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 (*PfAMA1*), 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 *PfAMA1* alleles share epitopes to which functional cross-strain antibodies can be induced, ii) increasing the number of *PfAMA1* 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 *PfAMA1* 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 *PfAMA1* 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 *PfAMA1* 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 *PfAMA1* [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 *PfAMA1* alleles (HB3 and CAMP) has been described. This suggests that these
four alleles, and possibly other PfAMA1 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 PfAMA1 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 PfAMA1) 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 PfAMA1 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 PfAMA1 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 PfAMA1 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 PfAMA1 alleles to cover polymorphism that occurs extensively in PfAMA1 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 PfAMA1 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 PfAMA1 as well as other malarial antigens [21-26]. Though formulations of PfAMA1 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 PfAMA1 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-PfAMA1 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-PfAMA1 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 PfAMA1 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 PfAMA1-based vaccine formulations have entered human trials and a summary of these trials is presented in the Table below. Some single PfAMA1 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 PfAMA1 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 PfAMA1 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), PfAMA1 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 PfAMA1 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 PfAMA1 alleles that have been designed specifically to cover polymorphism in naturally occurring PfAMA1, 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 PfAMA1 vaccine responses is the observed immune interference from responses to other malarial antigens.
Chapter 7

Table. *PfAMA1*-based vaccines that have gone into clinical trials (from published work)

<table>
<thead>
<tr>
<th>Candidate</th>
<th>Expression system</th>
<th>Adjuvant(s)/delivery system</th>
<th>Trial stage(s)</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><em>PfAMA1</em> (3D7)</td>
<td><em>E. coli</em></td>
<td>Montanide ISA 720</td>
<td>Phase 1</td>
<td>[29]</td>
</tr>
<tr>
<td>PEV301/PEV3A (<em>PfAMA1</em>)</td>
<td></td>
<td>Virosomes</td>
<td>Phase 1/2a</td>
<td>[37,38]</td>
</tr>
<tr>
<td>AMA1-C1 (FVO+3D7)</td>
<td><em>P. pastoris</em></td>
<td>Alhydrogel ± CPG7909, Montanide ISA 720</td>
<td>Phases 1, 2</td>
<td>[21,24,28,35,36,39]</td>
</tr>
<tr>
<td>FMP2.1 (<em>PfAMA1-3D7</em>)</td>
<td><em>E. coli</em></td>
<td>AS02A, AS01B</td>
<td>Phases 1, 2a</td>
<td>[34,40,41]</td>
</tr>
<tr>
<td><em>PfAMA1</em> (FVO)</td>
<td><em>P. pastoris</em></td>
<td>AS02A, Alhydrogel, Montanide ISA 720</td>
<td>Phase 1</td>
<td>[26]</td>
</tr>
<tr>
<td>PfCP-2.9 (MSP1&lt;sub&gt;19&lt;/sub&gt;-AMA1 <em>DIII</em>)</td>
<td><em>P. pastoris</em></td>
<td>Montanide ISA 720</td>
<td>Phase 1</td>
<td>[42,43]</td>
</tr>
<tr>
<td>AdCh63 AMA1/ MVA AMA1</td>
<td></td>
<td>Simian adenovirus/ MVA</td>
<td>Phase 1</td>
<td>Hill (in preparation)</td>
</tr>
</tbody>
</table>

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-*PfAMA1* responses [35,44]. Naïve individuals and infants are less likely to have circulating anti-malarial antibodies, suggesting that *PfAMA1*, 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 \( PfAMA1 \). 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.

REFERENCES


