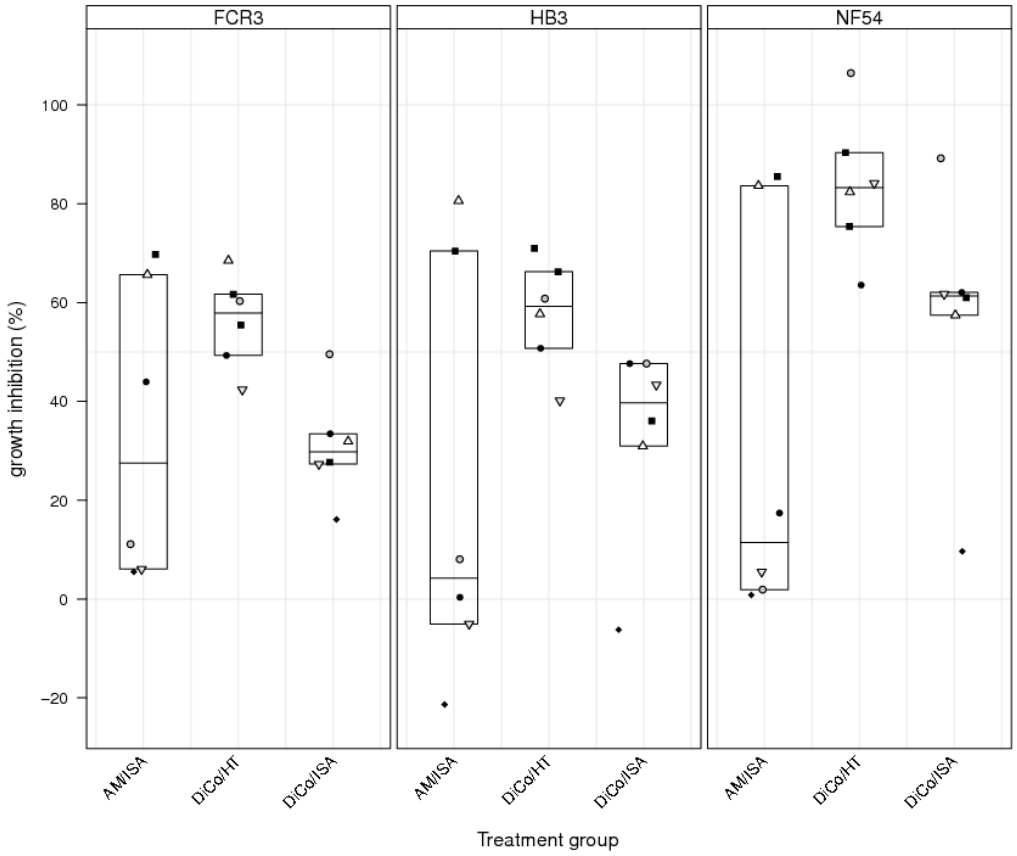

Figure 3. *In vitro* parasite growth inhibition levels of protein A-purified IgGs from day 70 samples. IgGs were tested in triplicate at a final concentration of 10 mg/ml against the FCR3, HB3 and NF54 culture-adapted strains of *P. falciparum*. The data presented is the average of two independent assays, and plot symbols represent data from individual animals within the same immunisation group in all panels. Plot symbols are also the same as that used for the same animals in other figures.

Figure 4. Protein sequence (aa 25 – 545) alignments for DiCo antigens and parasite AMA1 alleles. AMA1 protein sequences of malaria parasites used for *in vitro* growth inhibition assays were accessed from the GenBank database. The DiCo sequences contain point mutations at the cleavage (K376R) and potential N-glycosylation (N162Q, T288V, S373D, N422D, S423K, N499Q) sites and differ from parasite AMA1 sequences at these sites. Amino acid residues 25 to 96 represent the prodomain of the AMA1 transmembrane protein, residues 97 – 303 represent domain I, residues 304 – 440 represent domain II and residues 441 – 545 represent domain III of the protein ectodomain.

Comparison of mean growth inhibition per immunisation group amongst the three parasite strains showed that IgGs from the DICo/HT group inhibited NF54 parasites better than the other two strains ($p = 0.003$ against FCR3 and $p = 0.005$ against HB3, Tukey HSD). Mean inhibition for this group however did not differ between HB3 and FCR3 parasites ($p = 0.973$, Tukey HSD). Mean inhibition against the three parasite strains was similar for both the DiCo/ISA and AM/ISA groups ($p = 0.85$ for AM/ISA and $p = 0.076$ for DiCo/ISA, one-way ANOVA). In this study, inhibition of the growth of the NF54 parasite strain was unexpectedly high in comparison with the other two strains.

DISCUSSION

Multi-allele and multi-antigen malaria vaccine approaches have shown potential in inducing antibody responses with broad-strain inhibitory capacity in a number of rodent and rabbit studies [33,34,40]. In the current study, the immunological benefits of such immunisation strategies, as well as the safety of these formulations, were further analysed in a non-human primate model. Two

different adjuvants were used for vaccine formulation in this study; Montanide ISA 51 (w/o) is a proprietary adjuvant from Seppic (Paris, France) and has been used in a number of human studies [41-43]. CoVaccine HT™ (o/w) is also a proprietary adjuvant developed by Protherics Medicines Development Limited, a BTG International Group company, and has recently entered human trials [44] . In the current study both adjuvant formulations were well tolerated as most of the parameters (chemistry and haematology) measured to assess organ and systemic functions were well within normal values for healthy animals within the same facility. Palpable inguinal lymph nodes were the only local reaction to vaccination observed in study animals. Mean levels of alanine transaminase in the AM/ISA immunisation group and aspartate transaminase in the DiCo/ISA group increased a day after each immunisation but were back to baseline within a week. Transient increases were seen in individual animals in all immunisation groups but only those in groups immunised with Montanide ISA 51 showed mean levels higher beyond normal values. Hypotension and hypoxaemia induced following ketamine sedation have been associated with a release of these enzymes from the liver and heart muscle [45,46] and it is possible that Montanide ISA 51 enhanced this effect.

Mean levels of creatinine for the DiCo/ISA group were high throughout the study period, with four of the six animals in the group showing levels above normal prior to the first immunisation and throughout the study. As these levels remained largely unchanged throughout the study, it suggests that treatments did not alter renal function. Increased neutrophil counts in the DiCo/HT group following immunisation were concomitant with decreased levels of serum iron in the same group. This observation has also been made in previous studies and is suggestive of the possibility that these events are coupled. The limited leg movement experienced by one animal in the AM/ISA group was most likely due to physical injury from needles used to deliver the vaccine or for blood sampling and not vaccine-related. Moreover, such an outcome has not been observed in earlier studies in this lab with this adjuvant.

Previous studies with CoVaccine HT™ formulations at an SFASEs dose of 2 mg or 10 mg in rhesus monkeys [47,48] and at 2 mg or 4 mg SFASEs in rabbits [47] showed no local or systemic adverse events , while only minimal transient adverse events were seen in a small percentage of ferrets with formulations containing 0.125 - 4 mg of SFASEs ([47,49]). For Montanide ISA 51, though little to no reactogenicity has been reported in animal studies and in some human studies [41,50-52], other human studies have concluded that this adjuvant, with its current composition, might not be suitable for use in humans due to high reactogenicity [42,43].

Mean antibody responses against the AM/ISA vaccine were statistically similar on day 70 and 126 while responses against the two DiCo mix groups had declined significantly by day 126. This was due mainly to the exceptionally high titres in two of the six animals in the AM/ISA group (Figure 1) and does not

reflect a more durable IgG response against the AM fusion protein compared to DiCo mix, especially since the four low responders in the AM group also showed titre decreases on day 126. The AM/ISA vaccine also induced appreciable levels of anti-$MSP1_{19}$ IgG, especially in the two animals that also had the highest anti-AMA1 IgGs in this group. Vaccine design studies with $MSP1_{19}$ fusion proteins have shown that IgG responses induced against the $MSP1_{19}$ component increase several fold compared to IgG responses to formulations with $MSP1_{19}$ alone [35,53], suggesting that fusion enhances antibody formation against the $MSP1_{19}$ component.

Observations in ELISA were in agreement with the *in vitro* growth inhibition data as animals with high IgG levels had correspondingly high GIA activities and vice versa. Purified IgGs from all animals in the DiCo/HT group showed high mean growth inhibition of the three parasite strains compared to inhibition by IgGs in animals from the DiCo/ISA group (Figure 3). This suggests that the CoVaccine HT™ formulation was functionally superior and that this adjuvant may be most suitable for inducing the required high titres of functional IgGs. Inhibition of all three strains was similar for all IgGs from the DiCo/ISA or AM/ISA immunisation groups (Figure 3). Inhibition of NF54 parasites by IgGs from the DiCo/HT group was however slightly higher compared to that of the two other strains (Figure 3). This can however not be attributed to greater sequence similarity between NF54 AMA1 and the DiCo antigens. Indeed the greatest sequence similarity for the AMA1 allele sequences indicated in Figure 4 is between the FVO AMA1 allele and DiCo 2 (12 amino acid differences apart from the one cleavage and six N-glycosylation site differences). This notwithstanding, the data demonstrate the specificity broadening benefits of DiCo mix formulations in non-human primates, and adds to the accumulating data on the strain-transcending properties of the DiCo strategy that have been demonstrated in rabbit studies [33,34].

Four animals in the AM/ISA group had very low IgG titres (Figure 2) with corresponding low inhibitory activities below 20%, especially against the HB3 and NF54 strains (Figure 3). The other two animals in this group had high vaccine responses in both ELISA and GIA (Figures 1 and 2) hence the weak responses in the four animals cannot be due to poor immunogenicity of the candidate antigens. Thus these animals were most likely non-responsive to immunisation, and this makes it difficult to draw firm conclusions on the effectiveness of the AM fusion protein candidate in this study. Parasite inhibition studies done *in vitro* with antibodies raised in rabbits as well as challenge studies in mice have however shown that vaccines composed of AMA1 and MSP1 either as a mixture or a fusion protein product induced functional antibodies [35,36,54].

The data presented shows that both adjuvant formulations were well tolerated upon administration to rhesus macaques. The data further demonstrates the specificity broadening benefits of multi-allele formulations compared to single *Pf*AMA1 formulation in non-human primates [55,56], and adds to the

accumulating data on the strain-transcending properties of the DiCo strategy. The three antigens, especially when formulated in CoVaccine HT™, induced IgG levels that inhibited multiple parasite strains *in vitro*. It is however difficult to draw firm conclusions on data from the AM fusion protein since most of the test animals immunised with this vaccine showed low vaccine responses. The DiCo antigens thus represent a unique strain-transcending strategy for developing a malaria blood stage vaccine with benefits for susceptible individuals in areas where *P. falciparum* is endemic.

COMPETING INTERESTS

Four of the authors are in the process of obtaining a patent for the three synthetic Diversity-Covering (DiCo) AMA1 proteins. This does not alter their adherence to any Malaria Journal policies on sharing data and materials. All other authors have no competing Interests.

AUTHOR'S CONTRIBUTIONS

Conceived and designed the experiments: EJR BWF CHMK AWT. Performed the experiments: KAK, VW. Analysed the data: KAK EJR BWF. Designed and produced recombinant proteins: BWF, VR. Wrote the paper: KAK EJR BWF CHMK AWT. All authors have read and approved the final manuscript.

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Chapter 6

Measurement of the plasma levels of antibodies against the polymorphic vaccine candidate apical membrane antigen 1 in a malaria-exposed population

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In press

ABSTRACT

Background

Establishing antibody correlates of protection against malaria in human field studies and clinical trials requires, amongst others, an accurate estimation of antibody levels. For polymorphic antigens such as apical membrane antigen 1 (AMA1), this may be confounded by the occurrence of a large number of allelic variants in nature.

Methods

To test this hypothesis, plasma antibody levels in an age-stratified cohort of naturally exposed children from a malaria-endemic area in Southern Ghana were determined by indirect ELISA. Titres against four single *Pf*AMA1 alleles were compared with those against three different allele mixtures presumed to have a wider repertoire of epitope specificities. Associations of antibody levels with the incidence of clinical malaria as well as with previous exposure to parasites were also examined.

Results

Antibody titres against *Pf*AMA1 alleles generally increased with age/exposure while antibody specificity for *Pf*AMA1 variants decreased, implying that younger children (≤ 5 years) elicit a more strain-specific antibody response compared to older children. Antibody titre measurements against the FVO and 3D7 AMA1 alleles gave the best titre estimates as these varied least in pair-wise comparisons with titres against all *Pf*AMA1 allele mixtures. There was no association between antibody levels against any capture antigen and clinical malaria incidence. Antibody levels in participants with higher parasite densities from previous exposure tended to be lower than the levels in participants with lower parasite densities, though this trend was also not statistically significant.

Conclusions

The current data shows that levels of naturally acquired antigen-specific antibodies, especially in infants and young children, are dependent on the antigenic allele used for measurement. This may be relevant to the interpretation of antibody titre data from measurements against single *Pf*AMA1 alleles, especially in studies involving infants and young children who have experienced fewer infections.

Background

Antibodies have a demonstrably crucial role in protection against clinical malaria and the measurement of malaria-specific antibodies and their correlation with protection against disease/infection is essential in field as well as vaccine trial studies. Anti-malarial antibodies participate in such effector mechanisms as complement-mediated parasite clearance, red cell invasion inhibition, direct neutralisation of parasites/toxins and antibody-mediated cellular inhibition/cytotoxicity [1-5].

Antibodies are naturally induced against a host of parasite antigens, and *in vivo* protection may generally be based on the cumulative/synergistic effect of relevant responses rather than responses to any single antigen. Additionally, at the peak of an infection, high levels of the relevant antibodies, rather than their generation from memory may be necessary for protection [6,7]. The precise determination of anti-malarial antibody levels in field and vaccine studies in disease-endemic areas is therefore very crucial to data interpretation as well as for identifying antigen correlates of protection.

For polymorphic parasite antigens, antibodies against one allelic form have been shown to react less with other alleles as a significant proportion of antibodies are directed against strain-specific epitopes. *Plasmodium falciparum* apical membrane antigen 1 (*Pf*AMA1), a type 1 integral membrane protein expressed in the merozoite and sporozoite stages of the parasite and a leading candidate for the development of a blood stage vaccine is one such antigen [8-15]. Polymorphism in *Pf*AMA1 is due to a number of non-random point mutations that occur in the antigen's ectodomain, an effect that has been associated with host immune pressure on the parasite [16,17]. Thus for a highly polymorphic antigen like apical membrane antigen 1 (AMA1), many variants of which are likely to be present in a single population, estimation of the true antibody levels can be quite tricky as antibody levels measured against any single *Pf*AMA1 allele may underestimate the true levels of persisting antibodies. This hypothesis was tested by comparing the plasma anti-*Pf*AMA1 antibody levels in plasma samples collected prior to the low transmission season in a naturally exposed population against four single *Pf*AMA1 alleles and three different *Pf*AMA1 allele mixtures. The antigen mixtures are expected to have a variety of unique epitopes that would enhance binding of the broad spectrum of polyclonal anti-AMA1 antibodies in naturally exposed individuals. The study further assesses the association of antibody levels with the incidence of clinical malaria during the low transmission season as well as with previous exposure to parasites.

Methods

Ethics statement

The current study used archived human samples from a longitudinal cohort study conducted during the malaria seasons of 1994 and 1995. The original study was approved by the Ministry of Health in Ghana and ethical clearance was sought from the ethics committee of the Ministry of Health. Written informed consent was obtained from parents of participating children for the original study, but sample analyses in the current study were done anonymously.

Study population and sampling

A random sample of 95 archived plasma samples drawn from the previous longitudinal cohort study (conducted at Dodowa, an area in Southern Ghana with seasonal, stable transmission of mainly *P. falciparum*) was used in this study. A detailed description of the study site and sampling procedures has previously been published [18,19]. The original study involved a total of 300 children between the ages of 3 and 15 years. Blood samples were drawn from study participants at the beginning of the high transmission season (April 1994) as well as at the end of the season, prior to the beginning of the low transmission season (November 1994). Plasma samples used here were prepared from blood samples taken before the low transmission season in November 1994. Clinical malaria was defined as having a fever and/or an axillary temperature above 37.5ºC, as well as parasitaemia above 5000 parasites/µl of blood.

Antibody determination

Anti-*Pf*AMA1 antibodies titres in plasma samples were measured using an antigen capture ELISA. Plates were coated separately with 1µg/ml of AMA1 alleles from the FVO (GenBank accession number AJ277646), HB3 (GenBank accession number U33277), 3D7 (GenBank accession number U65407) and CAMP (GenBank accession number M58545) parasite strains, as well as with 1µg/ml of three different antigen mixtures; i) a mixture of three Diversity covering (DiCo) antigens whose design is based on the amino acid sequences of 355 naturally occurring *Pf*AMA1 alleles [20], ii) a mixture of the FVO, HB3, 3D7 and CAMP alleles, designated as *Four*, and iii) a mixture of all seven allelic antigens, designated as *Seven*. All antigen mixtures had equal weights of the component alleles. All antigens were expressed in *Pichia pastoris* and potential N-Glycosylation sites were removed by methodologies that have been previously described [12,20-22]. Plates were blocked with 200 µl/well of 3% BSA in PBS-Tween 20 (0.05%) for 1 hour, after which 100 µl/well of plasma (diluted 1: 200 and titrated 3-fold over 8 duplicate wells) was added and incubated for 1 hour. Bound antibodies were detected by incubation with 100 µl/well of 0.8 µg/ml alkaline phosphatase-conjugated goat anti-human IgG for 1 hour. Colour development was with 1 mg/ml p-Nitrophenyl phosphate in DEA buffer (0.15%

$MgCl_2.6H_2O$, 0.01% diethanolamine, pH = 9.8) for 30 min and optical density (OD) was measured at 405 nm. ODs were subsequently expressed in arbitrary units (AU) by the calibrator (hyperimmune human serum pool) included on each ELISA plate using the 4PL-based ADAMSEL programme (Remarque©), a data management system that is accessible from the EMVDA website (www.malariaresearch.eu). One arbitrary unit (1AU) is equivalent to the reciprocal plasma dilution that gives an OD of 1.0 over background.

Statistical analyses

Antibody titres were log-transformed to achieve normality and stratified by age (3 to 5 years, 6 to 10 years and 11 to 15 years) in order to assess the effect of age/antigen exposure on the specificity of elicited antibodies. Antibody titres were compared for the same age group across capture antigens by one-way analysis of variance followed by Tukey Honest Significance Difference post hoc tests where necessary. The student t test was used to make pair-wise comparisons between titres of different age groups measured against the same capture antigen. Titres are also presented as boxplots per age group on each capture antigen. For each capture antigen, antibody titre variability amongst the different age groups was assessed by Levene's test for homogeneity of variances. The log-transformed titres against different capture antigen pairs were subsequently compared using Tukey mean-difference (TMD) or Bland-Altman plots [23], which assess the degree of agreement between same sample measurements by different methods (here different capture antigens). Since titres were log-transformed, the x-axis gives the geometric mean of the two antibody measurements for the sample against the capture antigen pair and the y-axis gives the ratio of titre measurements. Plots show a bold horizontal line (line of equality) indicating the geometric mean of titre differences (ideally at titre ratio = 1 or titre difference = 0) between the antigen pair and dotted lines indicate the 95% limits of agreement for the paired data distribution. The vertical axis has been modified to show fold difference instead of the absolute titre difference.

Association between antibody levels against each of the capture antigens and cumulative incidence of clinical malaria (with the corresponding 95% confidence intervals) was estimated by the Kaplan-Meier method. Clinical malaria incidence rate estimation included all malaria episodes that met the case definition. Data from all 95 children sampled for this study were included in the analyses. The association between antibody levels (and age group) with previous exposure to infection (defined as categorical variables) were assessed separately for titres against each capture antigen using a linear regression model. For each antigen, Cox regression with a robust standard error was used to estimate the rate ratio and its 95% confidence interval. The exposure of interest, antibody levels, was modelled as a continuous variable transformed to log base 2 so that rate ratios

indicate the decrease in incidence rate of malaria corresponding to a two-fold increase in antibody levels.
Analyses and plots were made using the R statistical package (version 2.13.0, 2011, R development core team) and STATA package (Statacorp, College Station, TX).

RESULTS

The hypothesis that measurement of antibody levels in a malaria-endemic population against a polymorphic antigen would be influenced by the specific allele used was investigated in this study. Anti-*Pf*AMA1 antibody levels in the plasma of naturally exposed children was measured against four single *Pf*AMA1 alleles and compared with titres against three different *Pf*AMA1 allele mixtures (Figure 1). Antibody levels against all capture antigens/mixtures increased with age and mean levels against all antigens were significantly higher in the 11-15 year olds compared to the 3 – 5 year olds ($p < 0.05$, student t test). No significant differences were observed in antibody titres between the 6 – 10 year olds and the other two groups in separate comparisons ($p > 0.05$ in all cases, student t test). Antibody levels against different capture antigens/mixtures did not significantly differ for the same age groups ($P > 0.05$, one-way ANOVA).
Results of the pair-wise comparison of antibody titres against the different capture antigens by TMD plots are presented in Figure 2. The more distant data points are from the line of equality, the greater the binding preference of the same antibodies for one allele over the other. On this premise, plot panels comparing antibody levels against single alleles indicate that younger children (3 - 5 years) showed a trend of greater strain-specificity, characterised by the slightly greater spread of data points (cross or "+" symbols) compared to that of older children in most panels (Figure 2). This greater variability of antibody titres in younger children is also obvious from Figure 1 (Levene's test, $p < 0.05$ for all capture antigens except for the antigen mixtures *Four* and *Seven*).
Titre measurements of the polyclonal pool of antibodies in naturally exposed individuals would require an antigen(s) that present a broad range of antibody epitopes, a condition that may be fulfilled with multi-allele formulations. Three different allele mixtures (a mixture of the three DiCo antigens, designated DM, a mixture of the four single alleles, designated as *Four*, and a mixture of all seven *Pf*AMA1 alleles, designated as *Seven*) were used for titre measurements in this study. The best single alleles for antibody measurement in this population were determined by pair-wise comparison with titres against these allele mixtures.

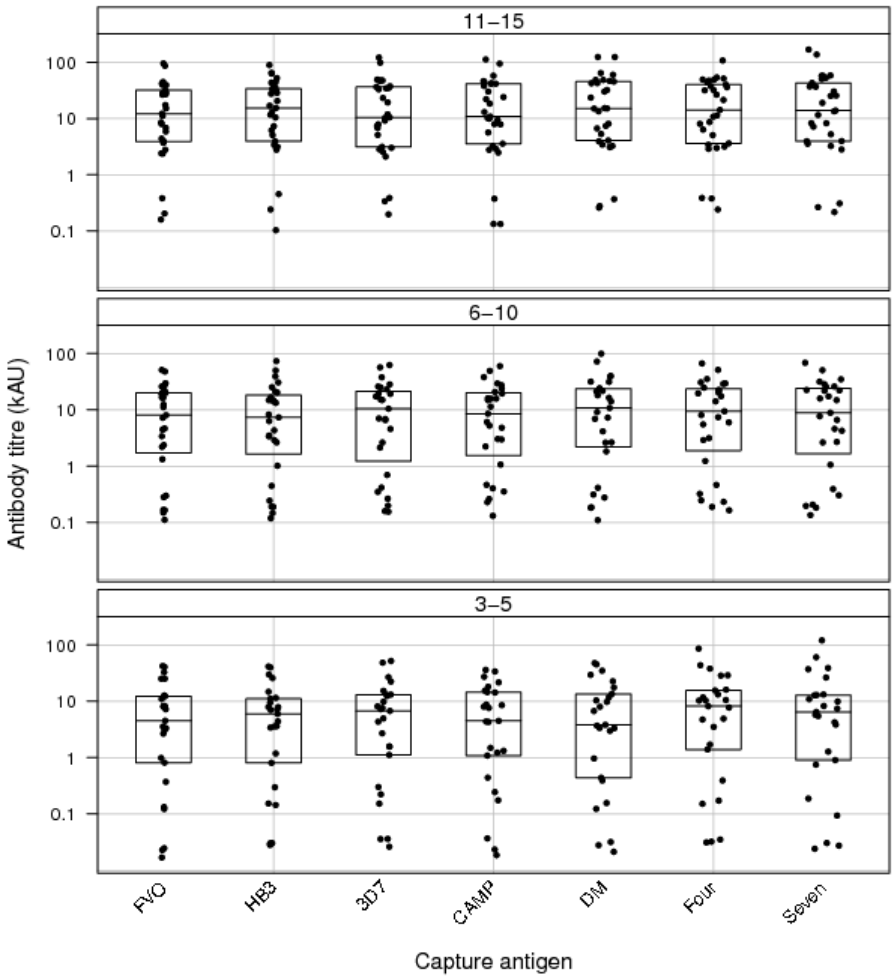

Figure 1. Plasma antibody titres against *Pf*AMA1 alleles and allele mixtures. Anti-*Pf*AMA1 antibody titres in plasma of malaria-exposed children were measured by an indirect ELISA using four *Pf*AMA1 alleles (from the FVO, HB3, 3D7 and CAMP parasite strains) and three different allele mixtures (DiCo mix or DM, a mixture of the four natural alleles, designated as *Four*, and a mixture of all seven alleles, designated as *Seven*) separately as capture antigens. Titres are presented for children ages 3 – 5 years (lower panel), 6 – 10 years (middle panel) and 11 – 15 years (upper panel). Each symbol represents plasma antibody titres of a study participant. Boxplots show the upper and lower quartiles as well as the median of each distribution. The vertical axis (antibody titre) is expressed in kilo arbitrary units (kAU), with one arbitrary unit being equivalent to the reciprocal plasma dilution that gives an OD of 1.0 over background.

Antibody titres measured against the FVO and 3D7 alleles were least variable as judged by the narrow width of 95% limits of agreement (Figure 2) in separate pair-wise comparisons with all allele mixtures. Limits of agreement for the pair-wise comparison of titres against FVO and 3D7 alleles was also very narrow (Figure 2). The number of *Pf*AMA1 alleles (domains I, II and III) identified in four countries in the Africa region and whose amino acid sequences are similar to those of the four alleles used in this study are presented in Table 1. The sequences are part of published *Pf*AMA1 sequences that were retrieved from

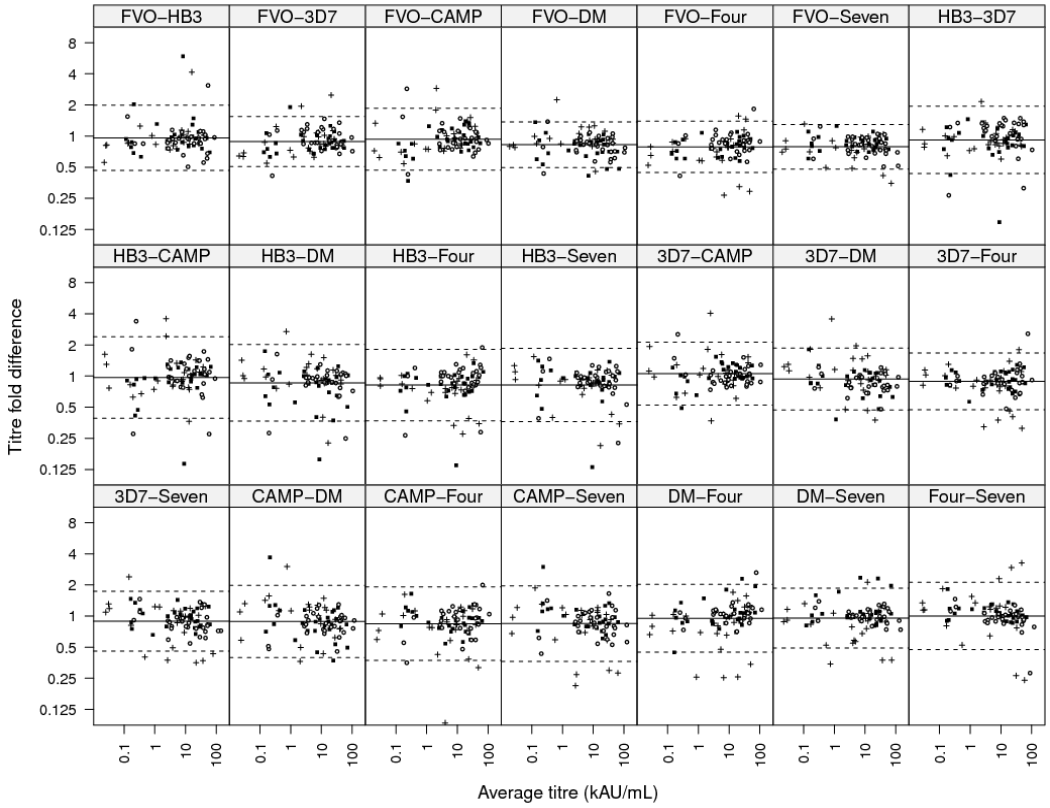

Figure 2. Pair-wise comparison of anti-*Pf*AMA1 antibody titres against different capture antigens/mixtures. Tukey Mean-Difference (TMD) plots were used to assess the level of agreement between antibody titres measured against pairs of capture antigens/mixtures. Each point represents a plot of the difference between two log-transformed titre measurements for a sample against the geometric mean of the same two measurements. For each panel, the bold horizontal line represents the average of all the differences between titres of the same samples against the indicated capture antigen/antigen mixture pair while the dotted horizontal lines represent the 95% limits of agreement for the distribution. Plot symbols represent individual data points; cross (or plus) symbols are study participants aged 3 – 5 years, filled squares are participants between 6 – 10 years and open circles are participants between 11 – 15 years.

GenBank as of January 2011 (Remarque, personal communication). It is clear that all four alleles used in this study occur at very low frequencies in these populations (Table 1), though the 3D7 allele seems more prevalent compared to the FVO allele.

The current study also investigated the association of antibody levels with the cumulative incidence of clinical malaria as well as with previous exposure to *Plasmodium* parasites. Of the 95 children whose samples and clinical data were available for analysis, 23 had at least one clinical episode during the low transmission season and the incidence rate of malaria decreased with the age of participants (Table 2). There was however no association between antibody levels against any of the capture antigens and the incidence of clinical malaria before and after correction for age (Table 3).

Table 1. Number of *Pf*AMA1 alleles with similar amino acid sequences to the alleles used in this study.

*Pf*AMA1 allele	Country			
	Gambia	Mali	Nigeria	Kenya
FVO	1	0	0	0
HB3	0	1	0	0
3D7	2	23	2	1
CAMP	3	6	1	1
*Number of sequences	127	923	52	143

*This is the total number of *Pf*AMA1 amino acid sequences from those countries based on published data as of January 2011.

Table 2. Characteristics of study population

Characteristics	Number of children (%)	Cumulative incidence (95%CI)	Child-months at risk	Malaria cases	Rate per 100 child-months (95% CI)
Age group					
3 - 5 years	39 (41)	20.1% (10, 38.1)	318.82	11	3.5 (1.9, 6.2)
6 - 10 years	27 (28)	22.4% (10.7, 43.2)	226.63	7	3.1 (1.5, 6.5)
11 - 15 years	29 (31)	17.6% (7.7, 37.3)	243.58	5	2.1 (0.9, 4.9)
TOTAL	95	19.8% (13, 29.7)	789.03	23	2.9 (1.9, 4.4)

Clinical malaria is defined as a history of fever or temperature >= 37.5 and parasite density >= 5000/µl

Association of anti-AMA1 titres against the different capture antigens with previous exposure to parasites within the preceding 6 months of the study was investigated using linear regression models. Participants were grouped by their geometric mean parasite density into four categories and the relationship with antibody levels against each capture antigen assessed. Participants who had no detectable parasites (None, n = 10) had the lowest anti-AMA1 antibody levels (Figure 3).

Table 3. Age-adjusted incidence rate ratio (IRR) for the association of anti-parasite antibody levels with malaria incidence

Antibody	Antigen	Crude HR (95%CI)	HR adjusted for age (95%CI)	P-value for adjusted HR
IgG	AMA1-FVO	1.06 (0.92, 1.22)	1.09 (0.93,1.27)	0.3
	AMA1-3D7	1.05 (0.92, 1.19)	1.08 (0.94, 1.24)	0.3
	AMA1-DM	1.05 (0.92, 1.21)	1.08 (0.93, 1.26)	0.32
	AMA1-Four	1.07 (0.93, 1.23)	1.09 (0.94, 1.27)	0.25
	AMA1-Seven	1.06 (0.92, 1.21)	1.08 (0.93, 1.26)	0.3
	AMA1-CAMP	1.08 (0.94, 1.24)	1.11 (0.95, 1.30)	0.19
	AMA1-HB3	1.04 (0.91, 1.18)	1.07 (0.92,1.24)	0.39

Participants in this group had had no infection during the preceding six months before the plasma samples analysed here were taken, but might have experienced infections prior to the start of the study. Participants who had experienced moderate parasite densities (two groups, 1 < 760 and 760 < 3000 parasites/µl of blood, n = 29 and n = 28, respectively) had the highest levels of antibodies when compared with those of participants with no exposure within the preceding 6 months. Participants who had previously experienced parasite densities greater than 3000/µl of blood (3000+, n = 28, Figure 3) however had antibody levels intermediate between those of the unexposed (*None*) and the other two exposed groups (1 < 760 and 760 < 3000). Despite these trends, there were no significant differences in antibody levels between any two parasite density groups.

DISCUSSION

An accurate estimation of antibody levels against malaria parasite antigens is necessary for establishing antibody correlates of protection against malaria in human field studies and clinical trials. A number of parasite antigens that are currently being assessed as vaccine candidates show polymorphism, and the estimation of antibody titres using a single allelic form may be confounded by the occurrence of a large number of allelic variants in nature. In this study, the effect of antigenic polymorphism on the measured levels of anti-*Pf*AMA1 antibodies in plasma samples taken before the low malaria transmission season

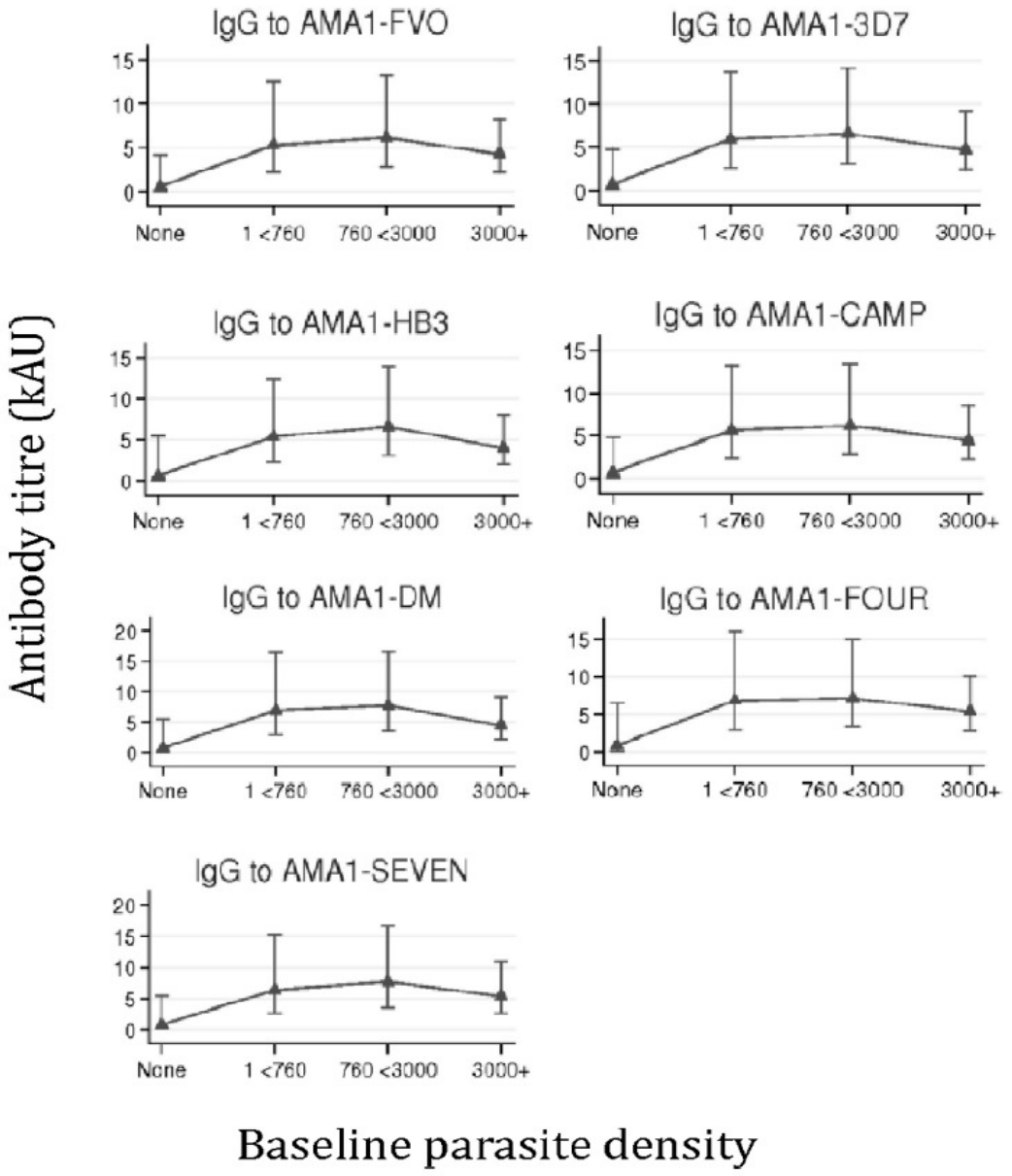

Figure 3. Geometric mean of antibody titres for study participants with different parasite densities. Error bars show the 95% CI of antibody titres per parasite density category. Sample size; n = 10 for the group "None", n = 29 for "1<760", n = 28 for "760 < 3000" and n = 28 for the group "3000+" in all panels.

in a previous study was assessed by comparing titres against single *Pf*AMA1 alleles and allele mixtures. The *Pf*AMA1 allele mixtures are expected to present a broad spectrum of antigenic epitopes that will be recognised by antibodies in the polyclonal pool. We also investigate the association of anti-*Pf*AMA1 antibody levels with the cumulative incidence of clinical malaria as well as with previous exposure to parasites.

For participants in any one age group, antibody levels against the different capture antigens/mixtures did not differ significantly. This observation is not

surprising as antibodies in the plasma samples from most study participants were most likely induced after repeated infection with diverse parasite strains over time. Several studies have indeed reported a large diversity of parasite strains within single communities in disease-endemic areas [17,24-26]. Genotyping of parasite strains in study participants here would have added to data interpretation, but this could not be done due to the unavailability of matching DNA samples.

The greater variability of antibody titres (against all but the antigen mixtures *Four* and *Seven*) observed in younger children (Figures 1 and 2) suggests that individuals with fewer parasite exposures might have a greater proportion of strain-specific antibodies compared to individuals who have had many parasite infections. It is therefore possible that the use of a single allele for antibody measurement, especially in infants/young children or individuals with limited parasite exposure, may under-estimate antibody levels and such data need to be interpreted cautiously. The data here also suggest that though humans discriminate between *Pf*AMA1 alleles and possibly other polymorphic parasite antigens, these effects may become less apparent with age and exposure to variant parasite strains. This observation is in agreement with previous observations in humans [27]. In a study involving participants from Papua New Guinea for example, Cortes et al. [28] showed that the majority of anti-*Pf*AMA1 antibodies directed against polymorphic epitopes were detected in younger age groups compared to older individuals. The development of a more cross-reactive profile with age/exposure may most likely be as a result of clonal imprinting, with antigens derived from every infection primarily boosting memory to previously encountered antigens. This phenomenon has been demonstrated in a controlled setting in rabbits after immunisation with different *Pf*AMA1 alleles in sequence [29].

Antibody measurement against a *Pf*AMA1 allele mixture is expected to give the best titre estimate of polyclonal anti-*Pf*AMA1 antibodies in the field since the mixture would have a large diversity of antigenic epitopes. Such an approach based on a practical number of alleles may be useful, especially since there is a high likelihood of many parasite strains occurring in a given community, and even when there are a limited number of strains, their specific AMA1 alleles may not be readily available for use as coating antigens. Three different allele mixtures were used for titre measurements in this study. DiCo mix (DM), one of the mixtures, consists of three DiCo antigens that were designed based on the sequences of 355 naturally occurring *Pf*AMA1 alleles to inherently cover naturally occurring polymorphism in *Pf*AMA1 [20]. The mixture has been shown in our laboratory to yield higher antibody titres in samples from naturally exposed individuals compared to titres against single natural alleles (unpublished data). Titres against the FVO and 3D7 AMA1 alleles were most comparable to titres against DM and the other two allele mixtures, suggesting

that the FVO and 3D7 alleles preferentially recognize a greater number of antibody specificities compared to the CAMP and HB3 alleles. This is against the observation that all four single alleles used in this study have only been found at very low frequencies in the Africa region (Table 1), though the 3D7 allele seems more prevalent compared to the FVO allele. Thus the measured levels of circulating antibodies in a population with multiple strain infections are dependent on the choice of *Pf*AMA1 alleles used for titre measurement.

The low prevalence of all four alleles shows the complexity involved in the measurement and evaluation of responses to *Pf*AMA1 in a naturally exposed population as even in the current study there may still be strain-specific anti-*Pf*AMA1 antibodies that may not be detected by the best single alleles. It is however worth mentioning that the levels of naturally induced cross-reactive antibodies seem to be the repertoire required for cross protection [29-31], and current vaccine strategies aim at inducing such cross-strain antibodies. Antibody measurement against a single allele could therefore give an indication of the levels of cross-strain (protective) antibodies. Data presented in Table 3 shows no association between antibody levels against any of the capture antigens and the incidence of clinical malaria before and after correction for age. Antibody levels against *Pf*AMA1 have been associated with a reduced risk of malaria incidence in other studies with comparable population characteristics [32-35], though titre measurements in these studies were made at the beginning of the high transmission season. Titres in this study were measured at the beginning of the low transmission season and it is possible that antibody-mediated clearance of infections during the preceding high transmission season would result in low antibody levels at this sampling time point. An earlier study in the same population measured anti-$MSP1_{19}$ antibodies and reported similar levels of antibodies at the beginning and end of the high transmission season [18]. That study also showed no antibody correlation with protection from disease, but the explanation given for the observations here, if proven, would suggest different decay rates for antibodies against the two antigens. The limited sample size in this study is also likely to reduce statistical power required to detect significant associations in the current study. Additionally, the *in vitro* functionality of antibodies could not be assessed as the amount of plasma available was very limited and assays would need to be performed with multiple strains in order to assess the specificity of functional antibodies.

There was a trend of decreasing antibody levels with increasing parasite density from previous exposure, but this inverse association was also not statistically significant. This notwithstanding, the trend is in agreement with the observation that maintenance of immune effectors involved in conferring protection against disease requires the persistence of low levels of circulating parasites [27,36].

The data presented generally suggests a cautious selection of antigens for the measurement of naturally induced antibody levels, especially in samples from individuals with limited exposure to parasites. Though the study utilized

archived samples taken during the 1994/1995 malaria season, the findings have direct relevance for the assessment of naturally acquired antibodies of broad specificity since titres are measured against randomly selected (allelic) antigens and the exact circulating strains/antigenic alleles within study populations are usually not taken into account. Alternatively, DiCo mix may represent an ideal candidate for the measurement of antibody titres in naturally exposed populations, especially in infants and young children as the three DiCo antigens, apart from the effect of mixing, have been designed to cover polymorphism that is seen in naturally occurring *Pf*AMA1 alleles. The data also points to a trend of increasing proportion of antibodies against cross-strain epitopes with age, and suggest the involvement of clonal imprinting in the development of this antibody repertoire. Finally, though there was no association of antibody levels with either a reduced incidence of clinical malaria or previous exposure to parasites, individuals with moderate parasitaemia (< 3000/µl) had higher absolute antibody levels than those with parasitaemia greater than 3000/µl of blood. These findings are collectively relevant to the interpretation of data on antibodies against polymorphic antigens, especially in field studies involving groups with limited parasite exposure.

Competing interests

We have read the journal's policy and have the following conflicts; Three of the authors are in the process of obtaining a patent for the three synthetic Diversity-Covering (DiCo) AMA1 proteins. This does not alter their adherence to all *BMC Infectious Diseases* policies on sharing data and materials. All other authors have no competing interests.

Author's contributions

Conceived and designed the experiments: EJR KAK BWF DD. Performed the experiments: KAK. Analyzed the data: KAK EJR SB. Designed and produced recombinant proteins: BWF, MvdE. Wrote the paper: KAK EJR BWF CHMK DD. All authors have read and approved the final manuscript.

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Chapter 7

General discussion

GENERAL DISCUSSION

The search for an effective malaria vaccine has become ever important, as a vaccine would greatly boost current coordinated efforts at malaria control and prevention. This thesis focuses on the formulation of a blood stage vaccine aimed at reducing parasite multiplication and thereby curbing disease symptoms and sequelae. The vaccines described in this thesis are based on the polymorphic yet promising candidate apical membrane antigen 1 (AMA1), an essential antigen expressed by *Plasmodium falciparum*. The thesis investigates multi-allele vaccine formulation as a strategy for overcoming the effect of allelic polymorphism on immune responses to *Plasmodium falciparum* AMA1 (*Pf*AMA1), as well as a dissection of the fine specificity of induced responses.

The main conclusions of the studies described in this thesis are that i) different *Pf*AMA1 alleles share epitopes to which functional cross-strain antibodies can be induced, ii) increasing the number of *Pf*AMA1 alleles in a vaccine from one to three results in an increased proportion of such functional cross-strain antibodies, iii) beyond three alleles the proportion of induced cross-strain antibodies reaches a plateau, iv) simultaneous or sequential administration of three *Pf*AMA1 alleles induces similar proportions of cross-strain antibodies, even though the latter was expected to induce a greater proportion of cross-strain antibodies since strain-specific responses would not be boosted, v) the proportion of cross-strain antibodies elicited by multi-allele vaccines is independent of the adjuvant used, though adjuvant choice determines the absolute levels of elicited antibodies, and vi) three *in silico*-designed, *Pichia pastoris*-expressed *Pf*AMA1 antigens represent a multi-allele candidate with broad allelic coverage for a human blood stage vaccine.

Multi-allele formulations and antibody response broadening

Vaccines based on single AMA1 alleles have been shown to confer solid protection against homologous parasite strains in animal models [1-4]. The many *Pf*AMA1 variants found in nature (649 alleles from a database of 1778 published valid sequences accessed from GenBank as of January 2011) may however complicate the design of single allele vaccines against human malaria as anti-AMA1 responses show strain specificity [5,6]. Vaccines based on conserved epitopes, or epitopes that are shared amongst a wide diversity of parasite strains, are therefore necessary to eliminate the effects of strain-specificity on vaccine effectiveness. Allelic variation results from the extensive polymorphism found in *Pf*AMA1 [7-11], and this begs the question whether there are shared epitopes to which functional cross strain antibodies can be induced. In chapter 2 the finding that approximately 50% of anti-FVO AMA1 mono-specific antibodies recognize the 3D7 AMA1 allele, and up to 80% of these (representing 40% of anti-FVO mono-specific antibodies) cross-react with two other relatively distant *Pf*AMA1 alleles (HB3 and CAMP) has been described. This suggests that these

four alleles, and possibly other *Pf*AMA1 alleles, share epitope targets to which functional antibodies can be induced. Additionally, the data presented in chapter 4 show that immunisation of groups of rabbits with three distinct *Pf*AMA1 alleles (FVO, HB3 and 3D7) in different orders yield antibodies with comparable specificity profiles and similar *in vitro* functional inhibitory capacities against multiple parasite strains. Subsequently administered alleles most likely boosted responses to previous alleles, and this is a strong indication of the likely existence of a significant number of antibody epitopes that are shared between alleles. To date, only a single conserved inhibitory epitope (4G2 binding epitope in domain II of *Pf*AMA1) has been described [12-14], and the observations made in this study point to the possibility of additional strain-independent epitopes in AMA1 from diverse *P. falciparum* lines. Data from the sequential immunisation study (chapter 4) also support the hypothesis of the development of an increasing cross-strain antibody repertoire with exposure to different parasite strains in an endemic population, as opposed to that involving the accumulation of many different strain specificities [15]. The development of a cross-strain antibody repertoire with age/parasite exposure in humans was also demonstrated in chapter 6 of this thesis.

Allele requirements for achieving the broadest anti-PfAMA1 response

Even though mixed allele vaccine formulations have shown promise for overcoming allelic polymorphism, the number and specific type of variant alleles that are required for near universal coverage are still open questions. Studies that utilized molecular and sero-epidemiological approaches have proposed the inclusion of representative *Pf*AMA1 variants from six [16] to ten [17] allele families in a single formulation to achieve universal coverage. These estimates are based on a limited number of sequences (150 unique haplotypes and 214 haplotypes from the two studies, respectively), and estimates could be higher if more sequences had been available for analyses. The work involved in evaluating individual alleles and the associated cost of producing individual proteins before formulation might however make such approaches less desirable. The findings described in chapter 3 of this thesis that three *in silico*-designed *Pf*AMA1 antigens (known as Diversity-Covering antigens or DiCos) formulated as a multi-allele vaccine elicit responses that are functionally comparable with formulations incorporating the DiCo antigens and four other *Pf*AMA1 alleles (seven alleles in all) suggests a practically relevant limit to the number of component alleles. The DiCo antigens were designed based on the amino acid sequences of 355 naturally occurring *Pf*AMA1 alleles to cover polymorphism that occurs extensively in *Pf*AMA1 and were expressed in the yeast *Pichia pastoris* [18]. Though vaccines composed of three recombinantly-expressed natural alleles were shown to have similar effects, a direct comparison with the DiCo mix vaccine suggested a slight advantage for DiCo mix in terms of immune response broadening (Chapter 4 of this thesis). To further reduce the work involved in evaluating antigens

individually before mixing, the Parasitology Department at BPRC is currently exploring expression systems that would allow the incorporation of all three DiCo sequences either as one fusion protein, or separately in the same expression vector such that the three proteins could be purified from yeast culture supernatants in an already mixed form.

Choice of adjuvants for vaccine formulation

As has been demonstrated in this thesis (chapters 3, 4 and 5), the right quality of antibodies (cross-strain fraction) at low titres may not be enough to achieve the desired, strain-independent, vaccine effect. High titres of these antibodies are necessary for optimal inhibition of diverse parasite strains, and this has also been demonstrated in an *in vivo* challenge study in monkeys [19]. Thus the selection of adjuvants for vaccine formulation is crucial, and adjuvants with high immuno-potentiation properties will be most desirable. It has been shown in this thesis (chapter 2) as well as in other studies ([20]; Remarque et al., in preparation) that approximately 50% of anti-FVO AMA1 antibodies cross-react with 3D7 AMA1 and other alleles. In theory, a single *Pf*AMA1 allele vaccine that elicits very high antibody titres would be effective against a diversity of parasite strains since it would have high titres of the cross-strain fraction. These high titres may however be difficult to achieve with currently available adjuvants, and this theoretical titre threshold is most likely lowered by multi-allele formulations, which have been shown to increase the overall proportion of elicited cross-strain antibodies. Nevertheless, the requirement of highly potent adjuvants with little or no reactogenicity is still necessary for multi-allele formulations in order to achieve high titres of the broadest possible response. Currently, adjuvants that are available for use in human vaccines include Alhydrogel, MF59 from Novartis, AF03 from Sanofi Pasteur, the Montanides (ISA 51, ISA 720) from Seppic and the AS adjuvant series from GlaxoSmithKline (GSK), with all but the Montanides having been used in registered vaccines. Of these, Alhydrogel and the Montanides have been used in many malaria clinical trials with mixed results in terms of desirability. Alhydrogel has in most instances not shown much promise regarding the induction of high titres of long-lasting (protective) responses against *Pf*AMA1 as well as other malarial antigens [21-26]. Though formulations of *Pf*AMA1 as well as other antigens with the Montanides have been shown to induce high titre responses, the associated high reactogenicity observed in some instances makes these less desirable [27-30]. Thus though candidate antigens may per se be good enough, their vaccine effects may not be fully appreciated due to the choice of adjuvant.

While adjuvants are necessary for immune response potentiation in subunit vaccines, a few studies have reported that the specificity of antibody responses to certain parasite antigens is dependent on the type of adjuvant used for formulation [31,32]. In chapter 3 of this thesis, it has been shown that antibody responses to *Pf*AMA1 have similar antigen specificity profiles irrespective of the

adjuvant used. Multi-allele formulations with at least five different adjuvants have been tested in the work described in this thesis, and the specificity of induced antibodies in all formulations as assessed by a harmonized competition ELISA has been generally consistent. Antibody specificity profiles are also comparable amongst anti-*Pf*AMA1 responses in rabbits (chapters 2, 3 and 4) Rhesus monkeys (unpublished data) and humans (Remarque et al., in preparation), though optimal depletion of rhesus and human anti-*Pf*AMA1 antibodies required slightly higher concentration of competitor antigens. The significance of this outcome for vaccine development is that irrespective of the adjuvant ultimately selected for *Pf*AMA1 formulation, the same quality of antibody responses is likely to be elicited.

Current status of AMA1-based vaccines

Over the last few years a number of *Pf*AMA1-based vaccine formulations have entered human trials and a summary of these trials is presented in the Table below. Some single *Pf*AMA1 allele vaccines, tested in naïve populations, have shown signs of the possibility of vaccine ineffectiveness resulting from allelic polymorphism ([33]; Remarque et al., in preparation). A 3D7 AMA1 single allele vaccine that was tested in malaria-exposed adults however showed no such effects [34], and this was most likely due to existing levels of anti-malarial antibodies developed after repeated exposure to diverse strains. There is currently a single *Pf*AMA1 multi-allele vaccine (AMA1-C1) in human trials, and this has shown early indications of response broadening but mainly against parasite strains expressing the vaccine component alleles [21,24,35,36]. This confirms the inadequacy of two alleles for dealing with the effects of polymorphism in *Pf*AMA1 responses. The developers of AMA1-C1 (NIAID/NIH, USA) are currently exploring the possibility of including AMA1 from the L32 parasite strain to make a three-allele vaccine termed AMA1-C2. As has been described in this thesis (chapter 3), *Pf*AMA1 vaccines composed of three alleles seem to yield the broadest response possible, but the addition of L32 AMA1 to the 3D7 and FVO alleles raises the issue of which alleles to include in a multi-allele vaccine as L32 is a very low prevalence strain (~ 0.22%, based on published *Pf*AMA1 protein sequences accessed from GenBank as of January 2011). This argument, if proven, would indicate that apart from the number of alleles, the choice of alleles might also be important for achieving optimal vaccine responses. The DiCo mix vaccine candidate being developed by BPRC Rijswijk consists of three *Pf*AMA1 alleles that have been designed specifically to cover polymorphism in naturally occurring *Pf*AMA1, and are expected to induce very broad responses in humans. The DiCo mix candidate is scheduled to go into phase I trials later in 2011/2012, with earlier pre-clinical studies showing a good level of response broadening.

An interesting phenomenon regarding *Pf*AMA1 vaccine responses is the observed immune interference from responses to other malarial antigens.

Table. *Pf*AMA1-based vaccines that have gone into clinical trials (from published work)

Candidate	Expression system	Adjuvant(s)/ delivery system	Trial stage(s)	References
*Pf*AMA1 (3D7)	*E. coli*	Montanide ISA 720	Phase 1	[29]
PEV301/PEV3A (*Pf*AMA1)		Virosomes	Phase 1/2a	[37,38]
AMA1-C1 (FVO+3D7)	*P. pastoris*	Alhydrogel ± CPG7909, Montanide ISA 720	Phases 1, 2	[21,24,28,35,36,39]
FMP2.1 (*Pf*AMA1-3D7)	*E. coli*	AS02A, AS01B	Phases 1, 2a	[34,40,41]
*Pf*AMA1 (FVO)	*P. pastoris*	AS02A, Alhydrogel, Montanide ISA 720	Phase 1	[26]
PfCP-2.9 ($MSP1_{19}$-AMA1 *DIII*)	*P. pastoris*	Montanide ISA 720	Phase 1	[42,43]
AdCh63 AMA1/ MVA AMA1		Simian adenovirus/ MVA	Phase 1	Hill (in preparation)

Accumulating data on antibody responses in humans show that persisting levels of other anti-malarial antibodies in naturally exposed semi-immune adults interfere with the functional activity of anti-*Pf*AMA1 responses [35,44]. Naïve individuals and infants are less likely to have circulating anti-malarial antibodies, suggesting that *Pf*AMA1, when formulated on its own, may be best suited as a vaccine for infants/naïve individuals but not for semi-immune adults.

Malaria vaccines; promises and challenges

The acquisition of anti-disease immunity in adults living in malaria-endemic areas, the successful treatment of both children and adults with immunoglobulins purified from the sera of semi-immune adults [45-48] and the induction of sterile immunity with whole parasite vaccines [49,50] clearly

suggest the feasibility of developing a vaccine against malaria. Apart from AMA1, a number of other promising candidate antigens, administered either as single recombinant antigens, antigen mixtures or chimeric proteins have also gone into human trials. The most prominent of these are CSP, MSP1, MSP2, MSP3, EBA-175 and GLURP [22,25,37,51-57], and trial outcomes have generally shown limited successes in terms of immunogenicity and potential efficacy. RTS,S is by far the most advanced malaria vaccine in terms of protective efficacy and developmental stage. It consists of the C-terminal repeat region of *P. falciparum* CSP fused with the N-terminal region of the Hepatitis B virus surface antigen and formulated with proprietary adjuvants from GSK. RTS,S has been shown to offer at least 50% efficacy (time to first clinical episode) over the first 8 months of study follow-up and protection that persists for up to 15 months after vaccination of 5 – 17 month old children in two malaria-endemic areas [58,59]. Though modest, this outcome is encouraging, and RTS,S is currently being evaluated in multi-centre phase III trials in Africa. Trials are currently on-going at eleven locations with varying transmission intensities, and the first data from these trials is expected to be available by the end of 2011.

Despite these advances, the pace of malaria vaccine development has generally been retarded by a number of factors aside those described for *Pf*AMA1. Whole parasite approaches on the one hand have been shown to induce durable protection [49,50,60,61], but vaccine manufacture and delivery have presented unique challenges. Subunit vaccines on the other hand are relatively easier to manufacture and deliver, but have not as yet yielded the desired level of protective immunity. Responses against a number of parasite antigens are believed to contribute to protection from disease, but these targets are not well defined. Additionally, the importance of these antigens as targets of protective immunity seems to vary amongst individuals and is dependent on exposure to parasites as well as on parasite and host genotypes [62]. This lack of defined correlates of protective immunity implies that selection of the "best" vaccine candidates has been based on vaccine properties of these antigens in animal models, or on such antigen characteristics as their accessibility to the relevant immune effectors and their importance for the parasite's survival. The lack of correlates of protection is also linked to the limited functional assay options for down-selection of the best candidates for further development, as well as with options for assessing vaccine trial outcomes.

The inability of currently available adjuvants to induce durable, long-lasting responses presents yet another challenge to malaria vaccine development. Both antibody and T cell-mediated responses on their own have been shown to at least confer non-sterile protection from disease [46,48-50,63], and an effective vaccine would be expected to induce both adaptive responses. Antibody responses require the induction of strong CD4+ T cell responses to provide the necessary B cell help. Induction of functional antibody and T cell responses has been achieved with vectored vaccines (DNA, viruses) that especially employ

heterologous prime-boost vaccination regimens [64-69]. The inclusion of toll-like receptor (TLR) agonist as immune modulators in currently available adjuvants has also been employed to induce strong antibody and T cell responses [36,70].

Finally, recent findings that sporozoite antigens that remain in the skin after parasite passage into the liver induce immune tolerance that later dampens protective vaccine responses [71] suggest that vaccine delivery routes as well as candidate antigens may need to be carefully selected. These challenges notwithstanding, improvements in technology and increasing understanding of the complex nature of the parasite offer promise for a breakthrough, and malaria vaccine development remains an important public health objective.

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Summary

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Nederlandse samenvatting

SUMMARY

A vaccine against the blood stages of malaria caused by *Plasmodium falciparum* would be an important addition to the currently available arsenal for disease control. A handful of immunologically relevant parasite antigens are at various stages of pre-clinical and clinical development. Amongst these is *Plasmodium falciparum* apical membrane antigen 1 (*Pf*AMA1), an essential antigen initially expressed in micronemes of asexual blood stage parasites and believed to play a critical role in the parasite's invasion of red blood cells. A similar role for this antigen has been described in sporozoites, and this makes *Pf*AMA1 an important multi-stage vaccine candidate. The immune response to *Pf*AMA1 is mediated mainly by antibodies that prevent invasion of host cells by parasites. *Pf*AMA1 however shows extensive polymorphism, with anti-*Pf*AMA1 antibody responses exhibiting strain-specificity. One proposed strategy for dealing with strain-specificity to *Pf*AMA1 responses has been the formulation of vaccines with a number of *Pf*AMA1 alleles as components, and this is the focus of the current thesis.

In **chapter 2** of this thesis the mechanism underlying broadening of anti-*Pf*AMA1 vaccine responses through immunisation with multi-allele formulations was investigated by comparing responses induced in rabbits with vaccines containing one and three *Pf*AMA1 alleles (from FVO, HB3 and 3D7 parasite strains). A harmonized competition ELISA, together with an *in vitro* growth inhibition assay, was used to show that the three-allele vaccine induces a greater proportion of functional cross-strain antibodies compared to single allele vaccines. Cross-strain antibodies were induced against epitopes that are shared between the vaccine alleles. These would include epitopes that are conserved in all *Pf*AMA1 alleles as well as epitopes that are common only to the vaccine alleles. Only a single *Pf*AMA1 conserved epitope has thus far been described and the findings here suggest there may be a couple more of such conserved epitopes. The findings here also suggest that increasing the number of alleles included in a vaccine should in theory result in an increased proportion of the induced cross-strain antibodies, and this raises the question of how many alleles are required to yield the best vaccine response against a diversity of strains.

This question was addressed in **chapter 3** by comparing responses induced in rabbits with vaccines incorporating three (Diversity Covering antigens or DiCos, designed *in silico* based on sequences of naturally occurring *Pf*AMA1 alleles) and seven (the three DiCo antigens, alleles from strains FVO, HB3, 3D7 and CAMP) *Pf*AMA1 alleles. Specificity assays showed that the proportions of induced cross-strain antibodies were comparable between the two vaccines, both formulated with CoVaccine HT™ as adjuvant. Functional assays with protein G-purified antibodies confirmed this finding, suggesting that optimal broadening of the antibody response is achieved with three alleles, as there is a response plateau beyond this number. The effect of adjuvant choice on antibody specificity was

also investigated in this chapter. Responses against DiCo mix formulated with three different adjuvants (CoVaccine HT™, Montanide ISA 51 and Montanide IMS) showed only marginal differences in the proportions of induced cross-strain antibodies. Functional assays however showed no adjuvant effect on antibody inhibitory capacity but rather an effect of the quantity of induced antibodies. Antibody responses against DiCo mix in Montanide IMS were 2 – 3 fold lower compared to formulations with the other two adjuvants and this resulted in corresponding low growth inhibition capacities.

In **chapter 4**, sequential immunisation as a possible strategy for further increasing the proportion of induced cross-strain anti-*Pf*AMA1 antibodies was investigated. Groups of rabbits were immunised three times with three *Pf*AMA1 alleles in different orders and their responses compared to those of rabbits immunised three times with the same alleles as a mixture. It was established that immunisation with three *Pf*AMA1 alleles in sequence resulted in similar proportions of cross-strain antibodies as three immunisations with the allele mixture. The findings here were also suggestive of the accumulation of a cross-strain antibody repertoire with increasing exposure to variant parasites in the field.

In **chapter 5**, the safety and immunogenicity of DiCo mix vaccines formulated with two different adjuvants (CoVaccine HT™ and Montanide ISA 51) were assessed in rhesus monkeys. Safety and immunogenicity data in non-human primates may give a better indication of the vaccines' effects in humans compared to data generated in rabbits. Different groups of rhesus macaques were immunised three times with the two DiCo formulations and an AMA1-MSP1 (AM) fusion protein candidate formulated in Montanide ISA 51. Vaccine safety was assessed by physical and behavioural observation of animals as well as by measurement of chemical and haematological parameters as indices for organ and systemic functions. All vaccines were well tolerated with no vaccine-related adverse events, and DiCo mix in CoVaccine HT™ yielded the best immunogenicity data as assessed by indirect ELISA and *in vitro* growth inhibition assays. This data further demonstrates the specificity broadening effects of multi-allele formulations, and plans are underway to secure the proprietary adjuvant CoVaccine HT™ for testing DiCo mix in a phase I trial in a non-endemic population. Data from the AM fusion protein was however difficult to interpret as four of the six immunised animals were non-responsive for unknown reasons.

Chapter 6 of this thesis examined the effect of *Pf*AMA1 allele polymorphism on measurement of anti-*Pf*AMA1 titres in field studies. Antibody titres in a naturally exposed cohort of children between the ages of 3 and 15 years, living in an endemic area of southern Ghana, were measured against four *Pf*AMA1 alleles (from strains FVO, HB3, 3D7 and CAMP) as well as three antigen mixtures (DiCo mix, a mixture of the four natural alleles as well as a mixture of all seven alleles). The best allele for titre measurement in this population was deduced from comparisons of titres against single alleles with those against allele mixtures.

The mixtures are expected to have a wide repertoire of epitopes that would be recognised by antibodies of diverse specificities present in the polyclonal pool of naturally exposed individuals. Apart from that, the three DiCo antigens were designed specifically to cover naturally occurring polymorphism when used as an equimolar mixture. The FVO and 3D7 alleles were found to be best for use as capture antigens when measuring anti-*Pf*AMA1 titres in this population. Analysis of *Pf*AMA1 protein sequence data retrieved from GenBank however showed that the 3D7 and CAMP alleles were most prevalent of the four alleles in the Africa region. Findings here also suggest that the proportion of cross-strain antibodies most likely increases with age/exposure to different parasite strains and humans, especially infants and young children with limited parasite exposure, might discriminate between *Pf*AMA1 alleles. Anti-*Pf*AMA1 titres measured against single *Pf*AMA1 alleles may therefore need to be cautiously interpreted as they may be underestimated in such individuals.

Chapter 7 finally presents a general discussion of the findings in this thesis along with the current status of malaria vaccine development.

The data presented in this thesis shows that multi-allele formulations are an important strategy for *Pf*AMA1 vaccine design, and that *Pf*AMA1 must at least be a component of an effective universal multi-antigen subunit vaccine. There are currently a number of vaccine design strategies that aim to combine several proven candidates either as the recombinant DNA, as antigen mixtures or as fusion proteins in order to cover polymorphism as well as target different stages of the parasite's lifecycle. Other challenges such as availability of potent adjuvants, vaccine delivery systems and delivery routes will also need further investigation.

SAMENVATTING

Een vaccin zou een belangrijke bijdrage kunnen leveren aan de bestrijding van malaria tropica, veroorzaakt door *Plasmodium falciparum*, verantwoordelijk voor 95% van de mortaliteit van malaria. Momenteel zijn er maar een klein aantal immunologisch relevante parasietantigenen in ontwikkeling tegen de bloedstadia van deze belangrijke infectieziekte. Een van deze antigenen is *Plasmodium falciparum* apicaal membraan antigen 1 (*Pf*AMA1), wat beschouwd wordt als één van de meest prominente bloedstadium-vaccinkandidaten *Pf*AMA1 is een essentieel eiwit dat waarschijnlijk een belangrijke rol speelt in de invasie van rode bloedcellen. Het komt initieel tot expressie in de micronemen van de asexuele bloedstadia van de parasiet. Het eiwit is ook aangetoond in sporozoiten en het vermoeden bestaat dat *Pf*AMA1 ook betrokken is bij de invasie van levercellen. Dit maakt *Pf*AMA1 mogelijk een multi-stadium vaccinkandidaat.

De immuunrespons tegen *Pf*AMA1 is hoofdzakelijk antilichaam gemedieerd. Deze antilichamen binden aan *Pf*AMA1 en blokkeren zo de invasie van gastheercellen. *Pf*AMA1 is een polymorf eiwit (allelische variatie) en antilichaamresponsen tegen *Pf*AMA1 zijn grotendeels allel-specifiek. Een mogelijke oplossing om allel-specificiteit van antilichaam responsen te verbreden is vaccinatie met een mengsel van verschillende allelen. Dit laatste is het onderwerp van dit proefschrift.

In **hoofdstuk 2** worden mechanismen verantwoordelijk voor de verbreding van de immuunrespons na vaccinatie met meerdere allelen onderzocht door de antilichaamresponsen van konijnen gevaccineerd met slechts één *Pf*AMA1 allel te vergelijken met responsen van konijnen gevaccineerd met een mengsel van drie *Pf*AMA1 allelen (van de FVO, HB3 en 3D7 *P. Falciparum* parasietstammen). Met behulp van een geharmoniseerde competitie-ELISA en een *in vitro* groei inhibitie assay (GIA) werd aangetoond dat vaccinatie met een mengsel van drie *Pf*AMA1 allelen meer functionele antilichamen opwekt die met meerdere *Pf*AMA1 allelen reageren (cross-strain) dan vaccinatie met een enkel allel. Het merendeel van de antilichamen opgewekt door gelijktijdige vaccinatie met drie *Pf*AMA1 allelen reageerde met epitopen die op ieder van deze drie vaccinallelen voorkomen. Dit soort cross-strain epitopen kunnen ook voorkomen op *Pf*AMA1 allelen die niet tot de drie vaccinallelen behoren. Tot nu toe is slechts één geconserveerde epitoop beschreven voor *Pf*AMA1 en de bevindingen uit hoofdstuk 2 suggereren dat er mogelijk meer geconserveerde epitopen kunnen zijn. De bevindingen in hoofdstuk 2 suggereren ook dat de antilichaamrespons verbreed kan worden door het aantal allelen in een vaccin te vergroten. Dit resulteert in de onderzoeksvraag hoeveel en welke allelen in een *Pf*AMA1 vaccin dienen te worden opgenomen.

Deze vraag wordt behandeld in **hoofdstuk 3**. Hier worden antilichaamresponsen in konijnen geïmmuniseerd met drie synthetische allelen (Diversity Covering PfAMA1 Antigenen of DiCo, specifiek ontworpen om een zo breed mogelijke *Pf*AMA1 herkenning te geven) vergeleken met responsen in konijnen

geïmmuniseerd met zeven *Pf*AMA1 allelen (3 DiCo PfAMA1's aangevuld met 4 natuurlijke allelen; FVO, HB3, 3D7 en CAMP). Uit specificiteitsbepalingen bleek dat de proporties cross-strain antilichamen vrijwel gelijk waren voor beide vaccins. Dit werd bevestigd door functionele assays met proteïne G gezuiverde antilichamen. Deze waarnemingen suggereren dat een brede antilichaamrespons opgewekt kan worden met een mengsel van drie allelen en dat hierna een plateau bereikt wordt. De invloed van het adjuvant, werd ook in hoofdstuk 3 onderzocht. Antilichaamresponsen tegen DiCo mix werden vergeleken met drie verschillende adjuvantia (CoVaccine HT™, Montanide ISA 51 en Montanide IMS). Het adjuvant had slechts een marginale invloed op de breedte van de geïnduceerde respons, maar wel een duidelijke invloed op de antilichaamhoeveelheden. Met behulp van functionele assays (GIAs) werden ook geen verschillen in de breedte van de respons waargenomen. Wederom resulteerde lagere antilichaamresponses in lagere GIA titers.

In **hoofdstuk 4** wordt onderzocht of sequentiële immunisatie de proportie cross-strain antilichamen kan verhogen. Om dit te onderzoeken werden groepen konijnen drie maal geïmmuniseerd met verschillende *Pf*AMA1 allelen in verschillende volgordes en vergeleken met konijnen geïmmuniseerd met een mengsel van de drie allelen. De resultaten wezen uit dat simultane en sequentiële immunisatie vergelijkbare hoeveelheden cross-strain antilichamen opleveren. De resultaten suggereren ook dat de hoeveelheid cross-strain antilichamen toeneemt na blootstelling aan variant allelen in het veld.

In **hoofdstuk 5** wordt de veiligheid en immunogeniciteit van DiCo mix antigenen, geformuleerd met twee verschillende adjuvantia (CoVaccine HT™ en Montanide ISA 51), onderzocht in resus makaken. Veiligheids- en immuniteitsgegevens in niet-humane primaten geven mogelijk meer informatie dan gegevens uit studies met konijnen. Groepen resus makaken werden drie keer geïmmuniseerd met twee verschillende *Pf*AMA1 DiCo formuleringen (in CoVaccine HT™ en Montanide ISA51) of met een *Pf*AMA1-MSP1-19 fusie-eiwit, geformuleerd in Montanide ISA51. Vaccinveiligheid wordt bepaald middels gedragsobservatie, inspectie van de injectieplaatsen en bepaling van klinisch chemische en hematologische parameters als indices voor orgaanfunctie. Alle vaccinformuleringen werden goed verdragen zonder dat er vaccingerelateerde bijwerkingen werden waargenomen. Een mengsel van drie DiCo PfAMA1 eiwitten in het adjuvant CoVaccine HT™ gaf de beste immunogeniciteit, bepaald met ELISA en met *in vitro* groei inhibitie bepalingen. Uit de gegevens van de immunisaties met het *Pf*AMA1-MSP1-$_{19}$ fusie-eiwit konden geen harde conclusies worden getrokken omdat vier van de zes geïmmuniseerde dieren onverklaarbaar lage responsen te zien gaven. De gegevens verkregen met non-humane primaten bevestigen dat vaccinatie met multi-allel formuleringen de specificiteit van de respons verbreedt. Momenteel worden plannen gemaakt om DiCo Mix met Covaccine HT™ in een fase Ia studie in niet aan malaria blootgestelde volwassenen te testen.

In **hoofdstuk 6** wordt het effect van *Pf*AMA1 polymorfismen op antilichaambepalingen in malaria-endemische gebieden nagegaan. Hiervoor werden antilichaamtiters in een cohort van aan malaria blootgestelde Ghanese kinderen tussen 3 en 15 jaar bepaald met verschillende antigenen of combinaties daarvan. Voor dit onderzoek werden platen gecoat met een van de natuurlijke *Pf*AMA1 varianten FVO, HB3, 3D7 of CAMP, of met een mengsel van antigenen (een mengsel van DiCo PfAMA1 eiwitten, 4 natuurlijke allelen of en mengsel van alle zeven PfAMA1 eiwitten). Het beste coating antigeen werd bepaald door titers tegen enkele allelen te vergelijken met titers tegen allelmengsels. Van de mengsels verwacht men dat deze een breed repertoire van potentiële epitopen bevatten, die door polyclonale antilichamen van aan malaria blootgestelde personen herkend kunnen worden. Ook werd een equimolair mengsel van de drie DiCo PfAMA1's getest. *Pf*AMA1 van de FVO en 3D7 allelen bleken een goed beeld te geven van de anti *Pf*AMA1 antilichamen in de onderzochte populatie. Analyses van *Pf*AMA1 eiwitsequenties uit Genbank echter wijzen erop dat de 3D7 en CAMP allelvormen het meest prevalent zouden kunnen zijn in Afrika.
De gegevens laten verder zien dat de hoeveelheid cross-strain antilichamen toeneemt met leeftijd en/of blootstelling aan verschillende varianten. Peuters en jonge kinderen lijken meer onderscheid te maken tussen verschillende *Pf*AMA1 allelen dan oudere kinderen. Het verdient daarom aanbeveling om *Pf*AMA1 titers bepaald tegen slechts één variant voorzichtig te interpreteren omdat deze mogelijk de hoeveelheden antilichamen in jongere leeftijdsgroepen onderschatten.
Hoofdstuk 7 stelt definitief een algemene bespreking van de bevindingen in deze thesis samen met het huidige statuut van de ontwikkeling van het malariavaccin.
De gegevens in dit proefschrift maken duidelijk dat multi-allel formuleringen een belangrijke strategie zijn voor *Pf*AMA1 vaccins en dat *Pf*AMA1 een component van een effectief universeel multi-antigeen vaccin tegen malaria zou moeten zijn. Er zijn momenteel een aantal vaccinstrategieën gericht op het combineren van meerdere gevalideerde kandidaat antigenen zowel als DNA vaccin, antigeenmengsels als fusie-eiwitten om zo polymorfismen en verschillende levensstadia van de parasiet te bestrijden. Andere uitdagingen zoals de beschikbaarheid van potente adjuvantia, vaccin-delivery systemen en toedieningsroutes behoeven nog verder onderzoek.

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CURRICULUM VITAE

Kwadwo Asamoah Kusi was born in Accra, Ghana on 18th April 1977. He completed his secondary education at West Africa Secondary School, Accra in 1994 and proceeded to the University of Ghana, Legon, where he obtained a Bachelor's degree in Biochemistry in the year 2000. He undertook a mandatory one year national service after graduation and later worked as a research assistant in the Biochemistry Department of the University of Ghana till 2002 before starting post-graduate studies in the same Department. He graduated in June 2005 with a Master of Philosophy degree in Biochemistry, and his post-graduate research work was on markers of malaria immunity in paediatric patients. This was conducted at the Immunology Department of the Noguchi Memorial Institute for Medical Research (NMIMR), a biomedical research institute under the College of Health Sciences of the University of Ghana. After graduation in 2005, he worked as a senior research assistant on various projects in the Immunology Department of NMIMR for two years. In April 2007, he proceeded to undertake PhD studies at the Biomedical Primate Research Centre (BPRC) in Rijswijk, the Netherlands. His work at BPRC, which is the focus of this dissertation, has been on malaria vaccine formulation strategies based on the polymorphic candidate *Plasmodium falciparum* apical membrane antigen 1 (*Pf*AMA1). He has now returned to his home institution (NMIMR) and is currently in the process of taking up the position of research fellow in the Immunology Department. He is also a part-time lecturer in the Biochemistry Department of the University of Ghana, Legon.

SUPPLEMENTARY FIGURES

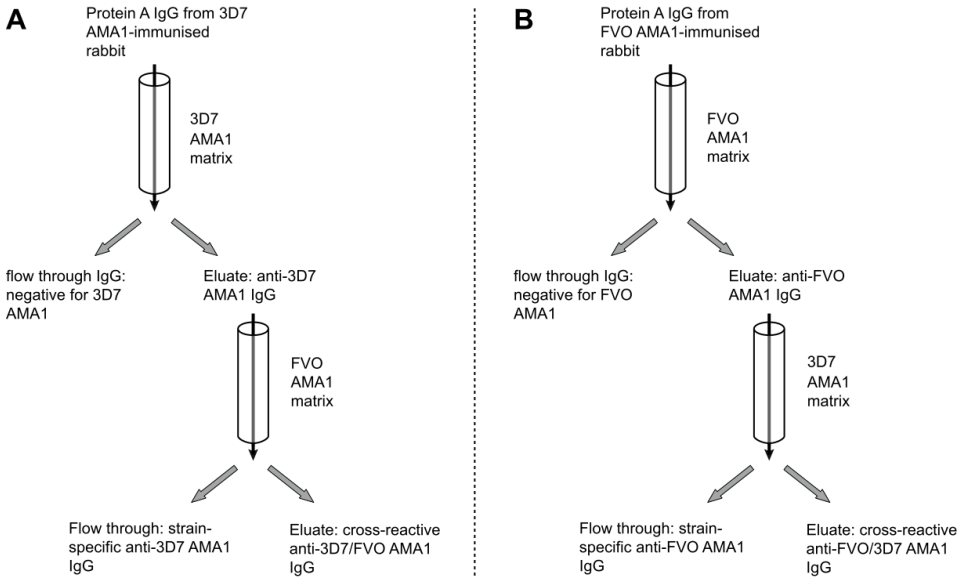

Figure S1 of Chapter 2. Schematic presentation of strain specific and cross-reactive anti-AMA1 antibody purification. Cross-reactive and strain-specific IgG fractions of anti-3D7 AMA1 IgGs (A) and anti-FVO AMA1 IgGs (B) were isolated from the sera of the respective mono-specific AMA1-immunised rabbits. Serum IgGs were first purified over protein A sepharose columns before affinity fractionation.

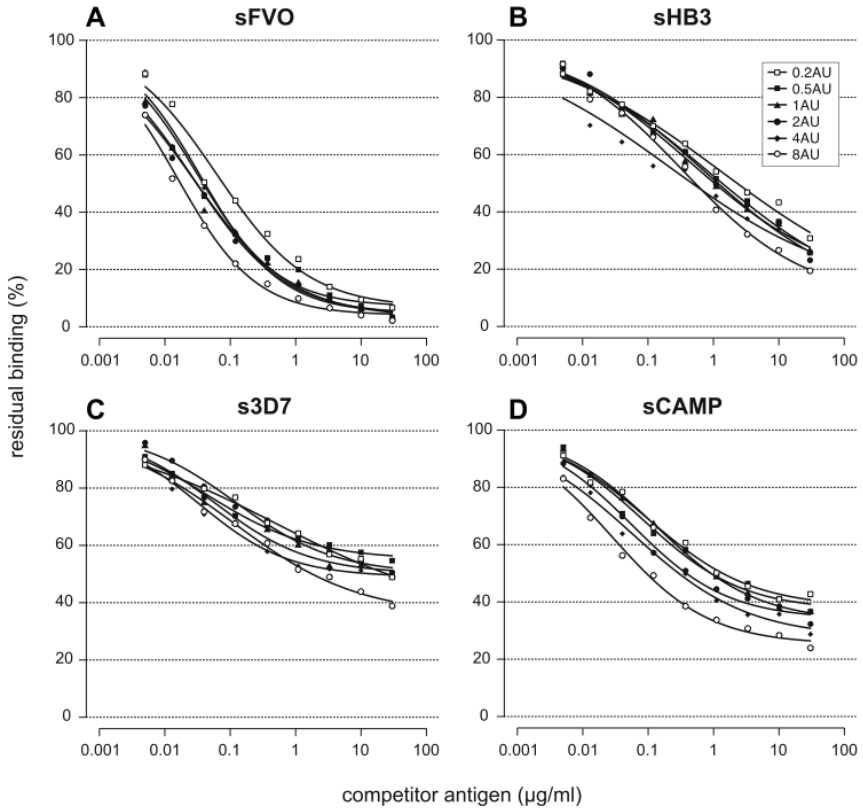

Figure S2 of chapter 2. Competition ELISA using different dilutions of anti-FVO AMA1 antibodies with FVO AMA1-coated plates. The assay involves co-incubation of a soluble/competitor antigen with antibodies in an antigen-coated plate such that there is competition between the coated and soluble/competitor antigens for binding to test antibodies. Protein A-purified anti-FVO AMA1 antibodies were used at dilutions equivalent to 0.2, 0.5, 1, 2, 4 and 8 times the antibody titre (1AU, the IgG dilution that yields an OD_{405} of 1.0). Each dilution of antibody was added to FVO AMA1-coated plates with soluble/competitor AMA1 antigens from the 3D7, HB3, FVO and CAMP parasite strains, each titrated from 30 – 0.005 µg/ml in duplicate wells. Antibodies that were not depleted by the soluble/competitor antigens bound to the coated antigen (residual binding), and the resulting optical densities (OD) were expressed as percentages of ODs from reagent wells with antibody but no competitor antigens. Competitor antigen concentrations (log transformed) were then plotted against the percent residual binding for all competitor antigens. Depletion patterns for competitor/soluble FVO or sFVO (A), sHB3 (B), s3D7 (C) and sCAMP (D) AMA1 antigens at the different antibody dilutions are shown.