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Rational selection of antigens for a blood-stage malaria vaccine

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Abstract

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Doctor of Philosophy

Rational selection of antigens for a blood-stage malaria vaccine

by Joe Illingworth

An efficacious vaccine against *P. falciparum* malaria would have great public health benefit. A non-sterilising vaccine against the blood stage would be particularly appealing, as it would prevent disease while allowing exposure to the pathogen, thus allowing the development of natural immunity. Vaccinologists have for many years targeted proteins that are immunodominant during natural infection. This approach has yielded disappointing results in clinical trials. An alternative approach is to target vulnerable antigens that are not necessarily immunodominant during natural infection. This is the approach taken by the two most successful subunit vaccines in clinical development, RTS,S and ME-TRAP, which target the pathogen in the skin and liver stages. RH5 is a blood-stage antigen which does not appear to be a target of natural immunity, but appears to be be highly susceptible to broadly-neutralising vaccine-inducible antibody. This Thesis aimed to discover more blood-stage antigens with the profile of RH5 by producing and then testing the activity of antibodies against a range of merozoite antigens. To facilitate production of pre-clinical vaccine, I established a platform for the production of recombinant protein. Using this platform in combination with virus vectors I was able to test 28 pre-clinical vaccines targeting 24 individual merozoite proteins. This approach yielded two 'hits': AARP and S-antigen. Antibodies to AARP were characterised and found to exhibit unusual properties in their reactivity to parasites, recombinant protein, synthetic antigen and the host RBC. Out of 51 proteins that were attempted, I was only able to produce recombinant protein corresponding to 18. In order to test antigens that I could not express as recombinant protein, I conceived, established and optimised a novel immunisation strategy that uses polyethylenimine to deliver protein-expression plasmids directly into the cells of small mammals, thus negating the requirement for recombinant protein. This platform was found to elicit high concentrations of functional antibodies. It is now a priority to screen the remaining 33 antigens with this platform.

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Abbreviations

ADCI	**A**ntibody **D**ependent **C**ellular **I**nhibition
AdHu5	**Hu**man **Ad**enovirus serogroup **5**
ADRB	**A**ntibody **D**ependent **R**espiratory **B**urst
AU	**A**rbitrary **U**nits
BAP	**B**iotin **A**cceptor **P**eptide, see Section 3.1.5
BCG	**B**acillus **C**almette **G**uérin
BCR	**B** **C**ell **R**eceptor
BSA	**B**ovine **S**erum **A**lbumin
ChAd63	**Ch**impanzee **Ad**enovirus serogroup **63**
cGMP	**c**urrent **G**ood **M**anufacturing **P**ractice
CDR	**C**omplementarity **D**etermining **R**egion, with reference to immunoglobulin binding loops
COS cells	A simian cell line, **C**V-1 **O**rigin, carrying genetic material from **S**V40 virus
CTL	**C**ytotoxic **T**-**L**ymphocyte
DDT	**D**ichloro-**D**iphenyl-**T**richloroethane
ER	**E**ndoplasmic **R**eticulum

ELISA	**E**nzyme **L**inked **I**mmuno**s**orbent **A**ssay
ELISPOT	**E**nzyme **L**inked **I**mmuno**s**pot assay
EPCR	**E**ndothelial **P**rotein **C** **R**eceptor
FcR	**Fc** **R**eceptor
GIA	**G**rowth **I**nhibitory **A**ctivity
GPI	**G**lycosyl**P**hosphatidyl **I**nositol, in the context of protein tethering to plasma membranes
GYPA	**G**ly**co**phorin **A**, the RBC receptor for EBA-175
HIV	**H**uman **I**mmunodeficiency **V**irus
ICM	**Inc**omplete *P. falciparum* culture **M**edium
IFA	**I**mmuno**f**luorescence **a**ssay
IFN-ɣ	**I**nter**f**ero**n**-gamma
i.m.	**I**ntra**m**uscular, with reference to an immunising injection
i.n.	**I**ntra**n**asal, with reference to an immunising injection
i.v.	**I**ntra**v**enous, with reference to an immunising injection
L25	**L**inear **25**kDa PEI, by Alfa Aesar, see Section 2.3.3
M2.5	"**M**ax" **2.5**kDa PEI, by Polysciences^{TM}, see Section 2.3.3
M40	"**M**ax" **40**kDa PEI, by Polysciences^{TM}, see Section 2.3.3
M160	"**M**ax" **160**kDa PEI, by Polysciences^{TM}, see Section 2.3.3
mAb	**m**onoclonal **A**nti**b**ody
MVA	**M**odified **V**accinia virus **A**nkara
MW	**M**olecular **W**eight

NAI	**N**aturally-**A**cquired **I**mmunity
NMR	**N**uclear **M**agnetic **R**esonance
OVA	Chicken-egg **Ova**lbumin
ORF	**O**pen **R**eading **F**rame
PAGE	**P**oly**a**crylamide **G**el **E**lectrophoresis, referred to as **SDS-PAGE** when performed under denaturing conditions due to the presence of SDS
PBMC	**P**eripheral **B**lood **M**ononuclear **C**ell
PBS	**P**hosphate **B**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PEI	**P**oly**e**thylen**i**mine
PFA	**P**ara-**f**orm**a**ldehyde
PMR	**P**arasite **M**ultiplication **R**ate
ROS	**R**eactive **O**xygen **S**pecies
RBC	**R**ed **B**lood **C**ell
RPM	**R**evolutions **P**er **M**inute
iRBC	**i**nfected **R**ed **B**lood **C**ell
SDS	**S**odium **D**odecyl **S**ulfate
SDS-PAGE	**SDS**-**P**oly**a**crylamide **G**el **E**lectrophoresis
SNP	**S**ingle **N**ucleotide **P**olymorphism
SPR	**S**urface **P**lasmon **R**esonance
TCR	**T** **C**ell **R**eceptor
TLR	**T**oll-**L**ike **R**eceptor

Chapter 1

Introduction

1.1 Hypothesis

Vaccine-induced protection against blood-stage malaria can be achieved by focusing immune responses against one or two proteins that are critical for parasite survival. RH5 was recently identified as one such critical protein and there may be others encoded in the *P. falciparum* genome. The discovery of RH5 as a vaccine antigen was delayed because only short fragments of the protein could be expressed. Establishing tools to facilitate the rapid production of antibodies against full-length protein could lead to the discovery of novel antigens that are highly susceptible to anti-parasitic antibody.

1.2 Vaccines

Vaccines have saved more lives than any other class of medicine [1]. The global eradication of smallpox in the 1970s was entirely dependent upon the deployment of a highly efficacious vaccine [2, 3], and poliomyelitis, while not yet eradicated, has seen a reduction in prevalence of over 99% [4]. Thus, a well-deployed vaccine can be an extremely powerful public-health tool. Recognition of this fact has lead to increasing investment in vaccine

research and development programmes, as well as more thorough public immunisation campaigns [5].

1.2.1 Whole-organism versus subunit vaccines

All vaccines work according to the same principle. The adaptive arm of the immune system is stimulated by administering the patient with material that resembles the infectious agent on a molecular level, but in a format that is either non-infectious, or considerably less harmful than the target pathogen. This leads to immunological memory, so that the next time the host encounters the target pathogen a protective effector or recall immune response can immediately begin. The first modern vaccination was performed over 200 years ago by Edward Jenner [6] using infectious vaccinia virus to elicit immunological memory that cross-reacted with small-pox virus. This approach of inoculating individuals with whole microorganism-derived material has been highly successful, and a large proportion of vaccines developed since have used this strategy [7]. For example, the BCG vaccine against tuberculosis consists of extensively passaged cells of *Mycobacterium bovis*, a species that predominantly infects cattle. The yellow fever vaccine consists of an infectious, but attenuated, version of the virus that does not causes disease. Most seasonal influenza vaccines consist of a mixture of three inactivated viruses. The immunological dynamics of the response to whole-organism vaccines differ widely between diseases. Generally, however, a common feature of the pathogens that can be targeted by whole-organism vaccines is that they tend to cause diseases with a definite period of infection, after which the infection is cleared (although the consequences of infection can endure—paralysis after an episode of poliomyelitis can last a lifetime, for instance). Furthermore, the protective immune memory after a single exposure to these pathogens is long-lasting. In these two regards they differ from many of the diseases that are yet to be targeted by a highly efficacious vaccine, in particular tuberculosis, malaria and HIV. The pathogens responsible for these diseases tend to establish chronic infections in the human host, and if the pathogen is cleared, the host is liable to become re-infected. This means that simply harnessing the natural immune response is not sufficient to provide durable protection. Therefore, in order to be protective, vaccines

against these diseases must elicit an immunological response that is *more* effective than the response to natural infection.

The 1970s ushered in the era of recombinant DNA technology, allowing vaccinologists to heterologously express and purify single proteins from microorganisms. This led to the development of subunit protein vaccines, which focus the vaccine-induced immune response against just one or two antigen(s) from the pathogen. Examples of this include the hepatitis B surface antigen that is produced in yeast [8] and the vaccine against human papilloma virus [9]. Genetic immunisation represents yet another degree of separation from the disease-causing micro-organism. Here, genes encoding the proteins from the pathogen are delivered into the cells of the mammalian host either in the form of plasmid DNA [10, 11] or as part of the genome of a recombinant virus [12]. The recombinant antigen is produced by the host protein expression machinery *in vivo*, leading to an immune response against the foreign antigen.

1.2.1.1 Mechanism of antibody induction

Although the precise mechanisms of protection vary between vaccines, in almost all cases it is the vaccine-induced antibody (also termed 'immunoglobulin') that provides protection from re-infection [13]. Protein vaccines constitute the simplest possible scenario for the evolution of antibody, as the immune system is exposed to just a single antigen. The process is still not fully understood at the molecular and cellular level, but is described in outline below.

Each B cell produces an antibody of a single binding specificity, and for most of the stages in their differentiation pathway, they express a non-secreted form of their antibody called the BCR upon their surface. Once introduced into the body, soluble antigen is transported through the lymphatic system until it reaches a secondary lymphoid organ, where naïve B cells are gathered for the purposes of sampling the draining lymph fluid. Upon contact between the antigen and its cognate BCR, the BCR/antigen complex is internalised into the B cell. This constitutes the first of two necessary conditions for the development of an antibody response. Inside the B cell, the protein antigen is cleaved

into short peptides which are presented on the surface of the B cell as peptide-MHC class II complexes [14]. As this is happening, the B cell moves toward the T cell zone of the lymphoid organ. $CD4^+$ T cells sample the peptide-MHC presented on the surface of the B cell, and when the B cell encounters a T cell that can recognise the peptide-MHC on the B cell surface, the T cell provides co-stimulatory signals to the B cell. This co-stimulation is the second necessary signal for B cell activation on the path to humoral memory, and causes the B cell to enter a germinal centre reaction. The dynamics of germinal centre reactions are complex [15], but its finished products are B cells whose immunoglobulin genes have undergone somatic hypermutation to increase the affinity of the antibody:antigen interaction. Whereas naïve B cell produce IgM and IgD, B cells that have undergone germinal centre reactions produce IgA, IgE and IgG. IgG is the predominant immunoglobulin found in plasma, and is the only class of immunoglobulin to be discussed or studied in this Thesis. After leaving the germinal centre reaction, there are two B cell fates of relevance to long-term protection from disease. Long-lived plasma cells migrate to the bone marrow, where they receive survival signals from stroma and secrete large amounts of antibody. These are the major contributor to the serum antibody pool which forms the primary line of defence against infection. A fraction of B cells leaving the germinal centre become memory B cells. These continue to circulate through the blood and lymphatic systems, and upon re-encounter with their antigen are able to rapidly enter germinal centres or differentiate into short- or long-lived plasma cells, this time without the requirement for co-stimulation from T cells.

When administered alone in aqueous solution, recombinant proteins are often poorly immunogenic. To elicit higher titres of antibody often requires that the protein be formulated with an adjuvant. The mode of action of many adjuvants is not well understood, but can include specific ligation of host pathogen-sensing receptors (e.g. activation of Toll-like receptor 4 by the endotoxin analogue monophosphoryl lipid A), non-specific activation of the inflammasome (e.g. aluminium-based salts [16, 17]), and formation of a 'depot' in the vaccinated host from which antigen is slowly released (e.g. incomplete Freund's adjuvant [18]). Selection of an appropriate adjuvant will be guided by a number of considerations: potency must often be balanced against reactogenic

side-effects; different adjuvants can favour qualitative biases in the antibody response; and some recombinant proteins may be unstable in certain adjuvant formulations.

1.2.1.2 Induction of cytotoxic T cells

Once an infection becomes established, cytotoxic $CD8^+$ T cells are indispensible for clearing intracellular pathogens [13]. In mammals, almost every cell type expresses MHC class I molecules on their surface (RBCs being a pertinent exception for malariologists). These MHC class I molecules are loaded with peptides generated by cleavage of the proteins expressed by the cell. Thus, the peptide-MHC class I repertoire found on the surface of a cell provides a sample of the repertoire of proteins found inside that cell, and the presence of foreign proteins inside a cell results in foreign peptides being presented on the cell surface. Via their TCR, $CD8^+$ T cells are able to sample the peptide-MHC class I complexes expressed on the surface of other cells, thereby indirectly sampling the repertoire of proteins found inside those cells. Because T cells that interact with peptide-MHC from self-proteins are deleted by central and peripheral tolerance mechanisms, in theory a TCR should only recognise a peptide-MHC complex if a foreign peptide is being presented. The presence of foreign peptide-MHC indicates the presence of foreign protein inside the cell, most likely because that cell is infected. In response to recognition of a peptide-MHC class I complex, and given appropriate conditions (e.g. the presence of antiviral cytokines), effector $CD8^+$ T cells are able to kill their target cells. This can occur through a number of mechanisms, including exposing the target cell to the FasL death ligand, thus inducing apoptosis, and through the release of perforin and granzyme proteins, which have direct killing activity.

The critical step to eliciting a cytotoxic $CD8^+$ T cell response is the presentation of antigen-derived peptide on MHC class I on the surface of dendritic cells. Dendritic cells are professional antigen-presenting cells who help initiate the cytotoxic cellular response by activating $CD8^+$ T cells that recognise peptide-MHC class I on the dendritic cell surface. Antigen-derived peptide can take one of two broadly-defined routes to be presented on the dendritic cell surface. The first route, 'direct presentation', is the same that can occur in any other cell type and is not specific to antigen-presenting cells:

antigen found in the cytoplasm of the dendritic cell may be cleaved into peptide and loaded onto MHC class I. This can occur when a dendritic cell is infected, or transfected with a protein-expressing RNA or DNA plasmid (see Chapter 6). The second route is known as 'cross-presentation', and can only occur in professional antigen-presenting cells such as dendritic cells. For this to occur, a dendritic cell must internalise antigen via the endocytotic pathway (for instance, soluble antigen found in the lymph fluid, or by engulfing an infected cell). By a largely uncharacterised mechanism, antigen is exported from the endosomal compartments into the cytosol, where it is cleaved, and then loaded onto MHC class I molecules and transported back to the cell surface. This second route is important in initiating responses to pathogens that do not readily infect antigen-presenting cells.

Cross-presentation of internalised, soluble antigen does not result in the efficient activation of cytotoxic $CD8^+$ T cells, so to induce a potent response, vaccines should deliver antigen directly to the cytoplasm of dendritic cells, to allow direct presentation to occur. This can be achieved by transfection with protein-expressing nucleic acids [11], but by far the most potent CTL responses in response to subunit vaccination arise following gene delivery by virus vectors [19–21].

1.3 Overview of malaria

There are an estimated 400-900 million cases of febrile malaria each year [22], accounting for at least 650,000 deaths annually [23], and potentially as many as 1.2 million [24]. More than 90% of these deaths occur in Africa [25] and 75% of these deaths are among African children [22]. The combination of mortality and morbidity associated with malaria is thought to be a major impediment to the economic development of some of the world's poorest regions [26, 27]. Five species of *Plasmodium* parasite are known to infect humans: *P. ovale*, *P. malariae*, *P. knowlesi*, *P. vivax* and *P. falciparum*. It is the last of these that causes the overwhelming majority of fatalities and is the subject of this thesis.

1.3.1 Life cycle

The life cycle of *P. falciparum* is complex and encompasses two host species: a vertebrate host, and a female insect vector of the genus *Anopheles* (Fig. 1.1). For *P. falciparum*, the most common vector is the mosquito *Anopheles gambiae*.

1.3.1.1 From the skin to the liver stage

This stage of the life cycle is difficult to study, because there is no straightforward way to culture parasites of this phase *in vitro*. Therefore, much has been inferred from studies of *P. berghei* and *P. yoelii*, species of *Plasmodium* that are infectious to laboratory rodents. As a female *A. gambiae* takes a blood meal from the human host, she injects approximately 15 malaria sporozoites into the skin [28]. Within half an hour, the highly motile sporozoites transport to the liver. Here they glide through a number of hepatocytes, repairing the breaches left in the hepatocyte membranes [29] as they transit from cell to cell, before eventually infecting the hepatocyte where they will remain for the next 5–7 days in the case of *P. falciparum* (for *P. berghei* and *P. yoelii*, the intra-hepatic stage lasts around 48 hours). During this period, the parasite multiples itself several thousand-fold inside a parasitophorous vacuole [30]. This vacuole forms a barrier between the multiplying parasite and the internal antigen-presenting machinery of the hepatocyte (see Section 1.2.1.2), possibly contributing to the observation that the intra-hepatic stage of the parasite life cycle is immunologically silent during natural infection [31]. It is only just prior to the release of parasites from the hepatocyte that the parasitophorous vacuole ruptures, allowing the contents of the vacuole and the host cell cytoplasm to mix [32, 33]. Parasites are then shuttled into the host bloodstream in merosomes. These are sacs derived from host hepatocyte lipids that are not recognised as foreign by innate immune cells such as dendritic cells, thus protecting the parasites from host immunity as they transition from the intra-hepatic stage to the blood stage [32].

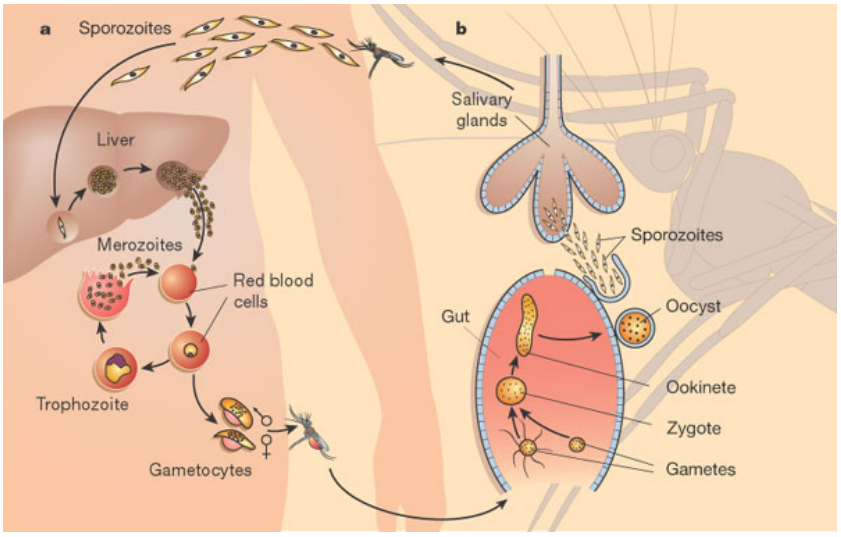

FIGURE 1.1: *P. falciparum* life cycle. Taken from [34].

1.3.1.2 The blood-stage: asexual amplification

The amplification of parasite biomass in host RBCs is the phase of infection that causes all of the symptoms of malaria disease [31]. Each infected hepatocyte releases thousands of merozoites into the bloodstream. Merozoites are cells of approximately 1 µm in length that are infectious to RBCs, meaning that just five infected hepatocytes can give rise to an initial liver-to-blood inoculum equivalent to tens of thousands of iRBCs. In the asexual growth pattern, after invasion into an RBC, *P. falciparum* parasites amplify around sixteen-fold over the next 40–48 hours (Fig. 1.2) although the multiplication factor and the duration are species-dependent. For the first 16 hours of intra-erythrocytic development, parasites are termed 'ring-stage', after their staining pattern when visualised microscopically with Giemsa stain. In the period 16–32 hours post-invasion the parasite becomes a trophozoite, before segmentation becomes apparent during the schizont phase (typically 32 hours post-invasion to rupture). After around 48 hours, the schizont-infected RBC bursts, releasing more merozoites into the blood plasma (or culture medium) and the asexual cycle begins anew, leading to exponential amplification.

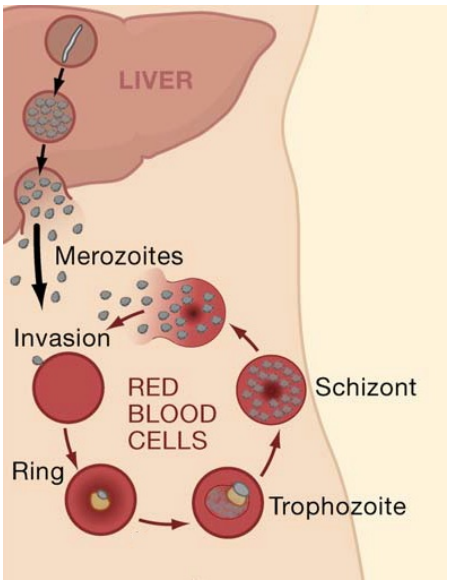

FIGURE 1.2: Blood-stage growth of *P. falciparum*. Taken from [35].

1.3.1.3 From the blood to the mosquito: the sexual stage

Alongside the asexual amplification of parasite biomass, a small fraction of the population develops into sexual gametocytes. Until recently, the signal for differentiation into the sexual forms was unknown. However, evidence appears to indicate a role for communication between iRBCs mediated by extracellular microvesicles [36, 37]. There are five microscopically distinguishable stages that a parasite must pass though on the path to becoming a mature gametocyte. When a female mosquito takes a blood meal containing a mature gametocyte, the change in pH and temperature acts as a signal for the gametocyte to shed the vestiges of its red cell membrane, becoming a gamete. The sex of the gametes is determined in the schizont before it embarks on gametocytogenesis, with a sex ratio of five females for every one male [38]. Whereas each female gametocyte gives rise to just on macrogamete, each male gametocyte produces 8 microgames [39]. Males gametes are distinguished by the presence of flagella, which allow them to move through the mosquito mid-gut and fuse with a female gamete. The resultant zygote is the first of the stages described here to have a diploid genome. This differentiates into an ookinete, which is able to form a hole in the wall of the mid-gut and form an oocyst. Over the next 6–10 days, the oocyst develops between the mid-gut wall and the basal lamina, with haploid sporozoites forming in the oocyst by mitosis. The oocyte

eventually ruptures and the free sporozoites migrate to the mosquito salivary glands, thus poised to infect a new vertebrate host at the next blood meal.

1.4 Malaria-control strategies

There is no vaccine that provides long-term protection against *P. falciparum* disease. However, the life cycle described above provides a number of opportunities for alternative control strategies that can disrupt transmission of the parasite in the insect host, and clear infection once it has established itself in humans.

1.4.1 The Global Malaria Eradication Programme (1955–1969)

In 1955, at the 8th World Health Assembly in Mexico City, the goal of global malaria elimination was established [40, 41]. This programme was remarkably successful given its almost total (and in retrospect naïve) reliance upon spraying of DDT. This was the first insecticide to have a residual effect, meaning that application was only required on an annual basis and thus permitting the elimination of insects from large rural areas. By the late 1940s and early 1950s, the successful use of DDT as an insecticide had generated optimism that malaria could be not just controlled, but eradicated by disrupting its mosquito-borne transmission [42]. This single-pronged approach was favoured because the Expert Committee viewed traditional methods, such as swamp draining to destroy mosquito habitats and physical prevention of bites through bed-nets, as out-dated and ineffectual [43]. The only second option that was approved by the Committee was mass administration of anti-malarial drugs (such as pyrimethamine) where spraying with DDT was impractical or not permitted by the local populace [40] .This campaign is often considered a failure, but this view does not do credit to the achievements of the programme. The geographical distribution of malaria was markedly reduced, although mainly in areas with well-functioning public health infrastructure. Localised eradication was also achieved, albeit only on islands or regions of sub-tropical climate [26]. However, in its central aim of global eradication, the campaign was unsuccessful.

1.4.2 Contemporary strategies to control malaria

By the late 1960s, the World Health Assembly had recognised that the Global Malaria Eradication Programme was unlikely to achieve its aims [43, 44], and by the mid-1970s, even modest goals of control had been virtually abandoned [43]. This led to malaria resurgences and a number of epidemics, with malaria mortality continuing to rise into the early 1990s [43, 45, 46]. However, even accounting for this spike in deaths between the 1970s and 1990s, the overall trend over the last century has been a reduction in malaria mortality [47]. There may be a number of factors contributing to this, including economic factors and climate change [47]. However, the sharp declines in mortality seen over the last 15 years across pockets of Asia, [48], East Africa [49, 50] and to a lesser extent, West Africa [51] are almost certainly attributable to more direct interventions. Calls for eradication have begun anew, and the Roll Back Malaria partnership has provided the funds and direction to guide more thorough malaria surveillance and the deployment of modern malaria control tools developed since the 1970s. The most important of these are the class of antimalarial drugs known as artemisinins [52, 53], and insecticide-treated bed nets [54, 55]. This has led some to question the necessity of developing a malaria vaccine [56]. Used in combination with indoor residual spraying of insecticides (in contrast to blanket coverage, as in Section 1.4.1), one mathematical model indicates that modern drugs and insecticide-treated bed nets have the potential to reduce the malaria prevalence rates to below 1% in low- and moderate-transmission settings [57]. However, the authors of this study state that this model assumes unrealistically high coverage levels [57]. Furthermore, even where models assume indefinitely sustained high levels of coverage, including twice-annual mass drug administration, in high-transmission areas parasite prevalence is not predicted to fall below 10% [57].

A concerning feature of current malaria-control programmes is that they tend to assume that non-vaccine malaria control measures will retain their current levels of efficacy and financial support [58]. However, history suggests that this is unlikely to be the case. There are precedents for mosquitoes becoming resistant to insecticides [59] or changing their feeding habits to lessen the impact of bed nets [60]. *P. falciparum* has developed resistance to antimalarial drugs such as chloroquine in the past [61, 62], and the evolution

of resistance to artemisinin-based therapies appears to in progess [63, 64]. Thus, the development of new tools to combat malaria is an essential component of malaria-control policy.

1.4.3 The utility of a vaccine

Continued vaccine research therefore remains vital. In my view, the strongest arguments for this come from modeling studies showing that substantial benefit could arise from the deployment of a vaccine that is only *partially* protective [65–67]. Typically, models assume that vaccine efficacy will be 50%, and the protective half-life will be 3 years. Prior to Phase III testing, this was the assumed profile of RTS,S, the subunit malaria vaccine in the most advanced stages of clinical development—it in fact fell somewhat short of this projection [68]. These models predict that vaccines may have variable impacts on mortality, morbidity and parasite prevalence, depending upon the intensity of transmission, deployment of non-vaccine control methods, the rate of immune decay and the access to healthcare provison [57, 65–67]. However, they generally agree that even the modest levels of efficacy seen from RTS,S could facilitate reductions in parasite prevalence if used in combination with other control strategies (having presumed 50% vaccine efficacy and a 3-year half-life). For example, one model found that mass vaccination with RTS,S could drive parasite prevalence below 1% in low-transmission areas, assuming a realistic level of insecticide-treated bed net usage. The role of RTS,S in malaria control would therefore be different to the 'magic bullet' vaccine envisaged 15 years ago, forming one arm of an integrated programme making use of indoor residual spraying, long-lived bed nets, destruction and draining of mosquito habitat and provision of artemisinin-based therapies [56].

These arguments illustrate the considerable utility of a vaccine of only modest efficacy such as RTS,S. However Griffin *et al.* also modelled the effect of a hypothetical vaccine with 95% efficacy and a protective half-life of 10 years (see supplemental protocol S5 in [57]). In this model, a single mass administration of such a vaccine would be sufficient to eliminate all malaria from low-transmission zones, and if used in combination with other methods could eliminate malaria from moderate-transmission zones and reduce

prevalence to below 5% in high-transmission zones. These studies illustrate the enormous public-health potential of a highly efficacious vaccine. Of note, the ability to prevent disease is not a necessary condition for a vaccine intended to be used as a tool to reduce malaria prevalence [56]. Recognition of this has led to development of vaccines that primarily aim to interrupt parasite transmission (transmission-blocking vaccines) from the infected human to the mosquito (see Section 1.6.2) [69], with the course of disease in the infected human a secondary consideration.

1.5 Naturally-acquired immunity (NAI) to malaria

All observable protection from malaria appears to arise from immunity against the blood-stage parasite [31]. Humans experiencing their first episode of blood-stage parasitaemia almost always become sick, initially experiencing symptoms including fever, fatigue and headaches that often progress to severe malaria. This is associated with respiratory distress, blackened urine and cerebral malaria, where parasites sequester in the blood vessels of the brain leading to coma. However, a patient that has survived his or her first malaria episode generally has a better prognosis upon re-infection [70], and as exposure is repeated, layers of immunity begin to develop [31]. The first level of immunity provides protection against severe disease. Among children living in higher-transmission areas, this typically develops in the first five years of life [70]. The next level of immunity is against mild disease, and tends to develop during the teenage years. This can provide protection against all clinical symptoms, whilst maintaining blood-stage infection. The extent to which asymptomic parasite carriage constitutes a transmissible reservoir in the community is not known, and the proportion of individuals classified as parasitaemic can vary widely depending upon the sensitivity of the detection method [71]. The final degree of immunity, termed sterilising immunity, confers the ability to completely clear parasites from the blood. This hierarchical arrangement has led some to suggest that there may be distinct mechanisms that confer immunity to severe disease, mild disease and asymptomatic infection. It should be noted that the immune situation is fluid, and a patient able to carry parasite asymptomatically for months may suffer an occasional febrile episode. Further, the distinction between sterilising immunity and an ability to

control parasitaemia without symptoms is difficult to establish because even the most sensitive diagnostic tests may miss a very low density sub-patent (i.e. non-microscopically detectable) blood-stage infection. Immunity does not appear to be permanent: it is commonly thought that after one or more years away from a malaria-endemic area, a previously immune individual will become susceptible to reinfection.

Immunity against the pre-erythrocytic stages of the life cycle probably has only a minor role in natural protection. Studies of hyperimmune adults tend to show rapid re-establishment of blood-stage infection after drug clearance, suggesting functionally non-existent liver-stage immunity [72]. Moreover, work in the 1930s on asymptomatically infected humans found that re-exposure (via mosquito bite) to the homologous parasite strain was followed by a spike in blood-stage parasitaemia around one week later [73], indicating unhindered liver-to-blood inoculation. That the breakthrough infections reported in both studies were asymptomatic indicates that protection against clinical disease is occurring at the blood stage [72, 73].

1.5.1 Innate immmunity

In malaria, the innate immune response has two functions: first, the detection of an infection in order to stimulate an adaptive immune response; and second, a degree of direct parasite killing. In the former category, for example, parasite-derived haemozoin can be indirectly detected by TLR9 through its ability to present parasite DNA [74], and possibly through direct interaction with TLR9 itself [75]. In addition to the TLRs, a number of other innate pathogen-sensing receptors have been implicated in disease outcome through associative genetic studies [76]. In the latter category, macrophages and dendritic cells are phagocytes that have an essential role as antigen presenting cells, vital to the induction of $CD4^+$ and $CD8^+$ T cell responses. However, phagocytosis by macrophages has also been ascribed a role in parasite clearance in the absence of parasite-specific antibody, probably due to an interaction between CD36 on the macrophage and the EMP1 family of proteins found on the surface of iRBCs (see Section 1.5.4) [77, 78]. Neutrophils, specialised phagocytes of a different lineage to macrophages, have also been implicated in the early stages of blood stage immunity to *P. berghei* in rats

[79]. This may be important in controlling parasitaemia shortly after the liver-to-blood inoculation, where the parasitaemia is well below 0.1%. Components of the innate immune system may also potentiate elements of the adaptive immune response. For example, the presence of antigen-specific antibody on gametocytes can direct their lysis by complement [80], and opsonisation (i.e. coating) of microbes with antigen-specific antibody leads to enhanced phagocytosis by macrophages and neutrophils.

1.5.2 Adaptive cellular immunity: $CD4^+$ and $CD8^+$ T cells

Since infected hepatocytes are capable of presenting antigen on MHC class I complexes, they are theoretically a target of $CD8^+$ T cells. However, as discussed in Section 1.5, pre-erythrocytic immunity appears to play only a minor role in naturally-acquired immunity. RBCs are one of the few populations of cells not to express MHC class I on their surface, meaning that $CD8^+$ T cells cannot play a direct role in clearance of blood-stage parasites. Indeed, mice deficient in MHC class I, and thus exhibiting a 40-fold reduction in $CD8^+$ T cell numbers, can resolve infection with a range of rodent *Plasmodia* [81]. Furthermore, the passive transfer of $CD8^+$ T cells from mice immune to *P. yoelii* to naïve mice does not confer any degree of protection [82]. Thus $CD8^+$ cells are unlikely to play any direct role in NAI.

The role of $CD4^+$ T cells in NAI is also not clear. Mice deficient in B cells (and therefore antibodies) are capable of developing immunity to *P. chabaudi adami*, which is considered a model of chronic malaria infection, but not *P. berghei* or *P. yoelii* [83]. Vaccine-induced protection from *P. chabaudi adami* in mice can be reversed by depletion of $CD4^+$ cells, and conferred onto naïve mice by passive transfer of $CD4^+$ T cells from vaccinated mice [84]. Thus, $CD4^+$ T cells can have a protective role in some species of rodent malaria. However, the evidence for a similar role against *P. falciparum* disease is weaker. A virally vectored genetic vaccine that elicited unprecedentedly high levels of antigen-specific $CD4^+$ T cells failed to protect naïve human subjects in a controlled human malaria infection delivered by mosquito bite [85]. The only convincing evidence for cell-mediated immunity to blood-stage infection in humans came from a trial where humans were administered ultra-low doses of iRBCs (around 30 per dose) and then cured

with atovaquone and proguanil 8 days later [86]. After three rounds of infection and drug-cure, patients were challenged with an equivalent dose of iRBCs and then observed without drug-treatment. In the absence of detectable parasite-reactive antibodies, all of the patients were protected from the fourth challenge. However, this study appears to have been compromised by the unexpectedly long half-life of atovaquone, which was probably still present in the plasma of volunteers at the time of the fourth challenge [87].

Altogether, there is limited evidence of a direct role for effector $CD4^+$ and $CD8^+$ T cells in protection against *P. falciparum* malaria. This does not rule out the indirect role played by $CD4^+$ helper T cells in the development of the humoral immune response. For example, the importance of a functioning $CD4^+$ T cell–B cell axis in mice for protection against *P. yoelii* and *P. chabaudi chabaudi* has been recently highlighted [88].

1.5.3 Humoral immunity

In 1960, Cohen *et al.* published a landmark article demonstrating that purified IgG from the serum of hyperimmune adults is sufficient to control acute episodes of human malaria [89]. In this remarkable study, Gambian children aged 4–30 months presenting with disease were either left untreated altogether, administered with IgG from hyperimmune Gambian adults, administered with IgG-depleted serum from hyperimmune adults, or administered with IgG from UK adults. The children that received IgG from hyperimmune adults essentially made a full and immediate recovery from both parasitaemia and clinical symptoms (Fig. 1.3). In the presence of hyperimmune immunoglobulin trophozoites declined in number, while gametocytes continued to rise [89]. This suggests that protective immunity is directed against the asexual blood-stages only.

These results were confirmed in similar studies, including one that used IgG purified from West African donors to treat infections in East Africa [90], and another using IgG from West African donors to treat Thai patients [91]. These demonstrated that hyperimmune IgG is capable of protecting against parasites that are phylogenetically distant from the immunising strains. While these studies do not demonstrate conclusively that

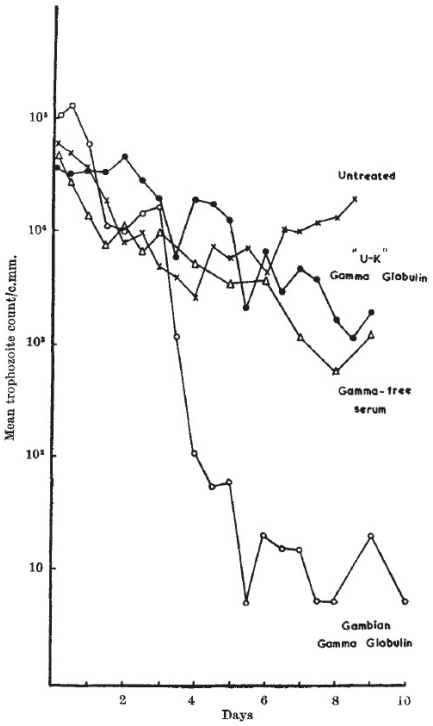

FIGURE 1.3: IgG purified from the serum of adults immune to malaria can reverse clinical malaria. Children in the Gambia presenting with clinical malaria were either left untreated, or administered with one of the following: IgG purified from the serum of hyperimmune individuals; IgG purified from malara-naïve UK adults; serum from hyperimmune adults depleted of IgG. Children receiving IgG purified from the serum of hyperimmune individuals rapidly cleared parasitaemia and recovered clinically. Taken from [89].

parasite-reactive IgG was necessary for protection (i.e. there may be other mechanisms of protection beside that provided by antibodies), it did prove that anti-parasite IgG can be sufficient to achieve protection against malaria disease[1]. Over the last 50 years, much effort has gone into dissecting the mechanisms of this protective humoral immunity.

1.5.4 RBC surface proteins

The blood-stage parasite spends the majority of its life inside RBCs, somewhat protected from immune surveillance. However, as intra-erythrocytic growth progresses, the parasite causes the expression of a suite of proteins on the iRBC surface, many

[1]Of note, passive transfer rarely resulted in complete elimination of parasites from the blood [92], leaving open the possibility that other mechanisms (such as $CD4^+$ cells) are necessary for sterile protection.

of which have a role in adherence of the iRBC to the endothelia of blood vessels (sequestration), or attachment of uninfected RBCs around an iRBC (rosetting). Adhesion to vascular endothelia promotes parasite survival by reducing shear forces, providing a more constant environment with respect to O_2 and CO_2 partial pressures, and avoiding passage through the spleen. The iRBC protein family that is largely responsible for cytoadherence, EMP1 (erythrocyte membrane protein), is a major virulence factor of *P. falciparum* [93]. It is encoded by the *var* family of genes, of which each parasite carries around 60 different copies. Each iRBC expresses just one var gene allele at a time, but is able to change alleles over time, meaning that as an immune response develops to one EMP1 isoform, another isoform can take its place. Compared to other children in their community, children infected with *P. falciparum* are less likely to have pre-existing antibodies recognising the EMP1 isoform expressed by the infecting strain [94]. This suggests selective immune pressure against EMP1 isoforms, indicating that EMP1 is a major target of strain-specific immunity.

The variant of EMP1 expressed by the infecting parasite can markedly influence the course of clinical disease. A subset of EMP1 variants that binds with high affinity to chrondroitin sulfate is observed almost exclusively during infections of women having their first pregnancy, and the resultant cytoadherence within the placenta is the cause of placental malaria [95]. The presence of antibodies that can inhibit this interaction are predictive of improved natal outcomes [96, 97]. Recently, it was reported that severe malaria is associated with the expression of EMP1 variants that contain a particular combination of binding cassettes that allow adherence to EPCR [98, 99]. That specific host-pathogen molecular interactions can cause particular syndromes is highly consistent with the observation that patients gradually become immune to different clinical manifestations, and immediately suggests an immune mechanism whereby antibodies directed to the specific EMP1 variants either disrupt the relevant host-pathogen interactions or lead to the phagocytosis of the parasites expressing those variants [100].

1.5.5 Anti-merozoite antibodies

The merozoite is the only form of the asexual blood-stage parasite that is completely exposed to the extracellular environment and thus vulnerable to attack by the immune system. This vulnerability is somewhat mitigated by the fact that the entire process of RBC egress and invasion takes less than a minute when observed *in vitro*, and possibly occurs even faster under the conditions of high haematocrit and agitation seen *in vivo* [101]. In order to achieve this rapid invasion, the merozoite must be highly dynamic, and during the course of invasion a number of different antigenic targets are exposed in a manner that is temporally well coordinated [35, 102–104] (the process of merozoite invasion into RBCs is discussed in detail in Section 1.7.1).

While EMP1 variants on the RBC surface are an established target of NAI, the contribution of anti-merozoite antibody is less clear. During the historic passive transfer studies conducted in the 1960s, it was observed that intramuscular administration of hyperimmune antibodies had little effect upon parasitaemia until schizonts ruptured and a new generation of ring-stages was formed [92]. It was inferred that antigens present on the late schizont or merozoite were the major targets of the passively transferred IgG [92]. This effect was not observed in a later study where antibody was administered intravenously [91]. It may be that the delay observed in the earlier studies was an artefact of the slower pharmacokinetics of IgG administered intramuscularly. On the other hand, it would be surprising if the strain-transcending benefit of passively transferred IgG [91, 92] arose solely by binding to hyper-variable proteins found on the iRBC. Strain-transcending blood-stage protection presumably involves antibody responses against comparatively invariant antigens, such as those found on the merozoite.

Elucidation of the role of naturally-acquired antibody against iRBC surface proteins is somewhat faciliated by the ease of studying iRBCs *ex vivo*. Although viable merozoites can be isolated, the process is technically demanding and the resultant merozoites are only viable for a matter of minutes [103, 105]. Crucially, the protocol requires large amounts of synchronised asexual culture. For this reason, the field tends to characterise antibody responses to merozoite antigens by measuring their activity against parasites

cultured *in vitro*, rather than against *ex vivo* isolates. The performance of naturally-acquired antibodies in these anti-merozoite assays often correlates (observationally) with protection against malaria in the endemic setting, and could be considered evidence that anti-merozoite antibodies contribute to NAI. On the other hand, it is a fallacy to assume that since an antibody sample has activity in an assay on a laboratory bench, a corresponding mechanism must contribute to *in vivo* protection. *In vitro* assays generally measure just one aspect of the overall functionality of an antibody sample [106], and positive readout assay generally associates with the degree of host exposure to malaria. It is therefore difficult to determine whether the activity measured in assays is simply colinear with the true mechanism of protective immunity. The most prominent anti-merozoite assays used by the field, and the *in vivo* activities they are thought to reflect, are considered below.

1.5.5.1 Opsonisation assays

Opsonisation is the process where antigen-specific antibody binds to a microorganism, marking it as foreign so that innate phagocytotic cells can recognise and engulf it. Subclasses of human that IgG efficiently opsonise pathogens are termed 'cytophilic' and include IgG1 and IgG3. Assays to measure to opsonisation of both RBCs and merozoites have been described, but only the latter are considered here. The ability of an antibody sample to opsonise merozoites *in vitro* and induce FcR-dependent phagocytosis by monocytes and neutrophils [107], or FcR-dependent release of destructive ROS from neutrophils in the assay of ADRB [108], appears to be strongly associated with protection from clinical malaria. Aside from IgG subclass, the attributes of an antibody sample that impart efficient opsonisation properties are somewhat ill-defined. It is not known, for example, if some antigens are more prone to induce functional opsonisation than others. Given that the phagocytotic cell does not directly 'see' parasite antigen, but rather the Fc regions of the parasite-bound antibody, it is tempting to speculate that the molecular identity of the antigen is of less relevance than localisation (presumably on the merozoite surface is preferable) and a high surface-density (known to be important in Fc-dependent activation of innate cells [109]). If is the case, the prospect of designing a

vaccine with good opsonising qualities seems remote: against the mouse malaria parasite *P. yoelii*, vaccination with MSP1, the most abundant protein on the surface of the merozoite, was not able to elicit antibodies with ADRB activity against a mixture of lysed merozoites and iRBCs, whereas blood-stage infection of mice with *P. yoelii* was sufficient to elicit antibodies with strong ADRB against the same mixture (although much of the ADRB activity following infection may have been against iRBC surface proteins) [110]. The major conceptual arguments against phagocytosis of merozoites playing an important role in protection against the blood-state are the relative lack of abundance of the phagocytotic cells themselves in blood and the extremely rapid rate of merozoite invasion (on the order of seconds): neutrophils, the most abundant phagocytes in blood, are outnumbered 1000:1 by RBCs, and it is difficult to understand how a neutrophil could locate and engulf a merozoite before the merozoite successfully invades.

1.5.5.2 Assays of GIA

The assay of GIA involves culturing asexual blood-stage parasites in the presence of anti-parasitic antibodies, and then measuring the resultant parasite growth [111]. When performed with purified IgG, only the immune cell-independent effects of the antibodies are measured, which is to say neutralisation, inhibition of function and processing, agglutination or possibly cross-linkage of parasite proteins (as opposed to ADRB and phagocytosis, for example). It is widely presumed that the antibodies contributing to the observed GIA are directed against proteins found on the merozoite. Certainly, human antibodies specific to AMA1 (apical membrane antigen 1, a protein found on merozoites and sporozoites) that are elicited during natural infection are capable of neutralising parasites *in vitro* [112–114]. This said, merozoite proteins are not the only antigenic targets of growth-inhibitory antibody. There are reports of proteins involved in the process of RBC egress being susceptible to neutralising antibody acquired as a result of natural infection, such as the recently described protein SEA1 (schizont egress antigen 1) [115]. More surprisingly, it has also been found that when added to ring-stage parasites, heat-inactivated (i.e. complement-deficient) serum from immune monkeys and humans

can inhibit the progress of intra-erythrocytic growth in the absence of any cellular effector [116, 117].

The extent to which immune cell-independent growth inhibition contributes to protection, and whether *in vitro* GIA can predict vaccine efficacy, are not clear [106]. As mentioned above, antibodies against AMA1 that are elicited during natural infection have *in vitro* GIA, and the AMA1 gene also appears to be under balancing selection [118, 119], presumably reflecting immune pressure on this locus. On the other hand, AMA1 vaccines capable of eliciting antibodies with potent *in vitro* neutralisation activity [114] have shown little effect on the *in vivo* parasite multiplication rate in Phase IIa blood-stage challenge trials [120, 121]. In fact, the clinical trials that have shown some evidence of reduced *in vivo* blood-stage parasite multiplication rates after vaccination have eiher been negative for *in vitro* GIA [122], or not reported any measurement of it [123, 124]. On the other hand, it has long been known that antibodies isolated from both immune monkeys [111] and humans [112] can inhibit the growth of parasites in this assay. In the field, level of GIA is associated with decreased risk of infection and does *not* appear to increase with age [117, 125–127], suggesting the GIA is not a simple marker of cumulative malaria exposure and has independent protective benefit.

By studying malaria-naïve individuals that have been vaccinated to elicit growth-inhibitory antibody, it should in principle be possible to delineate the mathematical relationship between *in vitro* GIA and the course of disease *in vivo*. A recent study performed by our group attempted to do this with *Aotus nancymaae* monkeys infected with the FVO strain. After vaccination with RH5 (see Section 1.7.3), we found that when the total IgG concentration in plasma was greater than five times the concentration of total IgG from the same monkey needed to give 50% GIA *in vitro*, self-cure was observed (Douglas *et al.* in press). There is no conceptual reason why a similar relationship should not exist between *in vitro* GIA with human IgG, and protection from malaria. It cannot yet be defined, because no anti-merozoite vaccine has yet shown a convincing decrease in parasite multiplication rate in humans, and it is impossible to dissect out the contribution of GIA from all of the other anti-parasitic activities of naturally-acquired antibodies. The best GIA-inducing vaccine to be tested in humans was able to elicit

total IgG with 63% *in vitro* GIA at 10 $\mathrm{mg\,mL^{-1}}$ (i.e. physiological concentration), but failed to show any efficacy [120]. However, the *Aotus* result implies that had 63% *in vitro* GIA been observed at 2 $\mathrm{mg\,mL^{-1}}$, some impact on parasite growth rate may have been observed.

Neutralising antibody differs from opsonising antibody or cellular immunity in that its general mechanism of action is to interfere with specific molecular interactions important for parasite survival (see Section 5.1.2, and [128–132]). The function of the antigenic protein, and the epitopes targeted by the immune response, are therefore highly relevant. Of note, the concentrations of merozoite-specific antibody required to achieve significant *in vitro* GIA appear to be extremely high—antibody concentrations required to give total growth inhibition *in vitro* are typically equivalent to those found in the serum of animals at the peak of a vaccine-induced immune response [133–135]. The antigen-specific EC50[2] for rabbit IgG raised against full-length RH5 is 55–114 $\mathrm{\mu g\,mL^{-1}}$, and against AMA1 is 70–156 $\mathrm{\mu g\,mL^{-1}}$ (both IgG samples raised using virus vectors) [136]. The requirement for extremely high concentrations of antibody is likely due to the kinetic constraint imposed by the very short period during which merozoites are exposed to the extracellular milieu [137]. These observations have clear implications for vaccinologists attempting to induce protective immunity through this mechanism.

1.5.5.3 The assay of ADCI

The assay of ACDI measures a mixture of neutralisation and opsonisation, in that it essentially constitutes a GIA assay performed in the presence of monocytes [138]. The addition of monocytes is reported to result in enhanced sensitivity, so that antibodies from malaria-exposed donors that achieve only moderate growth inhibition when applied alone to asexual cultures yield potent growth inhibition when monocytes are present [138]. It is uncertain whether sensitivity above that seen in the GIA assay is a relevant predictor of protection, given that the GIA assay already appears to be insufficiently stringent: for example, vaccines capable of eliciting rabbit antibodies with potent *in vitro* GIA [139] failed to elicit a protective immune response in humans [85]. However, the

[2] i.e. the concentration required to give 50% *in vitro* GIA

presence of monocytes may well allow the detection of additional protective mechanisms that are immune cell-dependent. For example, SERA5 is an antigen thought to exert its protective effect at least in part via ADCI [140, 141] and a vaccine containing a fragment of SERA5 appeared to show some level of protection in a *post-hoc* analysis of a Phase Ib clinical trial [142]. It should be noted that the precise role of the monocytes in inhibition is not known, but is purported to encompass phagocytosis as well as other undefined mechanisms [143]. The assay of ADCI has not been widely adopted, mainly due to technical problems establishing the assay. In particular, monocytes from different donors are not consistent in their activity, contributing to wide variability in assay readout. As with phagocytosis assays, it is also unclear why only certain antigens are reportedly susceptible to ADCI—in theory the only relevant determinants of ADCI induction should be the class of IgG involved, the localisation of the antigen, and its surface density (see Section 1.5.5.1).

1.5.5.4 Merozoite protein ELISAs

Protection from clinical malaria appears to correlate with serum reactivity to recombinant merozoite proteins, and this is often considered evidence that the merozoite is a target of protective immunity (recently summarised in [144]). Antibody responses to some antigens correlate more strongly with protection than others, presumably reflecting different levels of involvement in protection from disease and susceptibility to antibody. Although there are at least 100 proteins predicted to be localised to the surface of the merozoite [145], these studies typically focus on a narrow panel of antigens that were historically identified due to their immunodominance. However, immunodominance by no means reflects parasitological importance or susceptibility to immune attack. Antigens typically ascribed a protective role include MSP1, AMA1, MSP2 and MSP3 [144, 146], although the correlations are generally weak. This does not mean that these proteins are not targets of NAI, but probably reflects the fact that NAI arises cumulatively from responses to a large number of different blood-stage antigens expressed on the merozoite and elsewhere, with responses against individual proteins dispensable for protection. In a 2013 paper, Murungi *et al.* finally explored whether threshold

antibody concentrations to specific antigens were predictive of protection [147]. This advanced a field that has often relied on a simplistic postive/negative readout for antigen-specific antibody.

The focus on a narrow range of target proteins has arisen partially due to the difficulty in producing recombinant protein antigens, which are necessary for the detection of antibody in ELISAs and other types of assay (discussed in Section 3.1.1). A recent study reported the measurement of antibody titres to 39 full-length merozoite proteins [148], representing a considerable improvement upon previous efforts. This study found that whilst responses to a single antigenic target did not appear to reliably predict protection from malaria, responses against a combination of any 5 of the top 10 most protective antigens was strongly predictive—although as with every other metric described in this Section, this may be a simple marker of exposure to malaria. However, it does lend weight to the idea that naturally-acquired immunity is unlikely to be a product of a single antibody specificity acting exerting its anti-parasitic effect through a single mechanism.

1.5.6 Lessons for vaccinologists from NAI

To conclude this Section on NAI, it appears that humoral immunity is the major (or at least a sufficient) contributor to protection, but this does not rule out minor roles for innate immunity and cellular effectors. Protection appears to require that antibody responses are both of high magnitude, and against a broad range of targets. That no single assay of antibody activity is fully predictive of NAI reflects the fact that antibodies can contribute to immunity through a variety of mechanisms, any one of which may be partially dispensable for overall protection. Dissecting out just one aspect of the antibody response to natural infection is therefore challenging and does not reflect the fact that immunity probably arises through multiple mechanisms. This said, the development of immunity to specific disease manifestations is consistent with the hypothesis that protection from disease arises primarily from the slow accumulation of antibodies against EMP1 variants. Unfortunately the variability seen across the *var* gene family makes it unlikely that sufficient humoral breadth could be imparted with a subunit vaccine (although it may be possible to provide protection against syndromes

arising from specific host-pathogen molecular interactions, such as placental malaria [149]). Merozoites represent a less variable target than the iRBC surface, but the extent to which antibodies against merozoites contribute to NAI is uncertain. The occurrence of disease manifestations attributable to specific EMP1 variants (such as placental malaria), despite the presence of anti-merozoite responses, argues that these responses do not contribute heavily to protection. The rapid invasion of merozoites into RBCs necessitates extremely high concentrations of neutralising antibody to achieve measurable *in vitro* GIA, at least against the antigens that are immunodominant in natural infection. When assessing vaccine efficacy, it is important to recognise that there are degrees of protection against malaria, with numerous intermediate levels between total vulnerability and sterile clearance. This means that a vaccine that has potential to be highly effective in field studies (e.g. Phase IIb or Phase III clinical trials) might show no detectable efficacy in a Phase IIa trial where patients are immediately treated upon microscopic detection of blood-stage parasites [85].

1.6 Development of malaria vaccines

Although the asexual blood stage seems to be the major target of NAI, the *P. falciparum* life cycle presents numerous opportunities for disruption by immune attack. There are vaccines currently in development that target every stage of the parasite life cycle (see Section 1.3.1.1), and the achievements and prospects for these are summarised in this section.

1.6.1 Pre-erythrocytic vaccines

Conceptually, the sporozoite and the infected hepatocyte are appealing vaccine targets. In contrast to the merozoite, which is visible to the immune system for only a matter of seconds as it transits to a new RBC, the sporozoite must transit from the skin to the liver and is vulnerable to immune attack during this entire period. Likewise, the infected hepatocyte is visible to the cellular immune system for around a week. It seems

plausible that the weak immune responses against the pre-erythrocytic stages during natural exposure are a product of the very small number of foreign cells involved: only around 15 sporozoites are injected by the mosquito, and only a few infected hepatocytes are required for progression to the blood-stage. The tolerising environment of the skin, the site via which the infection is delivered, has also been implicated [150].

Improving the immune responses to the infected hepatocyte has yielded the most impressive efficacy yet seen against human infection. The delivery of whole sporozoites attenuated by irradiation [151–153], or viable sporozoites under the protection of prophylactic chloroquine [154] have both achieved 100% protection rates against subsequent challenge by infectious mosquito bite, probably through the induction of $CD8^+$ cells that recognise infected hepatocytes. Neither of these strategies represents a practical approach to vaccinating a population in a tropical, resource-constrained setting [155], but they do provide proof-of-concept data supporting the development of a vaccine targeting the intra-hepatic parasite. The most advanced subunit vaccine that attempts to do this is virus-vectored ME-TRAP. This consists of thrombospondin-related adhesion protein (TRAP) fused to an N-terminal string of epitopes (multi-epitope or ME) [156], delivered by a virus vector platform designed to effectively induce $CD8^+$ CTLs [157, 158]. At 21% sterile protection [19] this vaccine is considerably less efficacious than the whole sporozoite immunisation strategies. It is not clear whether the difference between the response to the ME-TRAP vaccine compared to whole sporozoite immunisation is quantitative (i.e. the number of CTLs that can recognise antigens presented on the infected hepatocyte surface) or qualitative (i.e. the affinity of the TCRs, the breadth of different proteins recognised by the immune response, the effector functions of the cells elicited). TRAP took a somewhat tortuous route to become a candidate vaccine antigen. It was initially discovered due to its sequence homology to circumsporozoite protein [159]. Around the same time, a *P. yoelii* protein called sporozoite surface protein 2 was shown to provide sterile protection against challenge when formulated as a vaccine in combination with *P. yoelii* circumsporozoite protein [160]. Eventually it was realised that TRAP is the *P. falciparum* orthologue of sporozoite surface protein 2, and that humans immunised with large numbers of irradiated sporozoites mounted relatively strong immune responses against it [161].

The sporozoite itself is the target of the most clinically advanced and efficacious malaria subunit vaccine, RTS,S, under development by GlaxoSmithKline [162]. This protein is based upon the hepatitis B surface antigen, which spontaneously particularises into multimeric particles. A set of repeating B and T cell epitopes from circumsporozoite protein has been fused onto the N-terminus of the hepatitis B surface antigen, and when this fusion protein is expressed with the unfused hepatitis B surface antigen in yeast cells, the result is a particle displaying circumsporozoite protein epitopes on its surface. The organised array of antigen that this comprises, in combination with a potent system of adjuvants, results in exceptionally high anti-circumsporozoite protein antibody titres [162]. These antibodies appear to be the main effector contributing to the moderate efficacy of RTS,S [163]. Vaccination with RTS,S generally results in a sterile protection rate of 30–40% in malaria-naïve subjects [164], and a disease-protection rate of 30–40% in malaria-exposed populations [68]. Extremely high antibody titres against the circumsporozoite protein epitopes contained in RTS,S seem to be required for protection against disease. This probably explains the rapidly waning efficacy that is observed in the field using this form of vaccine delivery [68], as the necessary titres of antibody are only maintained for a short period. Circumsporozoite protein was first identified by analysing the antibody response in mice repeatedly exposed to the bites of irradiated mosquitoes infected with *P. berghei* [165] and the human orthologue was cloned shortly after [166].

It seems unlikely that any adjuvant system will prove capable of improving upon and then maintaining the level of antibody titres induced by the most recent formulations of RTS,S. Future efforts to improve upon this vaccine should therefore focus on improving the antibody quality—either by targeting more susceptible antigens than circumsporozoite protein, or by focusing the antibody response onto more susceptible regions of circumsporozoite protein than the NANP repeats currently targeted. Other pre-erythrocytic antigens under consideration include liver stage antigen 1 (LSA1) [167] and the cell-traversal protein for ookinetes and sporozoites (CelTOS) [168]. However, there is a dearth of well-defined antigenic targets for inclusion in a pre-erythrocytic vaccine. This is largely due to the absence of any straightforward *in vitro* culture model for this stage. Laboratory-reared mosquitoes must be fed on blood-stage cultures

manipulated to produce sexual forms, a procedure that is often problematic [169]. After an appropriate incubation period sporozoites can be dissected from the mosquito salivary gland, a laborious task. Finally, these can then be used to infect cultured human hepatocyte cells. Thus, the identification of sporozoite proteins that are vulnerable to neutralising antibody, or those proteins that are well presented by the antigen presenting machinery of the infected hepatocyte, is extremely challenging. Furthermore, once identified, the pre-clinical testing of vaccines containing these proteins often necessitates the production of transgenic rodent *Plasmodium* sporozoites engineered to express the pre-erythrocytic antigen of interest [170, 171]. These are time-consuming to produce and offer only an indirect prediction of efficacy against human malaria.

1.6.2 Transmission-blocking vaccines

Transmission-blocking vaccines aim to prevent the productive infection of a mosquito in the first place by the induction of antibodies against sexual stages of the parasite. As with pre-erythrocytic vaccines, proof-of-concept data have come from whole-microorganism immunisation [172–174]. However, since then only Pfs25 and Pvs25, orthologous versions of the same protein, have been progressed to clinical trials [175, 176]. Excitingly, both trials elicited antibodies that, when mixed with sexual-stage cultures, could block infection of the mosquito. Pfs25 was identified by its reactivity to monoclonal antibodies that had been elicited through whole-parasite immunisation [174, 177], as were Pfs230 [178] and Pfs48/45 [179], two other sexual-stage antigens undergoing assessment as transmission-blocking vaccine antigens.

1.6.3 Blood-stage vaccines

An efficacious but non-sterilising vaccine targeting the blood stage should allow mild exposure to asexual-stage antigens in a manner that does not cause disease. It would therefore allow naturally immunity to develop and compensate for waning of the vaccine-induced response. This constitutes a considerable advantage over a pre-erythrocytic

vaccine that would leave patients essentially malaria-naïve until their first breakthrough infection.

None of the antigens named in the previous two sections are sufficiently immunogenic during natural infection to be considered targets of protective NAI. Nonetheless, with the exception of TRAP, they were all identified due to their immunogenicity under artificial experimental conditions (e.g. direct venous injection with purified gametocytes). The transmission-blocking and pre-erythrocytic fields have therefore been able to follow a strategy of enhancing the naturally weak responses to these antigens with some clinical success. The asexual stage presents a different situation. The antigens expressed in this stage are extremely immunogenic. This is probably a result of their abundance during infection— in an adult human, 1% parasitaemia represents 2–3 $\times$ 10^{11} total iRBCs with a mass of more than 20 g. AMA1, MSP1 and MSP3 are proteins that were initially discovered due to their immunogenicity during during whole-parasite immunisation with purified, non-viable merozoites [180–182] or active infection [183–186]. There is no question that antibodies against these proteins can affect parasite growth *in vitro* and in murine models of malaria, but direct evidence that vaccine-induced antibodies against these proteins can lead to human immunity to *P. falciparum* is lacking. Clinical trials for AMA1 vaccines that have elicited high concentrations of antibodies (100-300 $\mu g\,mL^{-1}$) that are functional *in vitro* have shown little effect on malaria infection [120, 187]. Likewise, a virus vector platform that elicited protective immunity in mice when used to deliver the *P. chabaudi chabaudi* orthologue of AMA1 [84] showed no effect in humans against controlled malaria infection when used to deliver the *P. falciparum* form of AMA1 [85, 139]. The most encouraging result came from *post hoc* analysis of a field trial, which appeared to show a reduction in the number of cases of malaria carrying the vaccine-homologous AMA1 allele [187]. However this was a secondary endpoint and requires proper confirmation. Similarly, vaccines containing MSP1 that have shown efficacy in pre-clinical studies [157] or elicited functional antibody responses in Phase I clinical trials [188] have gone on to show no efficacy in Phase II efficacy studies [85, 189]. A *post hoc* review of a Phase Ib clinical trial of a vaccine encoding MSP3 appeared to show some efficacy [190, 191], but as with the AMA1 trial result, this requires confirmation in a properly powered Phase II trial. Even if these vaccines did

show efficacy in challenge trials against the vaccine-homologous strain, these antigens are sufficiently polymorphic that immunity would likely be strain-specific [113, 192–194].

1.6.4 Mimicking NAI with a vaccine

The major conclusion from the discussion of NAI (Section 1.5.3) is that the natural humoral response owes its protective effect to its breadth and its magnitude. Whilst advances in delivery platforms, including adjuvants and virus vectors, mean the subunit vaccines are able to match NAI in terms of antibody concentration against a single protein [195, 196], the documented phenomenon of 'immune interference' makes it difficult to achieve the same level of breadth as is seen in natural infection [85, 197, 198]. Targeting a single blood-stage antigen that has been identified as a target of natural immunity is thus unlikely to yield success—indeed, it is tempting to speculate that the parasite has had time to evolve mechanisms by which it can evade immune responses directed to these antigens (such as polymorphism). By contrast, the pre-erythrocytic protection observed after immunisation with whole-sporozoites, and to a lesser extent with ME-TRAP and RTS,S, suggests that high levels of efficacy can be achieved *without emulating the mechanism of naturally-acquired protection*. By analogy, a viable route to immunity may be to target an asexual-stage protein that is not substantially immunogenic during natural infection, but whose function is essential to parasite survival and which can be blocked by vaccine-induced antibody.

1.7 Parasitology, RBC invasion and the discovery of PfRH5

Vaccine design can be simplistically reduced to two aspects: first, the target of the immune response (i.e. the antigen), and second, the quality of the immune response (generally determined by the delivery platform). There is an interaction between these elements. For example, an antigen expressed during the intra-hepatic stage of parasite growth would be most appropriately targeted by a vaccine that elicits cytotoxic T cells. However, these aspects do stand independently to an extent: a vaccine designed to elicit

antibodies that neutralise sporozoites should contain an immunogen optimised to direct humoral immunity to the most neutralising epitopes of the most vulnerable proteins on the sporozoite, and to an extent any adjuvant that elicits high concentrations of antibodies to those epitopes will suffice (at least in the design phase). The last 20 years of malaria vaccine development have seen huge strides in the development of platforms to elicit very high titres of antibodies [162] and T cells [199]. By contrast the selection of target antigens has been somewhat neglected. All of the vaccines described in Section 1.6 are based on antigens described at least 20 years ago (1994 or earlier), and with the exception of TRAP, all were discovered due to their immunodominance in whole-parasite immunisation (but not necessarily natural infection). In the meantime, molecular parasitologists have made great strides in identifying proteins that perform an essential role in parasite growth. Many of these proteins are exposed to the extracellular environment and could have their function blocked by vaccine-induced antibody. Although these proteins are not necessarily targets of NAI, or even necessarily immunogenic during natural infection, this does not rule them out as vaccine candidates—as illustrated by the success of vaccines targeting circumsporozoite protein and TRAP.

1.7.1 Merozoite invasion into RBCs

The invasion of RBCs by merozoites has been the subject of considerable research. This is partly because invasion is a critical and intricate process that is theoretically possible to disrupt with vaccine-inducible antibody, but also a result of technical bias: *in vitro* culture of asexual blood-stage parasites is relatively straightforward by comparison to the sexual and pre-erythrocytic stages.

1.7.1.1 The late schizont: a bag of merozoites

In last hours prior to rupture, a schizont resembles a sack of merozoites. Two membranes separate the merozoites from the extracellular environment at this point: the RBC membrane, and the membrane of the parasitophorous vacuole. A mature merozoite is a pear-shaped structure approximately 1 µm in length. The majority of its body is taken

up with the nucleus and endoplasmic reticulum, but its defining features are its apical organelles. The membranes of these are derived from other organelles: lipids from the nuclear envelope bud off as vesicles and form cisternae resembling the Golgi [200]. This structure in turn supplies vesicles to the developing rhoptries (Fig. 1.4). Microscopically, the rhoptries can be further divided into a granular apical region (i.e. the rhoptry neck) and a more homogeneous rhoptry body [200]. Other, smaller organelles can also be discerned. These resemble one another microscopically, but on the molecular level may well constitute a heterogeneous population of distinct organelles. These are often grouped under the heading 'micronemes' and 'dense granules', but subpopulations such as exosomes [201] and mononemes [202] have recently been identified. These organelles contain different sets of proteins involved in RBC invasion, allowing them to be secreted and exposed in a temporally-coordinated manner. When the merozoites are mature within the schizont, rupture is initiated by the release of the SUB1 protease from the exosomes of the merozoites into the parasitophorous vacuole [201]. SUB1 cleaves at least two members of the SERA family, themselves proteases, thus initiating a protease cascade.

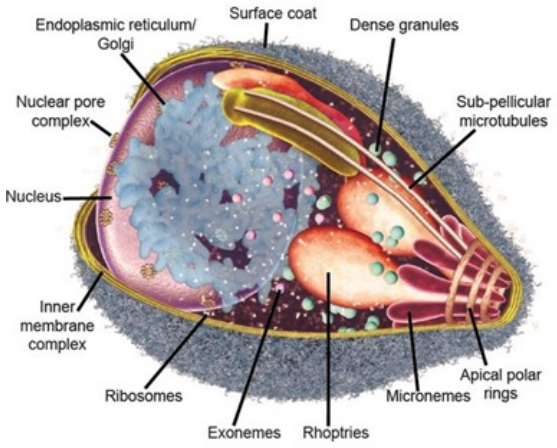

FIGURE 1.4: A *Plasmodium* merozoite. Taken from [102].

1.7.1.2 Initial contact with the RBC

Free merozoites invade the RBC through a series of distinct steps whose molecular components have not been fully dissected. Microscopically, initial contact between the

merozoite is accompanied by deformation of the RBC membrane, followed by reorientation of the merozoite so that its apical end points into the RBC [102]. Numerous proteins are found on the merozoite surface (see Table 1 in [102]). The source of many of these proteins are the micronemes, which are thought to be discharged in response to calcium signalling [103, 203]. Controversy surrounds the upstream events that lead to calcium signaling: one study implicated the change in $[K^+]$ as the merozoite leaves the RBC and is exposed to the plasma [103], while a conflicting report found that signaling was dependent upon signaling through the adhesin RH1 [203]. In any case, proteins that eventually make their way to the merozoite surface include members of the merozoite surface protein family [132], AMA1 [204, 205], proteases such as SUB2 [206], and (more controversially) members of the erythrocyte-binding ligand family such as EBA-175 [103]. The roles of these proteins are surprisingly poorly understood, but many of them cannot be genetically deleted, highlighting their importance to parasite survival.

1.7.1.3 Invasion into the RBC

After the parasite has reorientated, a short pause ensues before invasion itself begins. Two classes of protein are involved in this process. Adhesins interact with receptors on the RBC surface, while invasins are involved in the process but do not directly bind the RBC surface, instead interacting with other parasite proteins [102, 207]. The best known invasin partnership is between between AMA1, which is attached to the merozoite surface by a transmembrane domain, and the RON complex (RON2, RON4 and RON5), which is translocated across the RBC membrane by an unknown mechanism [208]. The importance of this is highlighted by observations that both soluble RON complex and an interfering peptide called R1[3] can block productive invasion [131, 208–210]. It is generally assumed that after the RON complex has been translocated across the RBC membrane, it acts as an anchor for the parasite to pull against as it enters the RBC. Through its interaction with RON2, AMA1 is thought to link this RBC anchor to the actomyosin motor which is found just beneath the apical plasma membrane of the

[3]The R1 peptide binds to AMA1 in the RON2 binding groove, thus blocking their interaction [131].

merozoite [211, 212]. In the presence of the R1 peptide, merozoites are seen to advance forward after re-orientation, but no invasion occurs [213]. This suggests that facilitating movement is not the sole function of the RON2–AMA1 partnership. High-resolution microscopy has determined that rhoptry release occurs aberrantly in the presence of R1, with rhoptry-associated protein 1 (RAP1) released onto the merozoite or erythrocyte surface [104]. This suggests that the AMA1-RON2 complex also helps with the formation of a tight junction between the merozoite and the RBC. During successful invasion, rhoptries are discharged into the RBC, followed by translocation of the tight-junction ring of proteins (as observed by RON4 labelling) around the body of the merozoite from the apical to the posterior end (Fig. 1.5). As the tight junction moves back, it draws the RBC membrane with it until the parasite is sealed inside the erythrocyte [104].

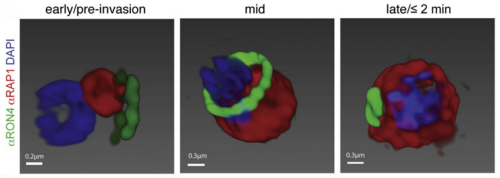

FIGURE 1.5: Merozoite invasion through the AMA1-RON complex tight junction, as observed by three-dimensional structured illumination microscopy. In the early stage of invasion, the tight junction complex (labelled with RON4) is apical to the rhoptry (labelled with RAP1) and the nucleus (DAPI). In the parasite captured midway through invasion, the tight junction complex has opened up, and moved backward toward the nucleus, while the rhoptry has been discharged forward into the RBC. In the late stages, the tight junction has sealed shut behind the nucleus, which has moved forward into the parasitophorous vacuole, derived from the rhoptry membrane. Taken from [104], Fig. 3C.

1.7.2 Invasion-associated candidate vaccine antigens

As of 2006 at least 50 proteins had been experimentally linked with the process of invasion [35], forming a shortlist of candidate vaccine antigens. Of these, by far the most promise is held by adhesins, proteins that recognise receptors of the RBC membrane to facilitate invasion. In *P. falciparum*, these were considered to fall mainly into two families, with parasites possessing at least one member of each. The erythrocyte-binding ligand family consists of EBA-175, EBL1, EBA-140, and EBA-181, whereas the (*P. vivax*) reticulocyte binding protein homologues consist of RH1, RH2a, RH2b, RH4 and RH5 [214]. Until recently, these ligands were considered to have a large

degree of redundancy, with invasion occurring through at least two major pathways. 'Sialic acid-dependent' invasion was defined as using a pathway that can be blocked by neuraminidase treatment of the RBC surface to remove sialic acid moieties. The chief mediator of this invasion pathway was thought to be EBA-175 through an interaction with GYPA, an RBC surface glycoprotein [215]. 'Sialic acid-independent' invasion was thought to rely predominantly on RH4 and its interaction with complement-receptor 1 on the RBC surface [130]. It seemed to be the case that blockade of any one pathway resulted in switching to another pathway [134, 216, 217]. Under this model, each individual ligand was thought to be dispensable for invasion and it was therefore assumed that an adhesion-blocking vaccine would have to include multiple components in order to block all these redundant invasion pathways [134, 217].

Mechanisms of adhesin blockade by antibodies are discussed in Section 5.1.2.

1.7.3 RH5 as a vaccine candidate

This view that all adhesins are individually dispensable for invasion prevailed until the discovery of RH5, which was the first adhesin reported to be refractory to genetic knock-out [218]. Within a few years, the RBC receptor for RH5 was discovered to be basigin, and the interaction between these two proteins was shown to be essential for invasion [128]. Blood-stage vaccine candidates have traditionally been criticised along the following lines: they usually require extremely high concentrations of antibody to achieve neutralisation *in vitro* and *in vivo*; target antigens generally possess problematic levels of polymorphism, meaning that any vaccine-induced antibodies suffer from strain specificity; and there is functional redundancy among invasion ligands. However, as a candidate vaccine antigen, PfRH5 transcended all of these criticisms. Antibodies raised to RH5 alone were found to be capable of completely inhibiting parasite growth, in contrast to antibodies raised against the other well-known adhesins [133]. RH5 displays very limited polymorphism [219], probably because it is not particularly immunogenic during natural infection [133] and is thus not under balancing immune pressure. As a result of this conservation and that fact that it appears to be universally essential [218], antibodies raised to the 3D7 isoform have proved highly functional against every strain

yet tested [133, 136]. Furthermore, antibodies against RH5 act synergistically with antibodies against RH4, resulting in the most potent polyclonal neutralising antibody mixture yet described ([136], and unpublished observations). Most importantly, monkeys vaccinated with RH5 are protected against heterologous blood-stage challenge (Douglas *et al.*, in press). Because RH5 is not immunogenic during natural infection, it is unlikely that it would have been discovered as a vaccine candidate antigen by associative sero-epidemiology (see Section 1.5.5.4). The three-dimensional structure of RH5 in complex with its RBC receptor basigin, and also a panel of inhibitory mAbs, was recently solved by X-ray crystallography [220]. This has defined some of the critical epitopes that are sensitive to neutralisation.

1.8 Project aims

1.8.1 Rationale for a blood-stage vaccine

Blood-stage vaccines hold substantial conceptual advantages over pre-erythrocytic and transmission-blocking vaccines. Immune escape from a pre-erythrocytic vaccine in a patient that had never experienced blood-stage parasitaemia would almost certainly lead to severe disease, while transmission-blocking vaccines offer no protection whatsoever against disease once infection has occurred. In contrast, an efficacious non-sterilising blood-stage vaccine would suppress but not totally eliminate parasitaemia, blocking disease and transmission while allowing the development of protective NAI. However, the field has been disappointed by poor efficacy from candidate blood-stage vaccines tested to date, and there has been a widespread expectation that any blood-stage vaccine would probably have to target multiple parasite proteins to overcome redundancy and polymorphism. The emergence of an asexual-stage antigen with the neutralising and vaccinological profile of RH5 was therefore surprising.

1.8.2 Merozoite-neutralising antibodies

Although neutralisation (as measured by the *in vitro* assay of GIA) does not appear to play the major role in NAI (Section 1.5.6), whole-sporozoite immunisation has shown that a vaccine does not need to emulate NAI in order to be protective. This could in theory be achieved by eliciting high levels of merozoite-neutralising activity. I therefore began this thesis by hypothesising that RH5 is not unique among malaria proteins. The aim was to take advantage of the wealth of parasitological information that has become available over the last 15 years to screen for proteins in addition to RH5 that are highly susceptible to broadly-neutralising antibodies. Specifically, I aimed to increase the potency of a final vaccine product by reducing the titre of antibody that must be elicited in order to achieve a protective level of growth inhibition. I hoped to achieve this either by finding another antigen which is more susceptible to antibody than RH5, or alternatively an antigen that elicits antibodies which act synergistically with anti-RH5 antibodies. With a multi-valent vaccine, it is important that the antibody responses to each antigen interact to produce a synergistic effect. This is because the responses to each antigen may interfere with each other, reducing the magnitude of each antigen-specific response [85, 197, 198] (measurement of synergy is discussed further in Section 4.1.2).

The discovery of RH5 as a vaccine candidate was delayed by three years, because antibodies elicited against recombinant fragments of the protein did not appear to have substantial anti-parasitic activity. This was a product of the notorious difficulty of expressing full-length *Plasmodium* proteins in *Escherichia coli* (see Section 3.1 and [221, 222]). In two independent studies, Baum *et al.* and Rodriguez *et al.* were only able to generate small fragments of RH5 using the *Escherichia coli* protein expression system, neither of which was able to induce potent growth-inhibitory antibodies when injected into rabbits [218, 223]. In a later study that used virus vectors to elicit antibodies (effectively mammalian *in situ* expression of the antigen) performed in our lab, we were able to recapitulate the negative result with the fragment described by Baum *et al.*, but went on to show that the full length RH5 antigen can elicit highly potent growth inhibitory antibodies [133]. In order to minimise the possibility of missing another susceptible antigen, we decided to only screen full-length constructs. This can be

achieved with virus vectors, but the production protocol is time-consuming and not wholly suited to screening large numbers of new antigens. Much of the work in this Thesis was therefore directed to the establishment and development of platforms to facilitate the induction of high-titre antibody responses to many different merozoite proteins. These high-titre antibodies against different antigens were then tested for their anti-parasitic activity.

Project outline

Chapter 3 describes how I established a protein expression system in our lab based on mammalian cells. This system had already been reported to yield high rates of success for the expression of secreted *P. falciparum* merozoite proteins fused to large tags unsuitable for vaccine antigen screening [224, 225]. It was hoped that a straightforward method for the expression and purification of soluble protein antigen (without large tags) would facilitate the elicitation of antibody via the protein-in-adjuvant approach.

Chapter 4 describes the production of a library of pre-clinical vaccines containing *P. falciparum* merozoite proteins, which were then screened for the ability to elicit neutralising antibodies. Although antibody elicitation depended primarily on vaccination with recombinant protein (produced using protocols established in Chapter 3), virus vector regimes were used in some cases as well. Two panels of antigens were selected for screening: one based upon published literature; one based upon genomic, transcriptomic and polymorphism data. The screen revealed two promising 'hits'.

Chapter 5 describes the characterisation of antibodies against AARP, the most promising 'hit' from the screen described in Chapter 4. It was found that the antibodies elicited have unusual properties, including a high level of cross-reactivity to proteins in whole parasite extract, and also to proteins on the RBC surface.

Chapter 6 The overall success rate for expression of novel proteins in Chapter 4 was 35%. This represented a considerable improvement upon previous attempts, but

still left a large proportion of candidate antigens unscreened. I therefore developed an enhanced genetic immunisation approach based on improved delivery of DNA into the mammalian host cells. This platform proved highly immunogenic and will facilitate future antigen screening projects.

Chapter 2

Materials and Methods

2.1 DNA

2.1.1 Sequencing

All new constructs produced during this Thesis were validated by sequencing across the full length of any new insert. Sequencing was performed by Source Bioscience (UK).

2.1.2 Plasmids

Maps of the backbones of plasmids used throughout this Thesis are shown in Figs. 2.1 and 2.2. pENTR4 is a commercial plasmid which has been modified to contain a long version of the CMV promoter incorporating intron A [226]. pOPINTT was a kind gift from Raymond J. Owens. pMOD (Fig. 2.2B) was cloned by removing the expression cassette from pCEP4 by SalI restriction (Fig. 2.2A), and replacing it with the expression cassette from Fig. 2.1A which had been amplifed with PCR. Insertion of the expression cassette into the pCEP4 backbone was performed by restriction digestion (Fig. 2.2) followed by In-Fusion cloning (Clontech USA), as per manufacturer's protocol.

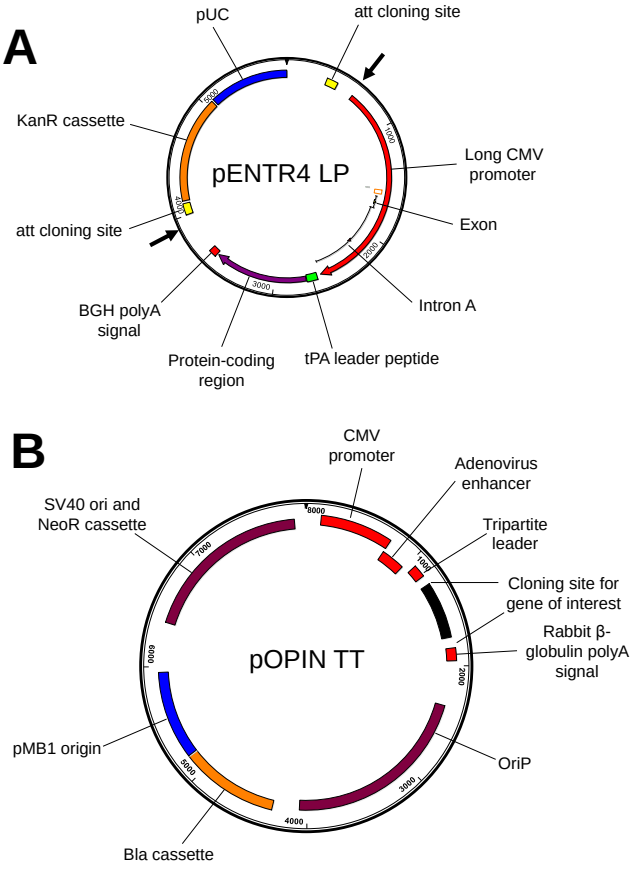

FIGURE 2.1: Plasmid maps for the backbones of pENTR4 LP (A) and pOPINTT (B) vectors. Specifics of the expression cassettes for the protein of interest are shown in the relevant sections of Chapter 3. Black arrows in (A) indicate boundaries of the LP expression cassette, which was amplified by PCR and cloned into pCEP4 (see Section 3.2.5 and Fig. 2.2.

2.1.3 Genes

An optimised gene for TRAP was produced by Matthew G. Cottingham. This encoded residues D27–K511, with PNP repeats from P330 to D371 removed. The amino acids encoded by all other genes are given in Table 4.1. All these genes encoded exactly the same amino acid sequence as that predicted in the 3D7 genome [227] with the following two exceptions which had already been produced and are described elsewhere: the gene for RH5 encoded residues E26–Q526, with substitutions N38Q and N214Q [133]; and the gene for AMA1 extends from Q25–K546 [139, 228]. All other genes were codon-optimised and synthesised by GeneArt®.

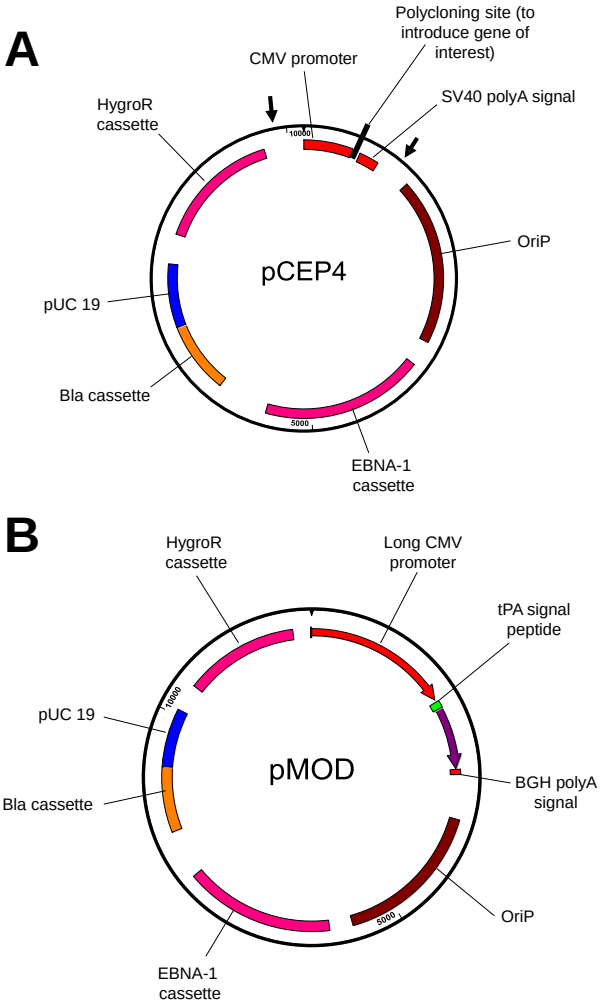

FIGURE 2.2: Plasmid maps for the backbones of pCEP4 and pMOD vectors. Expression cassettes for the protein of interest are shown in the relevant sections of Chapter 3. Arrows in (A) indicate SalI restriction sites, which were used to open up pMOD and allow insertion by In-Fusion of the PCR product described in Fig. 2.1A.

2.1.3.1 Construction of SNP-visualisation plots

The raw data for these plots (Fig. 4.5) came from a number of sources [219, 229–232] and was processed into a format that I could readily interpret by Jason P. Wendler. SNP data were downloaded from MalariaGEN and PlasmoDB. Coverage data were calculated using Samtools mpileup. Original data were parsed using Perl, and the analysis and plots were done in R.

2.1.4 DNA purification

For transfection of HEK293 cells for protein production, DNA was purified using QIAGEN (USA) Plasmid *Plus* Midi, Maxi and Giga kits (catalogue numbers 12943, 12963, 12991) depending upon DNA requirements, following the manufacturer's protocol. For mouse immunisation, initial preparations were performed using QIAGEN Plasmid *Plus* Midi Kits (catalogue number 12362), as per manufacturer's protocol. Regardless of the kit used for purification, DNA used for mouse immunisation was subsequently purified by phenol-chloroform extraction [233].

2.2 Virus vectors

The virus vectors encoding AMA1 and RH5 were kind gifts of Alexander D. Douglas and Sumi Biswas. Adenovirus vectors encoding ETRAMP and RIPr (Chapter 4) were produced by me exactly as described elsewhere [139, 234, 235]. In brief, genes for these proteins, having been cloned into pENTR4 LP for protein production purposes, were shuttled into the genomes of AdHu5 and ChAd63 by Gateway® cloning reaction (Fig. 2.1). These genomes were transfected into HEK293 cells, which were progressively passaged to allow viral amplification. Viruses were purified using AdenoPure kits (PureSyn, USA). Quantification of virus was by an immunostaining assay. Virus was titrated to almost-limiting dilution on beds of HEK293 cells. 48 hours post infection, cells were fixed and stained with primary antibody against adenovirus hexon protein (Cambridge Bioscience UK), and an HRP-conjugated secondary antibody. After development with ImmPact DAB reagent (Vector Labs, UK), spots corresponding to cells infected with a single founder virion were counted with an ELISPOT plate reader (AID, Germany).

2.3 Protein production in transiently tranfected HEK293 cells

2.3.1 Cell culture of suspension HEK293 cells

HEK293F suspension cells were obtained from Life Technologies®. HEK293E and HEK293T cells were obtained from Ray Owens in the Oxford Protein Production Facility. Cells were cultured in a temperature-controlled incubator at 37°C, with atmospheric air mixed with 8% CO_2, shaking at 130 RPM. Until late 2011, cells were cultured in Freestyle293 medium (Life Technologies™, UK), sometimes with the addition of 1% foetal bovine serum (batch number 30401, Labtech International™, UK). Twice weekly, cells were passaged to a density of 2.5×10^5 cells/mL. From early 2012, cells were passaged in Expi293 medium (Life Technologies™, UK). For routine culturing in Expi293, cells were passaged twice weekly to 1×10^6 cells/mL. Of vital importance, medium was always pre-warmed for at least 30 minutes prior to passage. HEK293E and HEK293T cells were grown in the presence of G418 antibiotic at a dilution of 1:1000 (Roche, catalogue number 4727878). HEK293E and HEK293T cells initially arrived as adherent cells grown in RPMI-based medium supplemented with 10% FCS. To adapt the cells to serum-free suspension culture, the cells were trypsin-treated and then put into suspension culture with Freestyle293 medium at 5% serum. At the next passage, serum concentration was reduced to 1%, and then at the next passage serum was removed entirely. The whole process took around 7 days.

2.3.2 Transfection

As my D.Phil. advanced, I modified and improved the transfection procedure.

In the initial stages, when cells were still cultured in Freestyle293 medium, I used both linear 25 kDa PEI (for details of formulation, see Section 2.3.3) and Freestyle™MAX (Life Technologies®, UK, catalogue number 16447100). Freestyle™ MAX was used as directed in the manual, using 1 µL of reagent per µg of DNA to be transfected. When

transfecting cells grown in Freestyle293 medium, transfection followed the following protocol. On the day before transfection, cells were passaged to 2.5×10^5 cells/mL. 24 hours later, transfection-competent DNA complexes were added to the culture.

When transfecting cells grown in Expi293 medium, transfection followed an identical protocol except that 24 hours prior to transfection cells were passaged to a density of 1×10^6 cells/mL. Around 84 hours later, supernatants were harvested by spinning cultures at 500 x g for 5 minutes. They were then filtered through a 0.22 µm vacuum filter.

2.3.3 PEIs

There are a few critical parameters that influence the performance of PEI. First, it can be branched or linear. Second, the mean molecular weight of the product and the width of the size distribution around that mean. Third, the extent to which the PEI polymer is contaminated by unwanted moieties (such as N-acyl groups [236]) that disrupt the string of protonable nitrogens and interfere with the ability of PEI to interact with DNA. In a given solution of DNA and PEI, N/P ratio refers to the ratio of nitrogen atoms found in PEI to the anionic phosphates found in the DNA. It is assumed that one in three nitrogen atoms in PEI is protonable, which means that charge neutrality of DNA/PEI polyplexes is achieved when the N/P ratio is approximately 3.

Linear 25 kDa PEI (herafter abbreviated to L25, Alfa Aesar, catalogue number 43896-01) was dissolved in ultrapure water at 1 mg mL^{-1}, stirring overnight on a heated block to promote dissolution, and stored as frozen aliquots. Once defrosted, aliquots were turbid due to PEI coming partially out of solution. Aliquots were stored at 37°C to promote dissolution for 1 month before being discarded. For transfecting HEK293 cells, this PEI was mixed with DNA in Freestyle293 medium at a ratio of 2 µg PEI to every 1 µg of DNA, in line with published protocols for *in vitro* transfection [237]. This corresponds to an N/P ratio of 15.5.

The PEI MAX range by Polysciences (USA) encompasses three separate PEIs with mean MWs of 2.5 kDa, 40 kDa and 160 kDa (hereafter abbreviated to M2.5, M40 and M160,

catalogue numbers 24885-2, 24765-2, 25439-2 respectively). These were dissolved in ultrapure water at 6.47 mg mL^{-1} and stored at 4°C for 1 year. For transfecting HEK293 cells, these PEIs were mixed with DNA in Freestyle293 medium at a ratio of 2 µg PEI to every 1 µg of DNA. This corresponds to an N/P ratio of 15.5.

For information regarding the formulation of PEI with DNA for vaccination purposes, see Section 6.2.1.

2.3.4 Protein purification

For purification of proteins C-terminally tagged with StrepII [238], supernatants were extensively dialysed into PBS using membranes with a 10 kDa molecular weight cut-off (Thermo Scientific UK) in a temperature-controlled room at 4°C. Strep-tagged proteins were purified using streptactin® sepharose™ resin as directed in the manual (IBA, Germany, catalogue number 2-1201-010).

For purification of proteins C-terminally tagged with 6His, purification was performed on the day of harvest. For the duration of the work supernatants were kept on ice. The NaCl concentration of the supernatant was adjusted to 400 mM (assuming a starting NaCl concentration in the medium of 150 mM), and imidazole was added to give a concentration in the supernatant of 10 mM. Supernatants were passed over 5 mL pre-packed HisTrap Excel columns (GE Healthcare, UK). Columns were then washed with a saline solution (400 mM NaCl, 20 mM NaPO4, pH7.4) containing 10 mM imidazole, before elution in a saline solution containing 500 mM imidazole.

2.3.5 Storage of purified protein

Purified protein fractions were pooled and exchanged into Dulbecco's PBS (Sigma, USA) using 10 kDa cut-off Amicon centrifugal filter devices from (Fisher Scientific UK Ltd, catalogue number 10026941) over two spin cycles. Protein concentration was estimated using a Nanodrop 2000 (Thermo Scientific, USA) based on an extinction co-efficient provided by the ProtParam tool of the Expasy server [239]. If necessary, protein

concentration was adjusted to 0.4 mg/mL. 100 µg aliquots (250 µL) were stored at -80°C.

2.4 Protein analysis and quantitation

2.4.1 SDS-PAGE and Western blots

15-well 4-20% gradient polyacrylamide gels (Fisher Scientific, UK) were loaded with 7.5 µL per lane of sample diluted in Laemmli buffer (BioRad, USA) containing 2-mercaptoethanol to reduce disulphide bridges. Depending on the application, 7.5 µL Precision Plus Protein Unstained Standards by BioRad (USA) or 5 µL Prestained Protein Marker, Broad Range (NEB, USA) were loaded as molecular weight markers. For visualisation of proteins, SimplyBlue^{TM} SafeStain (Life Technologies® UK) was used to detect protein in polyacrylamide gels (Pierce, UK). Western blotting was performed using the Turbo-Blot system (Bio-Rad, USA). Proteins were detected with a variety of primary antibody reagents including anti-His mouse monoclonal antibody (Qiagen Ltd, UK) diluted to 200 ng mL^{-1}, serum from a rabbit immunised with PfRH5 [133] diluted 1:4000, and streptactin-HRP conjugate diluted 1:10000 (IBA, Germany).

PNGase F (NEB, USA) treatment was performed as per manufacturer's protocol. Briefly, 800 ng of protein was added to 1 µL x10 glycoprotein denaturation buffer, topped up to 10 µL, and then heat-denatured for 10 minutes at 100°C. After denaturation, 2 µL of G7 reaction buffer, 2 µL 10% NP-40 and 4 µL of dH_2O were added. At this point 2 µL PNGaseF was added and the mixture was incubated for 1 hour.

2.4.2 Estimation of total protein concentration

Protein purity was estimated by reducing SDS-PAGE. If pure, and provided that the protein contained sufficient aromatic residues, protein concentration was calculated by OD_{280} as determined with a Nanodrop-2000, scaled for the extinction coefficient

calculated on the Expasy server [239]. For proteins that contained no aromatic residues, the bicinchoninic acid assay was used (Pierce, UK).

2.4.3 Sandwich ELISA to detect yield of recombinant PfRH5

This was performed to an almost identical protocol as the standardised direct ELISA in Section 2.6.2, with the following modifications: the anti-RH5 mAb 2AC7 [129] was coated onto plates at 5 µg mL^{-1}; test HEK293 supernatants were applied to supernatant at a 1:6 dilution in casein blocking solution (BioRad, catalogue number 161-0782) for 24 hour samples, and 1:50 dilution for 84 hour samples; detection was with rabbit anti-RH5 serum (immunised as in [133]), followed by anti-rabbit IgG (whole molecule) produced in goat conjugated to alkaline phosphatase (Sigma-Aldrich, UK, catalogue number A8025) diluted 1:5000 in PBS with 0.05% TWEEN-20.

2.5 Immunisations and serum harvest

2.5.1 Mice

For antigen screening and testing, I followed the following dosing strategies to immunise groups of 4 female BALB/c mice. For protein, 20 µg of protein was formulated into 50 µL of Dulbecco's PBS. This was then mixed with an equivalent volume of Addavax^{TM} adjuvant (Invivogen, USA). The resultant 100 µL was split two ways and administered into the left and right quadriceps of mice anaesthetised with vaporized isofluorane. For virus vectors, two regimes were used. In a standard adenovirus-MVA protocol, 10^{10} adenovirus particles (typically AdHu5) were administered in 50 µL PBS i.m. as the priming inoculation, and 10^{7} PFU of MVA was administered in 50 µL i.m. as the booster inoculation eight weeks later. This total volume of 50 µL was split across left and right quadriceps of balb/c mice anaesthetised with vaporized isofluorane. In the heterologous adenovirus protocol, 10^{10} particles of AdHu5 were administered in 50 µL PBS as the priming inoculation, and 10^{10} ChAd63 particles (encoding the same antigen)

was administered in 50 µL as the booster inoculation eight weeks later. This total volume of 50 µL was split across left and right quadriceps of balb/c mice anaesthetised with vaporized isofluorane. Each mouse immunisation schedule included a GIA positive control group immunised with PfRH5 and a GIA negative control group immunised with OVA, both using an adenovirus-MVA regime.

Blood (approximately 1 mL per mouse) was harvested from mice by cardiac puncture under terminal anaesthesia and serum collected the following day.

For both virus-vector regimes and protein-in-adjuvant regimes, blood was harvested 2 weeks after the final immunising injection. Blood was left at 4°C overnight to allow clotting factors to aggregate, then spun at 4000 rpm for 4 minutes in a microfuge. The supernatant (i.e. serum) was harvested and stored at −20°C until further processing.

2.5.2 Rabbits

Rabbit immunisation with Freund's adjuvant was outsourced to Biogenes GmbH (Germany). 50 µg of protein in 100 µL PBS was combined with 100 µL adjuvant, homogenised, and administered i.m.. The first immunisations on day 0 were with Freund's complete adjuvant, the second and third immunisations on days 28 and 56 respectively with Freund's incomplete adjuvant. The final serum bleeds were on day 70, and pre-immune serum was collected on day 0 just prior to the first immunising injection.

2.6 Antibodies

2.6.1 Purification of antibodies with protein G

Serum was diluted three-fold into 20 mM sodium phosphate, pH7.4, and applied to a column containing protein G-sepharose (GE Healthcare, UK). Columns were washed with 15 column volumes of 20 mM sodium phosphate and the antibodies eluted with 10 mL 0.1M glycine, pH 2.7. The pH of the elutate was restored to approximately neutral by addition of 350 µL 1M Trizma, pH 9.0. The antibodies were then buffer exchanged

using Amicon centrifugal filter devices, 10 kDa molecular weight cut-off (Fisher Scientific, UK). This was accomplished by topping the eluate up to 15 mL with RPMI-1640 (Sigma, UK) and spinning at 3000 x g until approximately 300 mL remained. This was repeated once, and then for a second time using *P. falciparum* asexual culture medium without serum or gentamycin (see Section 2.7.1). The antibodies-in-medium were then passed through a filter with a 0.22 µm pore diameter and the OD_{280} measured by Nanodrop-2000, with a conversion factor of 1.4 OD_{280} units equivalent to 1 $mg\,mL^{-1}$. For rabbit antibodies, IgG was typically stored at 20 $mg\,mL^{-1}$, for mouse antibodies, IgG was typically stored at 2 $mg\,mL^{-1}$. For RBC depletion, equal volumes of antibody sample and 100% Hct RBCs were mixed at room-temperature for 1 hour before removal of the RBCs by centrifugation at 830 x g for 4 minutes and collection of the supernatant.

2.6.2 ELISAs to detect antigen-specific IgG

For all ELISAs, 96-well Nunc MaxiSorp® plates (Thermo Scientific, UK, catalogue number 442404) were coated with antigen by overnight incubation with 50 µL per well of PBS containing recombinant protein at 2 $\mu g\,mL^{-1}$. A detailed protocol is described in Jenner protocol J048 (mouse whole IgG ELISA). Briefly, after coating, wells were blocked with 100 µL of 10% skimmed milk power in PBS. Serum was applied at an appropriate dilution (determined empirically) in 50 µL PBS with 0.05% TWEEN-20. After washing, mouse IgG was detected with anti-mouse IgG (whole molecule) produced in goat conjugated to alkaline phosphatase (Sigma-Aldrich, UK, catalogue number A3562) diluted 1:5000 in PBS with 0.05% TWEEN-20. Rabbit IgG was detected with anti-rabbit IgG (whole molecule) produced in goat conjugated to alkaline phosphatase (Sigma-Aldrich, UK, catalogue number A8025) diluted 1:5000 in PBS with 0.05% TWEEN-20. Alkaline phosphatase was detect with 1 $mg\,mL^{-1}$ pNPP substrate (Sigma-Aldrich, UK) in diethanolamine buffer and absorbance measured at 405 nm. In many cases, a high proportion of the antibodies detected by ELISA were directed to the .BAP.Strep C-terminally fused purification tags (Section 3.1.5) on the coating antigen and it was necessary to remove the anti-tag antibodies Section 4.2.4.2. Depletion of anti-tag antibodies was performed by incubating samples with a large molar

excess of peptide relative to assumed antibody concentration. Peptides of equivalent sequence to the .BAP.Strep (GSASGLNDIFEAQKIEWHEWSHPQFEK) and .BAP.His (GSASGLNDIFEAQKIEWHEHHHHHH) tags, including amino-acids encoded by the cloning sites at the 3' end of the gene of interest, were ordered from NeoPeptide (USA) to 70% purity. A typical IgG concentration in murine serum is 2.5 $\mathrm{mg\,ml^{-1}}$. As my depletion peptides have a MW of around 3 kDa, and bivalent IgG has a MW of around 150 kDa, I assumed that a 1:1 molar ratio of peptide:Fab requires 1 g of peptide for every 25 g of antibody. I added 1.25 µg of peptide for every 1 µL of serum (or per 2.5 µg of total antibody), which is a 12.5-fold excess of peptide. Where not reported as raw OD_{405}, data was converted into concentration units according to one of two methods:

Endpoint titres were obtained for mice vaccinated with five separate protein vaccines (see Section 4.2.4.2), and also rabbits immunised with AARP.BAP.His (Fig. 5.4). Serial dilutions of serum were produced and analysed by ELISA, with a plate development time of 30 minutes. The slope of the line connecting adjacent points on the serial dilution curve was calculated with linear interpolation and the endpoint titre was defined as the lowest (theoretical) dilution that would have given an OD_{405} above background (i.e. 0.15 units). Serum from mice vaccinated with OVA in addavax adjuvant was used as the negative control for the assay.

Results from **standardised ELISAs** are reported as 'arbitrary units' throughout this Thesis. These were used for all the antibody quantification reported in Chapter 6 and many mouse anti-PfAARP ELISAs described in Chapter 5. The basic principles are described in [240]. For these assays, a serum sample is defined as the assay standard, and an identical dilution series of this sample is run on every assay plate. The hyperbolic relationship between IgG concentration and OD_{405} can only be approximated to a straight line in a certain range of IgG concentrations, and test samples are diluted such that they give OD_{405} values that fall in this range. In this manner, the IgG concentration in a diluted sample can be interpolated, and the IgG concentration in the original sample back-calculated.

2.6.3 Epitope-mapping with linear peptides

28 biotinylated, linear peptides were ordered from Mimitopes (UK) to cover the full length of the recombinant polypeptide tPA.AARP.BAP.Strep. The first peptide corresponded to the first 20 amino acids of the recombinant antigen with a primary amine at the N-terminal methionine, and 4-residue GSGK spacer at the C-terminus with biotin conjugated to the C-terminal lysine. Peptides 2–28 had biotin-SGSG fused to the N-terminus, followed by 20 amino acids from the antigen, with a C-terminal $–NH_2$ to form an amide group. Each peptide was offset from the next by eight amino acids. Typical yield was 1.1 µmol, which was dissolved in 200 µL dimethyl sulfoxide. This was further diluted 1:1000 in PBS, and bound to a streptavidin-coated plate (Thermo Scientific, UK) at 50 µL per well (or approximately 275 pmol per well). Total IgG was assayed for reactivity to immobilised peptide at 3.3 $\mu g\,\mu l^{-1}$.

2.7 Parasitology

2.7.1 Continuous *in vitro* culture of asexual parasites in human RBCs

Parasites were cultured according to standard methodologies [241–243] following the Jenner SOP ML016. Briefly, parasites were grown in O+ red blood cells at 2% Hct purified RBCs, which were prepared twice monthly. Culture medium contained 10% heat-inactivated pooled human serum mixed with RPMI 1640 supplemented with 5.94 g HEPES and 0.05 g hypoxanthine per litre. Cultures were grown in the presence of 20 $\mu g\,mL^{-1}$ gentamycin.

2.7.2 Synchronisation of parasite cultures

Sorbitol treatment of mixed asexual *P. falciparum* cultures selects for ring-stage parasites [244]. Cultures were spun at 830 x g for 4 minutes to pellet the cells, and the medium removed. Pelleted cells were resuspended in 5% sorbitol warmed to 37°C and incubated for 10 minutes. After 10 minutes, cells were spun at 830 x g for 4 minutes

again and then resuspended in culture medium. This method of treatment is toxic to parasites and recovery may take several life cycles [245].

Percoll treatment of mixed asexual *P. falciparum* cultures selects for late trophozoites and schizonts [246]. Cultures were spun at 830 x g for 4 minutes to pellet the cells, and the medium removed. The cells were then layered onto 65% Percoll in PBS at 37°C, and spun at 1870 x g for 5 minutes. Because late trophozoites and schizonts have a lower density than 65% Percoll, these remain on top of the layer while the other cells pass through the Percoll. The late trophozoites and schizonts were then removed with a pipette, washed in warm medium (without gentamycin or serum) and returned into culture.

2.7.3 IFAs

Tightly synchronised asexual cultures containing predominantly late schizonts at a high parasitaemia (>20%) were smeared onto glass slides to make thin films. Slides were fixed in 4% PFA for 15 minutes. Between all steps, slides were washed in PBS. To permeabilise cell membranes, slides were exposed to 0.1% Triton-100 for 10 minutes. Slides were blocked with 1% BSA in PBS. Primary antibodies (i.e. test mouse IgG) was applied to slides at 20 $\mu g\,mL^{-1}$ in PBS for 30 minutes. Mouse antibodies were detected with goat anti-mouse IgG antibody conjugated to alexa488. Cover slips were mounted onto slides with MOWIOL-DAPI (recipe: 6 g glycerol, 2.4 g MOWIOL (Polyvinyl alcohol 4-88, Hoechst) and 6 mL distilled water incubated overnight at 50°C with agitation, plus 12 mL 0.2 M Tris HCl pH 8.5, incubated overnight again at 50°C with agitation, spun 1000 x g for 5 minutes to remove solid material, DAPI added to 1 $\mu g\,mL^{-1}$). Each independent IFA experiment always included a negative control (anti-OVA) and a positive control, typically mouse IgG raised against the F2 domain of EBA-175 (CAMP strain) by vaccination with adenovirus and MVA [133]. In each experiment, all samples were analysed microscopically with an identical exposure period for the camera.

2.7.4 *In vitro* assay of growth inhibitory activity (GIA assay)

GIA assays were performed to the protocol of the MVI international GIA reference centre [247]. Sorbitol and percoll synchronised parasite cultures (3D7) at the trophozite phase were cultured for approximately 40 hours at 1% haematocrit at a starting parasitaemia of 0.2–0.4% in the presence of test antibody purified as in Section 2.6.1. Detection was based on the lactate dehydrogenase assay. BG98 is IgG purified from the pooled serum of 98 rabbits immunised with AMA1, and was a kind gift from Edmond Remarque. At 6 $\mathrm{mg\,mL^{-1}}$ it typically yields 80–90% GIA against FVO and 3D7.

2.7.5 Schizont extract and RBC Western blots

Tightly synchronised asexual cultures containing predominantly late schizonts were treated with Percoll to isolate the iRBCs. To make the lysates, iRBCs or RBCs were washed (span at 830 x g for 4 minutes and resuspended) 3 times with PBS, then diluted 10:1 in 2x Laemmli buffer (BioRad, USA) without 2-mercaptoethanol. After three freeze-thaws, the schizont extract was vigourously drawn back and forth into a pipette tip to shear the genomic DNA. 2-mercaptoethanol was then added, samples boiled for 5 minutes, and 8 µL of lysate was run on a 15-well 4–20% gradient polyacrylamide gel (Fisher Scientific, UK). Total IgG was used as a primary antibody at 0.5 $\mathrm{\mu g\,mL^{-1}}$.

Chapter 3

Establishment of a platform for the production of *Plasmodium falciparum* merozoite proteins in mammalian cells

3.1 Introduction

PfRH5 was identified as a promising blood-stage malaria vaccine antigen as a result of an antigen screen that used adenovirus and MVA vectors administered in a prime-boost regime [133]. Whilst the virus vector system was successful in eliciting antibody responses against 10 antigens, it took 3 years to produce the requisite pair of virus vectors encoding each antigen. With the work described in this Chapter, I aimed to establish a more high-throughput system for the production of pre-clinical vaccines to elicit antibodies against *P. falciparum* proteins.

3.1.1 Difficulties associated with production of recombinant *Plasmodium* proteins

P. falciparum proteins are notoriously difficult to express in heterologous systems, particularly prokaryotic cells [222]. This complicates the production of vaccines that stimulate an immune response against *Plasmodium* proteins, impacting both pre-clinical immunogen optimisation and subsequent cGMP processes. These difficulties are thought to stem from a number of traits idiosyncratic to *P. falciparum*. First, the *P. falciparum* genome is 80% A-T rich. This means that the codon usage in *P. falciparum* ORFs tends to be incompatible with the tRNA abundances found in traditional expression systems such as *E. coli*, yeast and Sf9 insect cells. The low genomic complexity also makes PCR amplification of sequences directly from the genome challenging and can lead to genetic instability once the gene is cloned into non-natural hosts such as *E. coli* or virus vectors [248, 249]. Second, encoded proteins often contain long, disordered, repetitive regions consisting of only one or two different amino acids [250], especially asparagine [251]. Proteins of this type have a tendency to aggregate, making purification and long-term storage difficult [250]. Third, *P. falciparum* and other apicomplexa tend to encode a disproportionate number of intrinsically disordered proteins in their genomes [252] which are difficult to express heterologously and to functionally characterise. Fourth, the glycosylation patterns seen in *P. falciparum* proteins are far-removed from those seen in other species [253–255] and might contribute to the reduced solubility and stability of recombinantly-expressed *P. falciparum* proteins. Fifth, some *P. falciparum* proteins appear to be unstable in the absence of parasite-specific molecular chaperones [256, 257], requiring heterologous co-expression of *P. falciparum* heat-shock proteins to express useful levels of recombinant protein [257]. Sixth, *P. falciparum* proteins tend to have a longer primary structure than orthologous yeast proteins [258], with ramifications for subcloning the relevant genes. These problems are illustrated by the poor success rates of various structural genomic projects. The SGPP group in Seattle attempted to express 1000 *P. falciparum* genes that were bioinformatically predicted to encode small, soluble enzymes, as these were judged to be the most amenable to expression in *E. coli* [222]. Of these 1000 genes, 337 expressed insolubly in inclusion bodies, and only 63 expressed

solubly. A separate attempt to express 95 *P. falciparum* proteins in *E. coli* only yielded five soluble proteins [259], whilst the same system had previously yielded a success rate >60% when applied to a randomly-selected library of human ORFs [260].

3.1.2 Advantages of recombinant protein for vaccinological studies

Genetic immunisation approaches based upon plasmid DNA [11, 261–263] or virus vectors [133, 157] represent an alternative method of eliciting antibodies against proteins from eukaryotic species, including *P. falciparum*. However, protein-in-adjuvant is preferable when screening novel antigens because it allows administration of a controlled dose of immunogen. Furthermore, the protein antigen can be examined for the presence of post-translational modifications (such as glycosylation or cleavage) that would not be possible using genetic immunisation methods that involve expression of the genes *in situ*. Finally, a stock of recombinant protein antigen allows assessment of vaccine-induced antibody titre by methods such as ELISA and SPR [136].

3.1.3 New technologies to facilitate production of recombinant *Plasmodium* proteins

A number of advances in protein expression technologies mean that it is now feasible to attempt a malaria antigen reverse-vaccinology screen using protein-in-adjuvant preclinical formulations. Primer extension-based PCR to generate fully synthetic genes was first successfully deployed in 1990 [264], and has now become an affordable and widely available tool. This technology allows the production of cDNAs encoding an amino acid sequence identical to that found in the natural organism, but making use of a different codon repertoire (as well as allowing manipulation of potential glycosylation sequons and other sites of post-translational modifications). One strategy that uses synthetic gene technology to improve heterologous protein expression is codon optimisation. Underpinning this strategy is the correlation observed between codon usage in a particular organism, and the abundance of the cognate tRNAs [265]. Codon-optimised genes are designed such that codons that are rarely used in the heterologous

expression host are changed to more abundant codons that encode the same amino acid. Codon optimisation has been shown in some cases to improve expression of *P. falciparum* proteins [266, 267]. Codon harmonisation is a slightly different strategy, based on the notion that codons corresponding to more scarce tRNAs in the natural host may cause a 'pause' in translation, allowing separate domains of the protein to fold co-translationally. Codon harmonisation attempts to replicate this in the synthetic gene, so that positions incorporating a codon that is less abundant in the natural host incorporate a codon that is also rarer in the heterologous expression host [268]. Together, gene synthesis and codon alteration strategies eliminate many of the difficulties associated with the low complexity of the *P. falciparum* genome (enumerated above) [221].

Another technology that has recently been adopted by the malaria field is the use of mammalian cells, especially HEK293 cells, to express *P. falciparum* proteins [128, 225, 269]. This is particularly useful when attempting to express proteins that are naturally directed to the secretory pathway in *P. falciparum*, such as merozoite invasion ligands. An obvious explanation for the vastly improved rate of soluble protein expression in HEK293 cells, compared to prokaryotic systems, invokes the argument that the secretory pathway of HEK293 cells more closely mimics the natural trafficking of secreted *P. falciparum* proteins. Fusion of a mammalian signal peptide to the N-terminus of the recombinant protein causes the protein to be translated into the endoplasmic reticulum, where it is exposed to chaperones that facilitate disulphide bridge formation, glycosyltransferase enzymes, and the protein folding quality-control machinery [270, 271]. Whilst many of these features are also present in yeast, the misfolded-protein surveillance system (ER-associated degradation system, or ERAD) is considerably more complex in mammalian cells than yeast [272]. This may account for the failure to express both PfTRAP and PfRH5 in our laboratory in yeast-based systems (K. Collins and A. Douglas, unpublished observations).

3.1.4 PfRH5 and PfTRAP

Because PfRH5 and PfTRAP are both promising vaccine candidates [19, 133, 136, 273, 274] that have been refractory to expression in our laboratory, I selected them as model

'difficult' proteins to enable the optimisation of a new protein expression pipeline. Initial attempts to express PfTRAP resulted in yields dramatically higher than those seen in other heterologous expression systems, and thus encouraged an attempt to express a larger number of secreted merozoite proteins (Chapter 4). In contrast, recombinant expression of PfRH5 remained challenging. The results presented here in Chapter 3 summarise attempts to improve expression levels and cost-effectiveness that were ongoing throughout my D.Phil. project.

3.1.5 Abbreviations for encoded DNA elements

Throughout this Chapter and the remainder of this thesis, a number of abbreviations are used to refer to DNA and amino acid sequence elements. In many cases these abbreviations are described more briefly in the abbreviations section at the beginning of this thesis (page xi).

6His	A tag consisting of six consecutive histidine residues, which has affinity for metal ions such as Ni^{2+} and Co^{2+}.
BAP	**B**iotin **A**cceptor **P**eptide, amino acid sequence GLNDIFEAQKIEWHE.
CMV	Refers to the human **C**yto**m**egalo**v**irus immediate-early promoter. The 'long CMV' refers to a promoter that includes the intronA splice sites (approximately 1900 base-pairs in length), the 'short CMV' sequence does not include this intron (approx 600 base-pairs in length).
CD4	Domains 3 and 4 from rat CD4, used as an epitope tag in plasmids from the laboratory of Gavin Wright [275, 276].

Strep The strep-tag is an eight amino acid tag designed by IBA (Germany). This tag is selectively captured by a modified form of streptavidin called streptactin, and can be eluted with either des-thiobiotin or biotin [238].

3.2 Results

3.2.1 Initial establishment of a suspension HEK293 expression platform: PfTRAP and PfRH5

My initial aim was to characterise the ability of the suspension HEK293 system to express and secrete a protein which in our laboratory has been refractory to expression in *E. coli*, yeast and Sf9 cells infected with baculovirus: PfTRAP. An optimised gene for PfTRAP (Hodgson *et al.*, in submission) was kindly cloned into the pOPINTT vector by Alexander D. Douglas to yield pOPINTT TRAP, epitope tagged with rat CD4, BAP, and a 6His tag (Fig. 3.1A). To measure transfection efficiency, pOPINTT TRAP plasmid was mixed with a second plasmid expressing non-secreted GFP in a 19:1 ratio, and fluorescent cells were counted microscopically 24 hours post-transfection. I compared two transfection reagents: Freestyle^{TM}MAX, a proprietary transfection reagent produced by Life Technologies Corp., and L25 polyethylenimine (PEI) [277]. Using the recommended Freestyle^{TM}MAX protocol and a published protocol for PEI transfection [237], transfection efficiencies of 5% with PEI and 20% with Freestyle^{TM}MAX were generally obtained. Both transfection reagents led to the production of detectable PfTRAP protein in culture supernatant (Fig. 3.1B). PfTRAP migrated with an apparent molecular weight of >100 kDa, despite a predicted molecular weight of 80 kDa. The increased transfection efficiency obtained with Freestyle^{TM}MAX was to some extent associated with higher expression of PfTRAP (Fig. 3.1B).

The Freestyle^{TM}MAX manual states that transfection efficiencies of 80% are achievable using this reagent. As sodium chloride inhibits the activity of Freestyle^{TM}MAX, I

hypothesised that the failure to achieve these efficiencies was due to sodium chloride present in the plasmid DNA elution buffer. However, eluting DNA in pure water or a salt-containing elution buffer had no discernible downstream impact on transfection efficiency or production of PfTRAP (Fig. 3.1C).

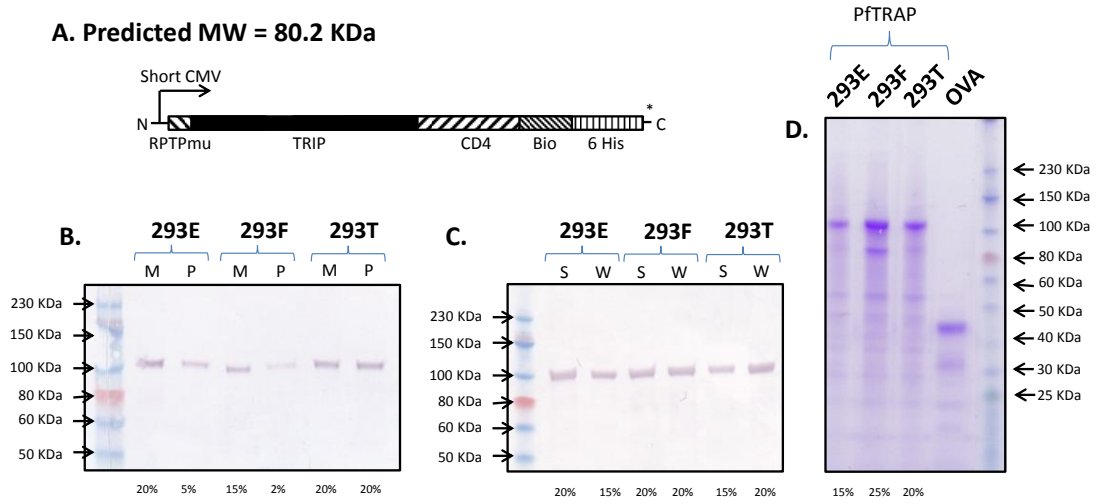

FIGURE 3.1: Initial establishment of the HEK293 system. (A) The PfTRAP construct used for the experiments in panels B, C and D (see Section Section 3.1.5 for key to abbreviations). (B) Western blot with anti-6His primary antibody. Transfections were performed in HEK293E, HEK293F and HEK293T cells using either L25 PEI (P) or Freestyle^{TM}MAX (M) as the transfection reagent. (C) Western blot as in panel B to determine whether the plasmid DNA elution solution affects protein yield (deionised water (W) or a salt-containing elution buffer (S)). (D) Coomassie-stained polyacrylamide gel. Supernatant from cultures of PfTRAP transfected HEK293E, HEK293F or HEK293T cells was concentrated 5-fold and loaded. The OVA band at 45 kDa was at 1mg/mL. Ladder is NEB Colorplus. Percentages below blots denote transfection efficiencies as assessed microscopically by counting GFP positive cells. * symbol denotes stop codon.

An additional variable that may improve protein yield is the use of Epstein-Barr Nuclear Antigen-1 (EBNA-1) expressing cells (HEK293E cells) or SV40 large T cell antigen-expressing cells (HEK293T) with plasmids that contain the Epstein-Barr virus OriP replicon or the SV40 virus origin of replication. These systems are reported to lead to replication of the plasmid within transfected cells and also aid nuclear import [237, 278]. The pOPINTT plasmid contains both of these DNA elements, and the OriP element has been shown to give a large improvement in protein production when using suspension HEK293E cells [237]. However, I was unable to detect any consistent differences between cell lines in protein expression when compared by semi-quantitative blot or gel (Fig. 3.1B–C). This may be because PfTRAP is produced so efficiently that the translation

machinery is already saturated. For future optimisation work, I continued using the HEK293E cell line as this seemed to produce the purest product (Fig. 3.1D), although at this stage the choice was essentially arbitrary.

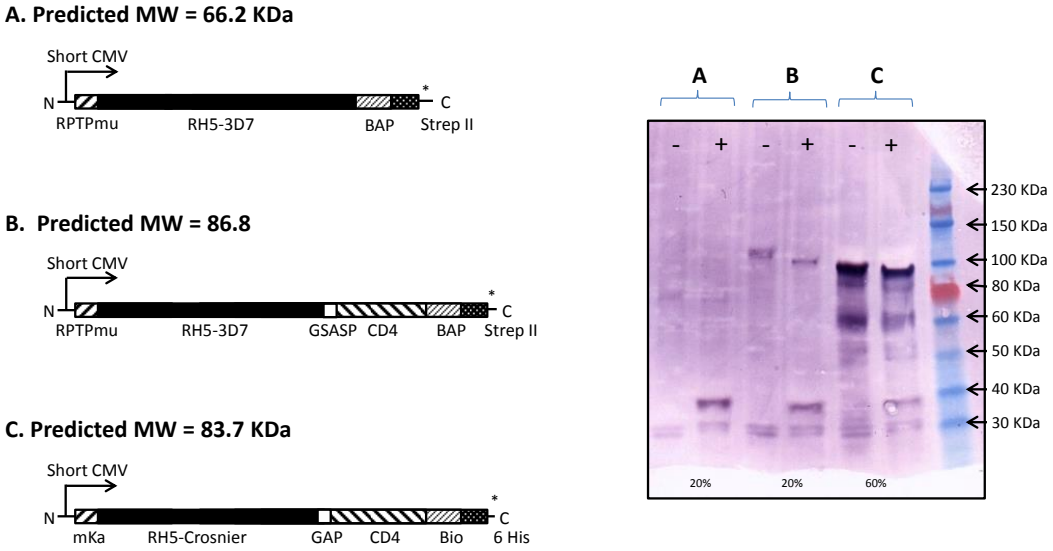

FIGURE 3.2: The effect of the rat CD4 tag epitope-tag on expression of PfRH5. Three constructs (A, B and C, see Section 3.1.5 for key to abbreviations) were transfected into HEK293E cells. Protein in the supernatants was detected by Western blot using anti-PfRH5 rabbit antibodies. + or – lanes show supernatant treated or untreated with PNGase F. Ladder is NEB Colorplus. Percentages below blots denote transfection efficiencies as assessed microscopically by counting GFP positive cells. A and B encode the PfRH5 polypeptide as it is encoded in the 3D7 parasite clone. C encodes the PfRH5 polypeptide as it is encoded in 3D7 parasite clone, but for four T to A amino acid substitutions to remove N-glycosylation sequons. * symbols denote stop codons.

I next attempted to express PfRH5 in HEK293E cells. Using the pOPINTT plasmid backbone, including the short hCMV promoter and the RPTPmu secretory signal, an expression plasmid encoding full-length PfRH5, C-terminally tagged with a biotin acceptor peptide and a strep-tag was generated and termed pOPINTT RH5.BAP.Strep (Fig. 3.2A). Unfortunately this expressed very poorly as compared to PfTRAP (Fig. 3.2) and it was impossible to purify any useful amount of recombinant PfRH5 using a streptactin column (less than 5 µg from a 150 mL culture of HEK293Es grown in Freestyle^{TM}293 medium). As Crosnier *et al.* have successfully produced PfRH5 tagged with rat CD4 domains 3 and 4 in HEK293E cells [128, 279], it was hypothesised that the presence of this 20.4 kDa scaffold might aid protein expression or secretion. The rat CD4 tag was cloned downstream of the PfRH5 coding region (Fig. 3.2B) and this significantly enhanced expression of PfRH5. Interestingly, whilst the predicted molecular weight of

this chimeric protein is 86.8 kDa, it migrated at approximately 100 kDa. This may be due to the presence of glycans attached to both the PfRH5 domain and the rat CD4 domain.

Cécile Crosnier kindly provided her PfRH5 expression plasmid, pTT RH5.CD4.Bio.6His (Fig. 3.2C). Using this plasmid, it was found that the product also migrated more slowly than expected (Fig. 3.2C), although to a lesser extent than for my construct pOPINTT RH5.BAP.Strep. This may be because the PfRH5 sequence in the native 3D7 construct (Fig. 3.2B) contains four predicted N-linked glycosylation consensus sites which are not mutated, whereas the Crosnier sequence contains A $\rightarrow$ T substitutions at these four sites (Fig. 3.2C). Thus, my construct may be more glycosylated. Consistent with this, PNGase treatment resulted in a larger change in electrophoretic mobility for my construct than for the Crosnier construct. However, even with PNGase treatment, both constructs had a higher apparent molecular weight than would have been predicted by their primary sequences. This may well be because PfRH5 has a grand average of hydropathicity score of -0.831, which is unusually low [280]. This is likely to impede the binding of SDS, resulting in a higher mass/charge value and decreased electrophoretic mobility. (For further information on plasmid design, see Section 3.2.5).

3.2.2 Improvements in cell culture and growth medium

Experiments designed to inform an optimised protocol for HEK293 culture and transfection were bedevilled by large natural variation in cell growth rates, amenability to transfection, and recombinant protein expression levels. For example, a single culture, just prior to transfection, was split into three equal volumes, which were transfected with three equal volumes of DNA and transfection reagent. 84 hours later, percentage transfection efficiencies were measured to be 30%, 25% and 10% in the three identically treated cultures.

In spite of the high level of noise, over 2 years of culturing HEK293 cells in suspension it was observed that the health of the culture in the days prior to transfection was the single most important factor to influence transfection efficiency and subsequent protein yield.

Culture health was primarily monitored microscopically, first by counting the proportion of viable cells using trypan blue, and second by assessing the tendency of cells to form clumps of more than 5 cells. Simple elements of cell culture, such as ensuring that all medium was thoroughly warmed up to 37°C prior to addition to the cells, were found to markedly improve transfection efficiency. This effect was particularly pronounced with respect to transfections using PEI as the DNA carrier (Table 3.2), suggesting that the mechanism of DNA carriage by PEI is more dependent upon culture health than the mechanism of carriage by Freestyle™MAX (see Section 3.3.2).

Table 3.2: Influence of serum on transfection and protein yields

	Experiment 1 (SERA5)	*Experiment 2 (PTRAMP)*	
	Transfection efficiency	Transfection efficiency	Purified protein
Freestyle + 293MAX	30%	33%	360 µg
Freestyle + PEI	60%	60%	360 µg
Freestyle (1%serum) + MAX	14%	-	-
Freestyle (1%serum) + PEI	60%	95%	1070 µg

Durocher *et al.* [237] have reported that expression of recombinant protein from mammalian cells growing in serum-free medium can be dramatically improved by the addition of 1% serum. Life Technologies® claims that serum-free Freestyle™293 medium is optimised for growth of HEK293 cell and CHO cells, and it was unclear if addition of 1% serum would improve yields in this advanced medium. In my hands addition of 1% FCS reduced the propensity of suspension HEK293E cells to form clumps, tending instead to grow as bunches of <4 cells. While cultures supplemented with serum were on occasion more readily transfected with PEI-DNA complexes, they appear to be less readily transfected by Freestyle™MAX-DNA complex (Table 3.2). Freestyle™MAX is a cationic lipid-based transfection reagent [281], a class of transfection reagent whose efficiency is thought to be diminished by the presence of proteoglycans [282, 283]. Given that serum contains a variety of proteoglycans, this decrease in transfection efficiency by Freestyle™MAX might have been expected. Importantly, and in line with the findings of Durocher *et al.* [237], the observed modest increase in transfection efficiency when using PEI in medium supplemented with 1% serum was paired with a more dramatic

increase in recombinant protein yield (Table 3.2). This suggests that the presence of serum results in an increased output of recombinant protein per transfected cell.

Late in 2012, Life Technologies®released a new line of HEK293 protein expression cells and reagents under the banner Expi293™. This system comprised a new culture medium, specially adapted cells, new transfection reagents and an expression enhancer reagent to be added to a culture 24 hours after transfection. The cost of the proprietary transfection reagents and cell lines was prohibitive to a moderate-scale protein production pipeline. However I felt that the medium, at double the price of Freestyle™293 (£150/L versus £75/L for Expi293 and Freestyle™293, respectively), might be a justified expenditure as it is designed to support four times higher cell densities than Freestyle™293 medium. In several independent attempts to express and purify PfAARP.BAP.Strep (see Chapter 5), the final yield of purified recombinant protein from cultures grown in Expi293 was double that from cultures grown in Freestyle™293 (Fig. 3.3). As this meant that culture volumes could be effectively halved to achieve equivalent protein yields, with corresponding reductions in incubator capacity and flask usage, Expi293 medium was used for all future protein expression work.

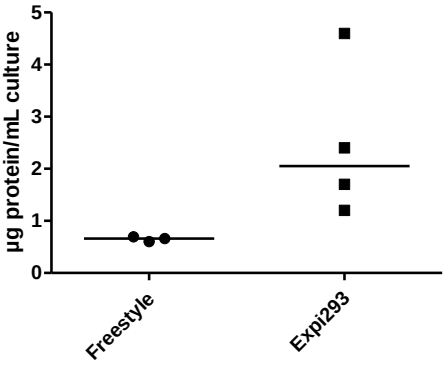

FIGURE 3.3: Final yields of purified protein from HEK293 cultures grown in different media. The same plasmid, encoding tPA.PfAARP.BAP.Strep under the control of a CMV promoter, was transfected into HEK293E cells grown in either Freestyle™293 medium or Expi293 medium (see text or Results and Materials and Methods for details). Culture supernatants were harvested, dialysed and the protein was purified using streptactin resin. As culture volume varied (150 mL – 250 mL), yield is expressed as the amount of protein obtained per mL of original culture volume. Each point represents an independent expression and purification; lines, medians.

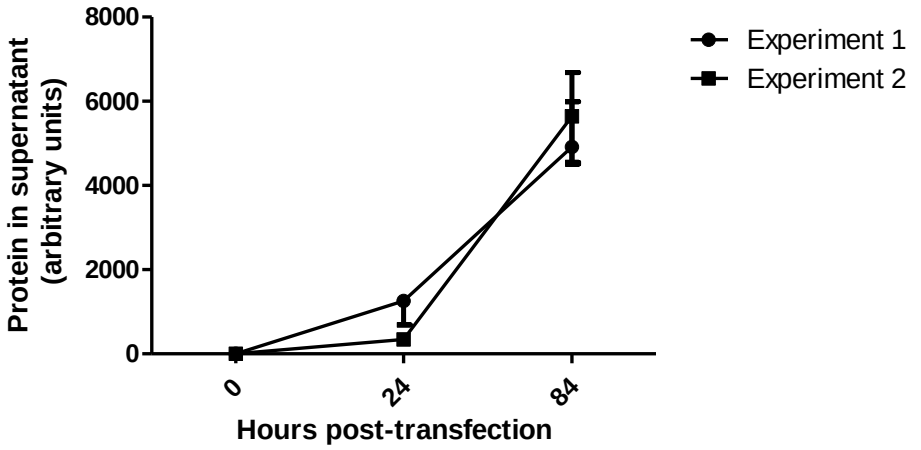

FIGURE 3.4: PfRH5 expression, as measured by anti-PfRH5 sandwich ELISA, increases over an 84-hour period. Results of two independent experiments are shown. Error bars represent the range of replicate wells in the anti-PfRH5 sandwich ELISA.

3.2.3 Kinetic of protein expression

An important variable when producing protein in HEK293E cells is timing the harvest of the supernatant. Durocher *et al.* [237] and the Freestyle^{TM}293 manual [284] report that protein levels can continue to rise for up to six and seven days after the day of transfection, respectively. In my experiments, PfRH5 concentration rose steadily over the first 84 hours after transfection (Fig. 3.4). However in my hands, regardless of the plasmid used for transfection, cell viability fell sharply between 84 hours and 108 hours from >98% viable to <30% viable. This is in accordance with a study demonstrating an almost complete depletion of L-glutamine and a complete depletion of glucose 3 days after transient transfection of suspension HEK293s (albeit using a different initial medium composition) [285]. Many recombinant proteins are resistant to protease degradation, and the presence of proteases released from dead or dying cells may not negatively impact subsequent protein yield. However, as the objective of this work was to establish a robust platform for expressing multiple proteins with little or no protein-specific optimisation, for routine preparations harvesting was carried out at 84 hours post-transfection.

3.2.4 Influence of PEI structure upon transfection efficiency and protein production

See also Chapter 6, Section 6.3.7

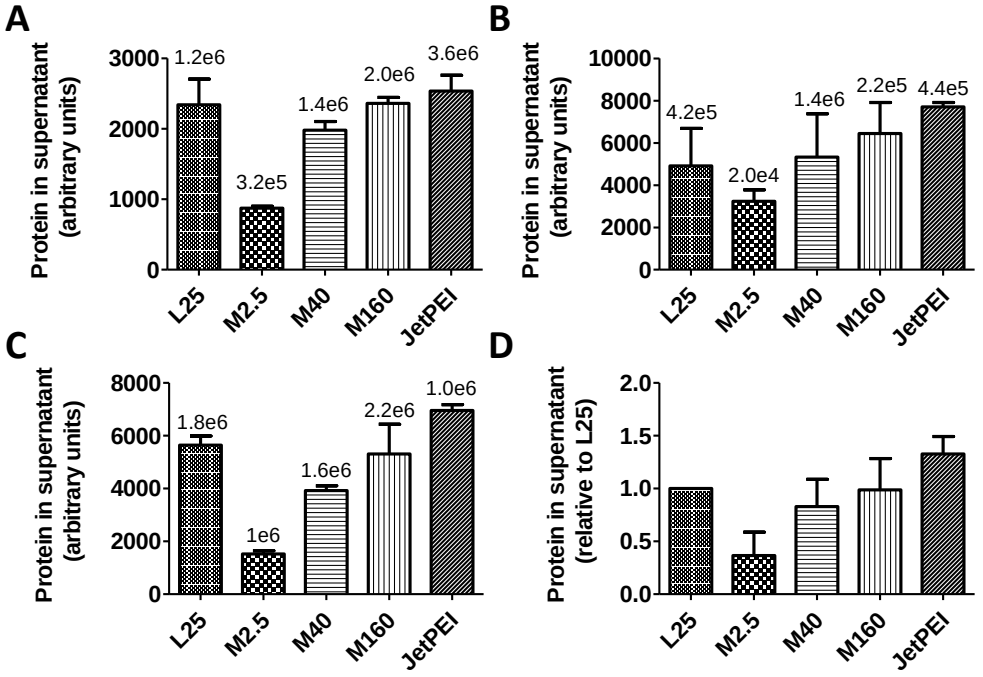

FIGURE 3.5: The influence of diverse forms of linear polyethylenimine on *in vitro* transfection efficiency. HEK293E cultures were grown in Expi293 medium and transfected with a DNA plasmid expressing secreted PfRH5. Supernatant was sampled 84 hours later when cells were still >95% viable and PfRH5 in the supernatant was quantified by sandwich ELISA (see Section 2.4.3). (A,B and C) PfRH5 concentration in supernatants from cultures transfected with four different forms of PEI (see Section 2.3.3). Each graph depicts results from an independent experiment. The height of bars represents the mean of three replicate wells on an ELISA plate and error bars represent the range from replicate wells. Numbers above bars represent the density of GFP+ve cells per mL of medium as assessed microscopically by counting GFP positive cells. Pooled data from the three experiments depicted in panels A–C is shown in (D). For each experiment, the concentration of PfRH5 in the supernatants of the culture transfected with linear 25 kDa PEI was defined as 1, and the concentration of PfRH5 in the other cultures was expressed as a fraction of this. Height of bars, mean of three independent experiments; error bars, range.

In light of the findings in Section 3.2.2, PEI was selected as the transfection reagent for all subsequent work. Various methods have been devised to produce PEIs of differing length and structure [236, 286]. Linear PEIs are generally less toxic and yield higher transfection efficiencies than branched PEIs [287]. Theoretically, the best predictor of the performance of a PEI as a DNA carrier is the extent to which the nitrogen atoms are available to become protonated. Contamination of the linear PEI chain

by unwanted functional groups that block nitrogen protonation is thought to decrease transfection efficiency, and efforts have been made by various academic groups and chemical companies to produce PEIs that contain a maximal proportion of protonable nitrogens. In another series of experiments, I compared the transfection efficiency and subsequent protein yield resulting from linear 25 kDa PEI from Alfa Aesar (USA) with the 'MAX' range of PEIs produced by Polysciences (Fig. 3.5, see Section 2.3.3 for detail of formulation). Keeping the N/P ratio constant (2 mg of PEI for every 1 mg of DNA, see Section 2.3.3), 25 kDa linear PEI consistently performed comparably to 160 kDa 'MAX' PEI, the best of the 'MAX' PEIs tested (Fig. 3.5), and even performed comparably to the proprietary reagent *in vivo* JetPEI (for more information on this reagent see Chapter 6, especially Sections 6.2.1, 6.3.7, 6.4.5 and Fig. 6.9). On this basis, linear 25kDa PEI was used for all subsequent transfections of HEK293E cells for protein expression.

3.2.5 Plasmid design

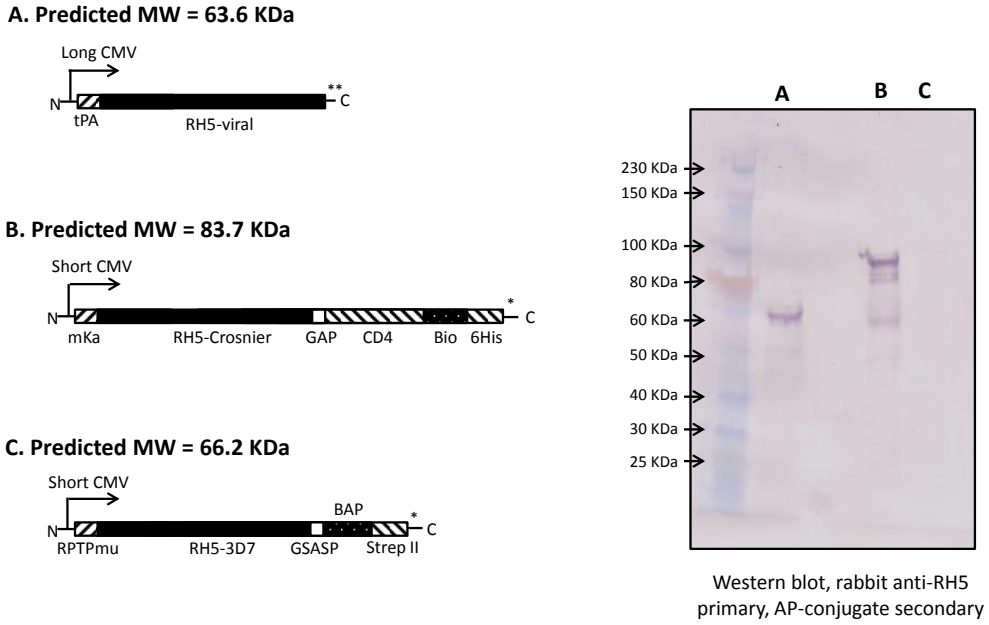

FIGURE 3.6: The effect of the long version of the hCMV promoter on expression of PfRH5. Three constructs (A, pENTR4 LP RH5; B, pTT RH5.CD4.Bio.6His; and C, pOPINTT RH5.BAP.Strep; see Section 3.1.5 for abbreviations) were transfected into HEK293E cells. Protein in the supernatants was detected after 84 hours by Western blot using anti-PfRH5 antibodies raised in rabbits and an alkaline-phosphatase conjugated secondary antibody (see Materials and Methods). Ladder is NEB Colorplus. Percentages below blots represent the transfection efficiency for each culture. * symbols denote stop codons.

Without modification to the polypeptide-encoding region, it is possible to dramatically improve the level of recombinant protein expression by including additional sequence elements in a vector [278, 288–290]. Plasmids incorporating different elements can produce large differences in protein yield. Using pOPINTT, it was not possible to produce any detectable PfRH5 protein when the rat CD4 tag was not present (Fig. 3.2). However, the immunogenicity of adenovirus and MVA vectors encoding un-tagged PfRH5 [133, 136] suggests that mammalian cells are capable of expressing this protein *in situ* without rat CD4 fused to the C-terminus. Given these and the earlier results in Fig. 3.2, plasmid design was further explored. The adenovirus entry vector pENTR4 LP RH5, which had been used as an intermediate to shuttle genes of interest into the adenovirus genome, was transfected into HEK293E cells and the level of PfRH5 found in the supernatant was compared to that from cultures transfected either with pOPINTT RH5.BAP.Strep or pTT RH5.CD4.Bio.6His (Fig. 3.6). As assessed by Western blot, pENTR4 LP RH5 yielded a similar concentration of untagged CD4-free PfRH5 as pTT RH5.CD4.Bio.6His (Fig. 3.6). On the basis of this result with PfRH5, and the useful possibility of this plasmid behaving as a dual use vector, capable both of acting as a recombinant adenovirus shuttle vector and a protein expression plasmid, pENTR4 LP was selected as the primary vector for expression of malaria proteins (see Section subsec:ProtDiscElem for further discussion).

The results in Fig. 3.1 indicated that HEK293E cells that carry a gene expressing EBNA-1 do not appear to produce any more PfTRAP from a plasmid containing the OriP element than HEK293F cells transfected with the same plasmid. However, this result had several caveats that made the OriP element worth revisiting with a more difficult protein such as PfRH5. First, at the time of the initial experiments it was not appreciated that compared to other malaria proteins, PfTRAP is expressed unusually well from HEK293 cells. Second, the initial experiments relied on HEK293F cells as a comparator, with the implicit assumption that HEK293E cells would give equivalent yields to HEK293F cells from a plasmid that does not contain the OriP element. The protein expression cassette from pENTR4 LP (shown in Fig. 3.6A) was thus cloned into the commercial plasmid pCEP4 (which contains the OriP element) [291] to yield pMOD. This plasmid was then transfected into HEK293 cells, and recombinant PfRH5 protein in the culture

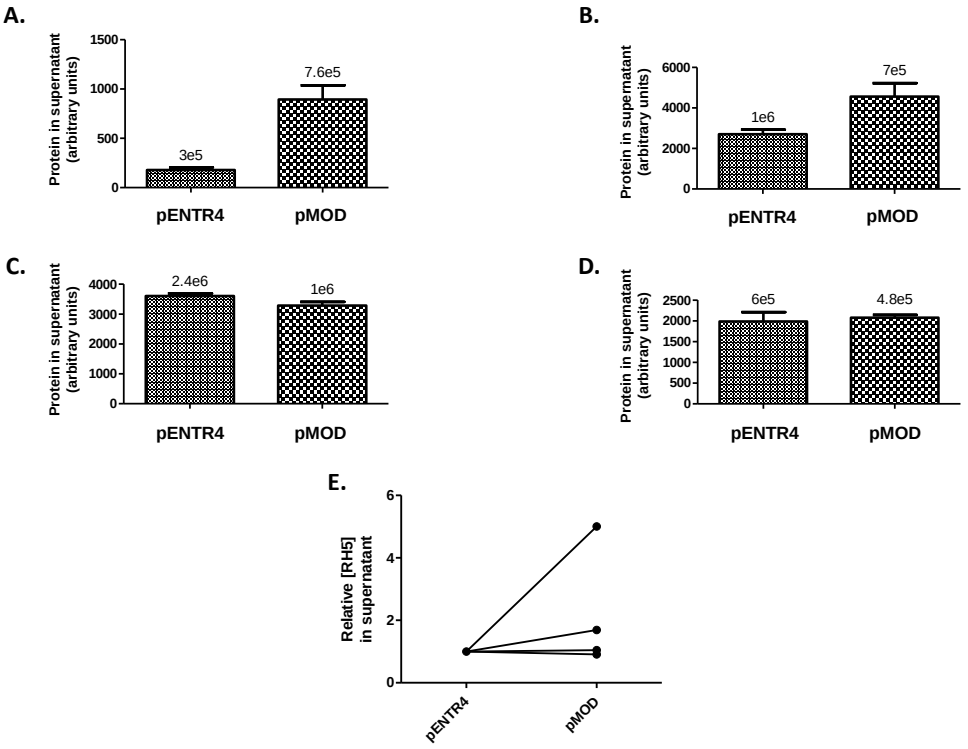

FIGURE 3.7: Expression of PfRH5 from the expression cassette shown in Fig. 3.6A, cloned into pENTR4 and a modified plasmid pMOD, which is based on the commercial plasmid pCEP4. (A–D) Concentration of PfRH5 in HEK293E culture supernatants 84 hours after transfection, from four independent experiments. The density of GFP +ve cells (as measured microscopically) per mL of medium is shown above bars. Error bars represent the range of replicate wells in the anti-PfRH5 sandwich ELISA. (E) Summary of results from panels A–D, with the PfRH5 concentration in the supernatant of the pENTR4 supernatant defined as 1 and the concentration of PfRH5 in the pMOD supernatant expressed as a fraction of this.

supernatant was quantified by capture ELISA (see Section 2.4.3). Over four independent experiments pMOD generally outperformed pENTR4 LP, although substantial noise in the system meant that the difference was not statistically significant (Fig. 3.7). However, given the labour and material costs associated with expression and purification of difficult proteins, these results provide a good rationale for further investigating the pMOD vector and its potential adoption as the standard HEK293E protein expression plasmid in our laboratory.

3.3 Discussion

A platform designed to screen antigens for their ability to elicit functional anti-parasitic antibodies must conform to a number of criteria. Minimising false negative results is paramount. In this regard, protein-in-adjuvant is the preferred platform for immunisation, because it allows a controlled dose of antigen. Correctly folded protein is also important, as the highest affinity (and most functional) antibody clones tend to recognise conformational epitopes. It is also desirable to elicit antibodies that bind across the full length of the protein, because only certain regions of the protein may be vulnerable to neutralisation and in the absence of detailed parasitological studies it is impossible to predict fragments of interest. Speed of production is also important. Protein antigen production in HEK293 cells is the only established platform that meets these criteria with a reasonable success rate for different proteins [225]. An important additional factor, where the suspension HEK293 system did pose some initial disadvantages, was cost effectiveness (for a full comparison of the three antigen-screening platforms considered in this thesis, see Section 6.5 and Table 6.1).

3.3.1 Suspension cells

The work in described in this Chapter was undertaken with the aim of establishing a versatile platform for producing a variety of secreted *P. falciparum* proteins in our laboratory, without needing a large scaffold tag such as CD4 domains 3 and 4. Accordingly, I sought at the outset of this thesis to optimise protocols to maximise yield in a non-protein-specific manner. It should be noted that up to this point, all vaccine development efforts in the group (and the Institute as a whole) had focused on viral vectored delivery and not production of recombinant malaria protein antigen. Effort was focused on enhancing the health and cell density of the HEK293 cell culture, improving the efficiency of the transient transfection process, and maximising the potency of the protein expression plasmid. I was also able, through some optimisation, to substitute the most expensive reagent in the protocol, Freestyle^{TM}MAX, with linear PEI—a far more affordable alternative.

Aside from the reduced labour associated with suspension culture as compared to adherent cell culture, it is possible to obtain higher yields of recombinant protein from cells grown in suspension [285]. Serum-free medium was preferred because of the tendency of serum proteins to complicate subsequent protein purification. The total protein concentration of serum is 60–70 mg/ml [292, 293], meaning that a standard RPMI- or DMEM-based medium containing 10% serum contains 6–7 mg of contaminant protein per ml of supernatant. Given that in my hands very few *P. falciparum* proteins expressed at more than 10 µg/ml of supernatant, this would require an enrichment factor of >600 to obtain a 50% pure sample. Although achievable with a multi-step protocol, this would be extremely difficult with a single-step purification process. By contrast, unconditioned Expi293 medium contains no protein at all, meaning that isolation of protein from cells grown in this medium at reasonable purity (>75%) is far less demanding using a single-step protocol.

3.3.2 Transfection efficiency and protein yield

Initial work, possibly carried out using sub-optimal culturing technique, appeared to show a consistent advantage for the cationic liposome formulation Freestyle^{TM}MAX over PEI. It was only after I was able to improve the growth-rates and overall health of the HEK293 cultures that I began to achieve high transfection efficiencies using PEI. Both PEI and cationic liposomes (such as Freestyle^{TM}MAX) use endocytosis as their primary mechanism for delivery of DNA to the host cell cytoplasm [294–297], but it has been suggested that cationic liposomes deliver DNA into cells via direct fusion with the plasma membrane [298]. This may mean a less healthy culture of cells that is not endocytotically active can still be transfected to a low level by Freestyle^{TM}MAX, accounting for the apparent superiority of Freestyle^{TM}MAX in earlier experiments. Only when the parameters for a healthy culture of suspension HEK293 cells were established could the additional gene delivery advantages of PEI (such as enhanced nuclear import [299, 300]) come to light.

Transfection efficiency, as measured microscopically by counting GFP positive cells, associated only weakly with protein output as measured by sandwich ELISA (Fig.

3.5). This is surprising, as more transfected DNA presumably leads to more copies of the relevant mRNA in the cytoplasm, which is known to correlate positively with recombinant production in mammalian cells [301]. This suggests that additional effort to improve transfection efficiencies using linear PEI to achieve 'more of the same' is unlikely to yield substantial improvements in protein yield. The inclusion of the Epstein-Barr virus OpiP element also had a disappointingly small effect on PfRH5 yield after transient transfection (Fig. 3.7). In the presence of the EBNA-1, this element is thought to aid in nuclear import of the plasmid, and also to lead to its replication in the nucleus [278, 302]. The net effect is analogous to improved transfection efficiency: more copies of the same expression cassette are transported to the nucleus. It may be that *PfRH5* mRNA under the control of the long CMV promoter is translated sufficiently slowly by the HEK293 protein expression machinery that all available ER-resident ribosomes are occupied even at comparatively low transfection efficiencies, so that delivering more PfRH5 expression cassettes to the nucleus has little effect. It is also unclear why fusion of rat CD4 to the C-terminus dramatically improved expression of PfRH5 (Fig. 3.2) and whether the effect may be specific to PfRH5. For this reason it was not investigated further.

3.3.3 Elements that improve expression of PfRH5 recombinant protein

Overall, this work did succeed in establishing a platform for expression of *P. falciparum* proteins in our laboratory. However there are still unanswered questions and areas that could benefit from further optimisation. The construct shown in Fig. 3.6A, pENTR4 LP RH5, yielded significantly more recombinant PfRH5 than that in Fig. 3.6C, pOPINTT RH5.BAP.Strep, for reasons that are not fully clear. An obvious explanation is that the construct in Fig. 3.6A contained the PfRH5 gene under the control of a CMV promoter containing intronA [226] and a tripartite leader. The theoretical benefits of including a promoter-proximal intron in eukaryotic expression constructs are well understood [303] and include enhancement of transcription [304] as well as improved mRNA export from the nucleus [305]. The use of different signal peptides [306–308] (Fig. 3.6A and C) may also have influenced protein yields. An alternative explanation is that the 20

amino acid C-terminal appendage constituted by the BAP and Strep tags in pOPINTT RH5.BAP.Strep impinges on the translation and secretion of PfRH5, and that the rat CD4 tag in pTT RH5.CD4.Bio.6His somehow mitigates this. Yet another factor that may partially account for the superiority of pENTR4 LP RH5 is the presence of a dual opal codon, which may reduce stop codon read-through. The eukaryotic stop codon is thought to consist of a four-nucleotide consensus sequence [309, 310] and suboptimal fourth nucleotide can lead to inefficient translation termination and reduced protein yields [285].

3.4 Future Work

There remain some potential modifications to the HEK293 protein production protocol which should be tested. The simplest of these is the addition of a tranfection enhancer such as sodium butyrate [311] or valproic acid [312] to the cells shortly after transfection. These compounds interfere with the packaging of plasmid DNA into chromatin and hence improve gene expression from the recombinant plasmid. The parent plasmid of pMOD, pCEP4, is also designed for the development of stably episomally transfected cell lines [278, 291]. As well as carrying an Epstein-Barr virus OriP sequence which allows for some plasmid replication, the plasmid carries a gene encoding a kinase that phosphorylates hygromycin, thereby inactivating it. This means that transfected cells have a selective advantage in the presence of hygromycin, thereby allowing the selection of cells carrying the plasmid. It would be worthwhile to establish a protocol in our laboratory for selecting transfectants with the use of hygromycin.

3.5 Summary of Method

To summarise the standard operating procedure as of February 2014:

- For general protein-production work HEK293E cells are used.

- For routine culture, cells are grown in Expi293 medium at a maximum density of 1.2×10^7 cells/ml and split twice weekly to a density not lower than 1×10^6 cells/ml.
- For transfections, cells were split to 1×10^6 cells/ml 24 hours prior to transfection. For every ml of culture to be transfected, 1 µg of DNA was pre-mixed with 2 µg of L25 PEI from Alpha Aesar (see Section 2.3.3) in 40 µl before addition to the culture.
- Protein-containing supernatants are generally harvested 84–96 hours after transfection.
- All protein-expression plasmids contained the gene of interest under the control of a CMV promoter containing intron A, typically pENTR4 LP.
- A variety of C-terminal tags remain under investigation.

Chapter 4

A merozoite antigen screen

Authorship statement

I selected the antigens for inclusion in the antigen screen, optimised the antigen screening pipeline, designed the pre-clinical vaccine antigen constructs and conceived all the experiments described in this Chapter. The only exceptions to this are the vaccines against EBA-175, RAP1, MSP3 and MSP4 which were designed by Alexander D. Douglas, produced by the Jenner Institute virus vector core production facility, and then tested by me.

All of the experimental procedures described in this Chapter were performed by me or under my direct supervision. Specific contributions by other laboratory workers were as follows: Daniel G. Alanine expressed and purified the proteins shown in Fig. 4.7, panels B and C following protocols devised by me; Rebecca E. Brown collected some of the IFA images in Fig. 4.8 and also performed the parasitological elements (Section 2.7.4) of the GIA assays shown in Fig. 4.10 panel C and Fig. 4.12 panel C.

4.1 Introduction

Until recently, blood-stage malaria vaccinologists had accepted the paradigm that invasion of RBCs by *P. falciparum* merozoites was mediated by multiple invasion ligands with redundant activity [134, 216], and that those proteins that were essential to parasite survival were highly polymorphic [313]. By such mechanisms did the parasite evade host humoral immunity. The discovery that strain-transcending neutralisation can arise following immunisation with just a single antigen, RH5, was therefore unexpected [133]. The work in this Chapter was designed to find a second antibody specificity with the following attributes: first, potent and broad neutralisation of *P. falciparum* laboratory isolates, and second, synergistic interaction with antibodies against PfRH5.

4.1.1 Vaccinological models of *P. falciparum*

The *P. falciparum* genome encodes over 5000 proteins [227], and somehow these gene products must be prioritised for inclusion in a subunit vaccine. At the core of any antigen screening programme is an assay that predicts the protective value of antigen [314–316]. Ideally, a full Phase II clinical trial would be performed using every possible vaccine antigen. However, this is financially and ethically not justifiable. A step below this would be non-human primate challenge studies using the same species of pathogen that causes the human disease, such as challenge of *Aotus nancymaae* monkeys with the FVO strain of *P. falciparum*. More practically, malaria vaccinologists have used rodent models of malaria to study immunity (using species such as *P. yoelli*, *P. berghei* and *P. chabaudi*) for around 50 years [317, 318]. However, differences between the course of disease progression in murine models and human infection have led clinical scientists to question the utility of mouse malaria as a research tool [319]. From a parasitological standpoint, murine models are of limited utility—the fact that a given protein is essential to the survival of one species does not necessarily extrapolate to include orthologous proteins in other species. Thus, while the presence of the Duffy antigen (the ligand for the *P. vivax* Duffy-binding protein) on reticulocytes is thought to be essential to the survival of *P. vivax* [320], *P.falciparum* contains four proteins that are orthologous to

PvDBP [321] which appear to have degenerate activity [134]. Likewise, PfRH5, although seemingly essential for *P. falciparum*, does not have any easily identifiable orthologues in other species of *Plasmodium*, including the rodent species, except *P. reichenowi* [218]. For these reasons, our laboratory has shifted focus towards cultured *P. falciparum* as a disease model.

4.1.2 Correlates of protection from clincal malaria

The field has devised a number of *in vitro* assays that detect that anti-parasitic activity of an antibody sample against cultured asexual *P. falciparum* through a variety of mechanisms, including ADCI [322–324], ADRB [108, 110] and GIA [247] (discussed in Section 1.5.5). Of these assays, the GIA assay is the most robust as it measures an activity whose mechanism does not require the addition of host immune cells. It is perhaps for this reason that it has become the most widely-used assay for measuring the activity of anti-merozoite antibodies, with a centralised GIA assay facility developed and funded by the PATH Malaria Vaccine Initiative based at the NIAID. However, it is far from clear that GIA of an antibody sample is predictive of protection from malaria for the antibody donor. Certainly, GIA does not appear to be the only mechanism by which natural immmunity to blood-stage malaria arises (discussed in Section 1.5.3) [106, 195]. This said, the mechanism of vaccine-induced immunity does not necessarily need to recapitulate the mechanism of natural immunity. Furthermore there is evidence that the GIA of an antibody sample can predict parasite growth rate in both animal models (A. Douglas, personal communication, and [325]), and to a lesser extent humans [120, 125, 126]. As far as I am aware, there is no established conceptual reason why the mechanism of parasite killing in the GIA assay (primarily neutralisation of merozoite proteins that are essential to parasite survival) should not translate directly into parasite killing in the blood of an infected human (although one cannot exclude the possibility that the invasion mechanism used by parasites cultured *in vitro* is markedly different to that seen *in vivo*).

A further reason for using the GIA assay as a primary readout of protective value relates to the objective of the study. The aim of the work in this Chapter was to find an antibody

specificity that worked synergistically with antibodies against PfRH5. Synergy can have various meanings with reference to vaccines, not all of which are useful in the pre-clinical context. To take a hypothetical example, antibodies induced by the sporozoite-targeting vaccines RTS,S [68, 326] in combination with antibodies induced by an MSP3 vaccine [190, 191] may work together to reduce the incidence of clinical malaria in a population by more than would be expected given their efficacies when administered alone. If this were the case, the interaction would be defined as synergistic. It would be also entirely unpredictable based on pre-clinical evidence, as the two antibody specificities would exert their effects on separate stages of the life-cycle via different mechanisms that are not measurable in a single assay. Pre-clinically, the interaction between antibody specificities can only be described with any degree of confidence when the effects of both specificities are measurable in the same assay. The action of the novel antibody specificity to be combined with RH5 would therefore have to be measurable by GIA.

4.1.3 Antigen selection and reverse vaccinology

Shortlisting the antigens to be screened is a vital part of a study such as this. Over the last thirty years, parasitologists and vaccinologists have used a variety of techniques to identify and characterise proteins that are important in parasite survival [35]. These proteins are an obvious starting point when drafting the list of antigens to be screened. However, I also tried to draw from the work of Rappuoli and colleagues, who have for the past 20 years pioneered the concept of 'reverse vaccinology' [314, 327]. This is based upon the notion that the number of protein antigens encoded in the genome of a pathogen is finite, because once the genome has been sequenced, the sequence of every possible protein antigen can be predicted. This concept was first applied to *Neisseria meningitidis* serogroup B [314]. The genome sequence of this species revealed 2158 protein-coding genes [328], of which 570 were bioinformatically predicted to encode features of surface-exposed proteins (such as leader peptides, transmembrane domains and host cell binding domains). Pizza *et al.* successfully elicited antibodies against over 300 of these proteins, and assayed the activity of the antibodies [314]. The work

eventually led to the design [329] and eventually the licencing in Europe [330] of a multi-antigen vaccine. The *P. falciparum* genome sequence was published in 2002, defining just over 5000 protein coding genes [227]. Fortunately, an array of proteomic [331–333] and transcriptomic [145, 334] studies have been published and provide information that can be used to narrow down the list of candidate antigens (for example, proteins encoded by genes that are not expressed at the asexual stage are unlikely to constitute protective blood-stage antigens).

4.1.4 An antigen-screening pipeline

The pipeline that I designed for screening new antigens is shown in Fig. 4.1. Antigen screens tend to be similar in form. First, a shortlist of gene products to be screened is drafted. Generally this relies on bioinformatic analysis of the predicted proteins encoded in the genome, or where available, is based upon published microbiological or parasitological literature. The next step depends on the method by which antigen susceptibility is to be measured. In the case of assays that involve animal models of disease, such as the mouse model of neonatal *Streptococcus agalactiae* infection [327, 335], generation of pre-clinical vaccines followed by immunisation of an animal is unavoidable. However, the primary readout of protective value in the present antigen screen is the *in vitro* assay of GIA, which is (for the most part) mechanistically dependent upon neutralisation of parasite proteins. Theoretically, any high-affinity binding molecule, including small-chain variable fragments generated by phage display [336, 337] or nucleotide aptamers [338, 339], could be used to probe the susceptibility of parasite antigens to neutralising antibody. However, it remains time-consuming to produce binding molecules using these techniques, and animal immunisation remains the gold-standard for eliciting binding molecules of nanomolar affinity. For this reason I followed the standard practice of producing mammalian polyclonal antibody against my proteins of interest, necessitating the production of pre-clinical vaccine.

Although it was known that the PfRH5 gene was essential to parasite survival in multiple strains [218], the discovery that it was susceptible to neutralising antibody was delayed by several years. This is because large, properly folded fragments of PfRH5

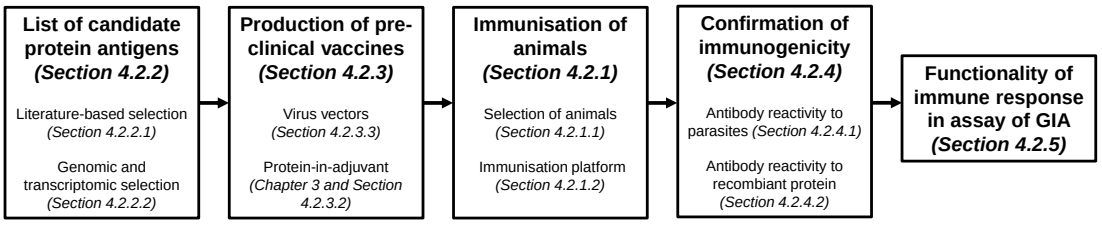

FIGURE 4.1: Pipeline for antigen screening.

could not be produced and refolded in the traditional systems for heterologous protein expression (namely yeast cells and *E. coli*), so the fragments that were included in the pre-clinical vaccines used to raise antibodies did not resemble PfRH5 as it appears in the parasite [218, 220, 223]. In a later study conducted in our laboratory using virus vectors to immunise animals we recapitulated the published negative results with one of the fragments, but went on to show that full-length PfRH5 can elicit potent growth-inhibitory antibodies [133, 218]. This discovery was enabled by recombinant virus vectors, which allow *in situ* expression of proteins from the cells of the vaccinated mammalian host. However, more recently, mammalian HEK293 cells have been shown to facilitate the production of full-length merozoite proteins [224, 225]. Given that one of these systems was already established in our laboratory [157], and having established the other myself (see Chapter 3), I planned a more comprehensive screen of antigens for inclusion in a blood stage malaria vaccine.

4.2 Results

4.2.1 Moderate-throughput antigen screening

In this antigen screen, the activity of antibodies in the *in vitro* assay of GIA is considered the primary correlate of protection. The induction of high-titre functional antibodies is therefore critical in order to minimise the possibility of false negative results (i.e. discarding a susceptible antigen because of a negative GIA result due to poor antibody quality or low antibody concentration). Having established a protocol for the production

of recombinant *Plasmodium* proteins in Chapter 3, it was necessary to optimise the protocol for the production of antibodies against said proteins.

4.2.1.1 Suitability of mice for antibody production

The ability of the adenovirus-MVA 10-week immunisation regime to reliably elicit robust concentrations of functional rabbit serum antibody to *P. falciparum* antigens, and the protocols for purifying and assaying the antibody, are well established in our laboratory [133]. However, the cage and handling costs alone for a 10-week immunisation schedule are around £300 per rabbit, or £900 for a dose group of three. An 8-group experiment (six test antigens and a positive and negative control group) would cost approximately £7200. This is prohibitively expensive for an academic project aiming to screen around 50 new vaccine antigens. Immunisation of mice represents a more affordable alternative. BALB/c mice cost £9.60 each, and a group of four mice costs £150 to house for 10 weeks. An 8-group mouse immunisation experiment with four mice per group therefore costs approximately £1500.

Before commencing antigen screening in mice I wanted to test the technical feasibility of purifying and assaying mouse antibody. The small volume of serum that can be obtained from a single mouse (typically no more than 400 µL) and the typical serum antibody concentration of 2 mg mL^{-1} makes purification of the requisite 1 mg of antibody difficult (this is the minimum amount of non-antigen-specific polyclonal IgG required to be reliably tested in the GIA assay). However, if serum from a group of 4 mice is pooled, the assay becomes straightforward. Assuming a typical serum yield of 1.5 mL per group of four mice, only a 30% yield is necessary, which is readily achievable with protein G resin. It was also not clear that mouse antibodies would perform comparably to rabbit antibodies. The mechanism by which rabbits generate immunoglobulin diversity differs from that seen in humans and mice [340, 341], and rabbits are known to have longer CDR regions which are the primary determinant of an antibody's affinity for its antigen. These differences likely contribute to the differential anti-parasitic activity of antibodies produced in mice and rabbits, as observed for the antigens PfAMA1 and PfMSP1 in the assay of GIA [247] and for Pfs25 in the standard membrane feeding assay [342].

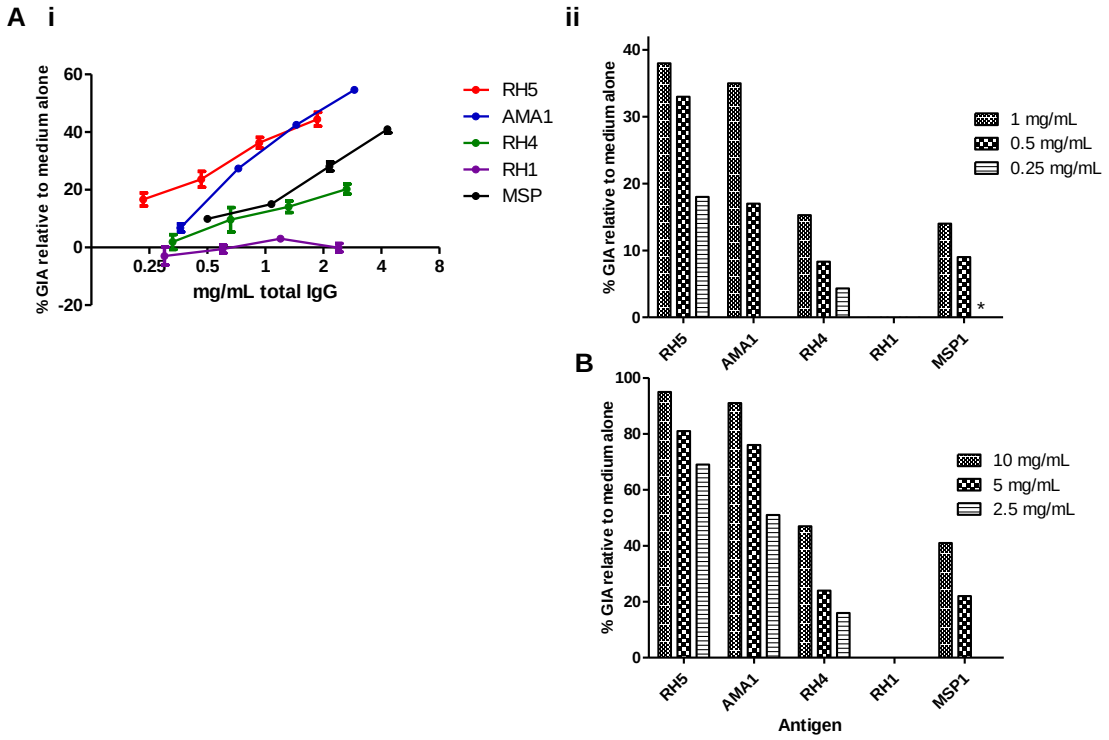

FIGURE 4.2: Rabbit and mouse antibodies give similar results in the *in vitro* assay of GIA. (A) Groups of female BALB/c mice (n=4) were immunised in a standard heterologous prime-boost adenovirus-MVA immunisation regime (Section 2.5.1). At day 70, serum IgG was harvested and assayed for GIA. To allow comparison to previously published GIA data with rabbit antibodies [133] (B), the results in (Aii) were interpolated from the dilution series shown in (Ai). * denotes a value that could not be confidently interpolated from the dilution series in (Ai).

To address these concerns, I immunised mice with a panel of virus vectors shown to elicit antibodies of varying neutralising capacity in a previously-published rabbit study [133]. Sufficient IgG to perform several replicate assays was obtained. Although the mouse antibodies did not reach such a high level of neutralisation as the rabbit antibodies, reassuringly the hierarchy of antigen susceptibility was identical between species (Fig. 4.2). In light of this result, all subsequent exploratory antigen and construct screening experiments were performed with mice.

4.2.1.2 Choice of adjuvant for pre-clinical protein-in-adjuvant immunisation

Live adenovirus and MVA vectors can be injected into mammals with no additional adjuvants and elicit a strong immune response. This is because adenovirus and MVA encode

a remarkably diverse range of molecules that are detected by the host's innate virus-sensing machinery [343–345]. On the other hand, most proteins are poorly immunogenic when administered alone without the addition of an adjuvant. Preparation of Freund's adjuvant, the most commonly used pre-clinical adjuvant, involves the emulsification of an aqueous antigen with mineral oil, which can be administered alone (Incomplete Freund's adjuvant) or further potentiated by the addition of inactivated dried mycobacterial components (complete Freund's adjuvant) [346]. Although developed almost 80 years ago, Freund's adjuvant system remains one of the most potent adjuvants available. Unfortunately a number of drawbacks make it unsuitable for a modern antigen screen. First, is is highly reactogenic, causing painful reactions and swelling that can last for many months and cause suffering to laboratory animals. Second, the high oil content of Freund's adjuvant (85% paraffin oil, 15% mannide monooleate, a surfactant) makes it an efficient disruptor of protein tertiary structure. Indeed, lysozyme and β-lactamase, proteins noted for the stability of their three-dimensional conformation, have been shown to rapidly denature and lose their biological activity in the presence of Freund's adjuvant [347, 348]. Paus *et al.* went on to demonstrate that formulation of β-lactamase with Freund's adjuvant leads to the evolution of antibodies that bind predominantly to internal linear epitopes that are not accessible in the correctly folded protein as it appears *in vivo* [348]. AbISCO®-100 is a less reactogenic adjuvant that has been shown to elicit comparable serum antibody concentrations to Freund's adjuvant [349]. Because AbISCO®-100 is currently in clinical development, supply is tightly controlled and it is now difficult to obtain this adjuvant for pre-clinical use. I therefore wondered if the pre-clinically adjuvant Addavax™ could be used in place of AbISCO®-100 or Freund's adjuvant. This adjuvant has been reported to induce potent immune responses in numerous studies [350–352], and is freely and commercially available. The composition of Addavax™ is based upon the Novartis clinical adjuvant MF59®: when formulated, it contains 2.5% squalene oil, 0.25% sorbitan trioleate, and 0.25% Tween 80. This represents a considerably less denaturing environment than Freund's adjuvant.

A study to assess the comparative immunogenicity of AbISCO®-100 and Addavax™ was devised by Simone de Cassan and performed by Rebecca Hillson (Fig. 4.3). Two weeks after the final immunisation, I measured the serum antibody titre by ELISA and

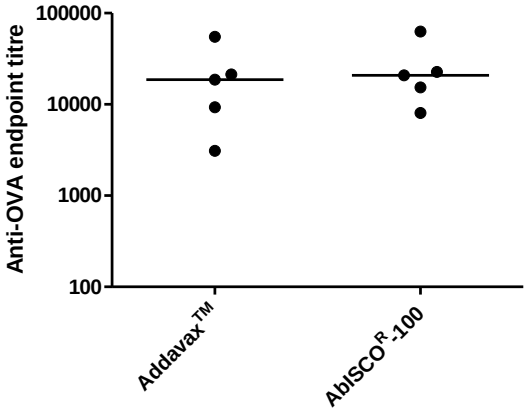

FIGURE 4.3: Addavax™ pre-clinical adjuvant is a licence-free alternative to AbISCO®-100. Female BALB/c mice were immunised three times with a 2-week interval between doses with 20 µg of chicken-egg ovalbumin mixed with either Addavax™ or AbISCO®-100 adjuvant according to a schedule devised by Simone de Cassan and performed by Rebecca Hillson. I determined the ELISA end-point titre of the anti-OVA serum antibody response two-weeks after the final immunisation. Data points, individual mice (n=5); lines, median; ELISA limit of detection = endpoint titre 100.

found no significant different between either groups (median endpoint titres, 4275 vs. 4156; ranges, 1302–25074 vs. 2796–20821, n=5). The apparent equivalence of Addavax™ and AbISCO®-100 with respect to potentiation of the humoral response can be with some confidence extrapolated to an equivalence between Addavax™ and Freund's adjuvant [349]. On the basis of this result, Addavax™ was selected for all future immunisations for screening of novel antigens and constructs.

4.2.2 Prioritisation of gene products for inclusion in an anti-merozoite antigen screen

The *P. falciparum* genome encodes around 5300 protein-coding genes [227], each one of which represents a potentially protective antigen. However, it is beyond the scope of this project to test each gene product. I therefore employed two strategies when selecting antigens for screening: literature-based selection; and an algorithm based upon bioinformatic data, including published transcriptomic and polymorphism data. The eventual list of antigens selected is summarised in Table 4.1 (page 97).

4.2.2.1 Literature-based gene selection

A number of antigens were selected based upon published reports describing properties that would be desirable in a neutralising antibody vaccine. Antigens had to conform to at least one of the following criteria:

- Susceptibility to neutralising antibody, either in the standardised *in vitro* assay of GIA, or other non-standard assays
- Demonstration of protection against *P. falciparum* in non-human primate vaccine efficacy studies
- Established involvement in the process of merozoite egress from schizonts or invasion into new RBCs
- Essential to parasite viability, generally defined by reported failures to disrupt the relevant gene
- Strong correlation with protection against clinical malaria in sero-epidemiological studies.

I selected a range of moderately well-characterised gene products that conformed to these criteria (for construct details and key to abbreviations, see Table 4.1, page 97).

S-antigen last received close scrutiny during the 1980s, primarily because of a highly repetitive central region that is allelically diverse between different circulating strains [353, 354]. However, Alan Cowman has repeatedly stated in review articles that S-antigen is refractory to disruption [35, 355].

GAMA is a protein with RBC-binding activity that is essential in the W2mef and 3D7 strains, and is reported to be susceptible to neutralising antibody [356, 357].

AARP is an apical protein that reportedly binds to RBCs and is susceptible to neutralising antibody [135, 358].

SERA5 is an essential protease involved in merozoite egress, fragments of which have shown at least partial protection against blood-stage malaria challenge [359, 360].

RIPr is refractory to knock-out and appears to be micronemally located prior to egress, before forming a complex with PfRH5 in free merozoites [361]. It is a strong candidate for inducing antibodies that could synergise with antibodies directed against PfRH5.

The function and physical location of **PTRAMP** are unclear [362–364]. However, its orthologue in *P. berghei* cannot be deleted by standard methods [363]. Furthermore, peptides representing fragments of PTRAMP have been shown to have *in vitro* GIA [365].

ETRAMP is a conserved protein which, like PfRH5, is only weakly immunogenic during natural infection [269], but which is highly conserved at the amino acid level [366].

Monoclonal antibodies directed against **CyRPA** demonstrated protective efficacy in an unusual passive-transfer experiment involving immunodeficient mice transfused with human RBCs and infected with *P. falciparum* [367].

RAMA is one of a group of GPI-anchored proteins that appear refractory to genetic disruption [368]. Furthermore, seroreactivity to RAMA has been observed to correlate with protection against clinical malaria in West Kenya [369] and Vietnam [370].

RhopH3 is refractory to genetic deletion [35], and naturally-acquired antibodies against this antigen have been correlatively implicated in protection against clinical malaria [148].

Pf92 is a surface protein that has been implicated in RBC binding [371], although by non-conformational peptide-based methods that are fraught with caveats. It is also reportedly refractory to deletion [35].

MSP3 has been clinically investigated as a vaccine antigen [190]. As part of the *post-hoc* analysis of a Phase Ib trial, the vaccine appeared to show significant protection against clinical malaria [191].

Antibodies from rabbits immunised with recombinant **MSP4** inhibited parasite growth in a dose-dependent manner [372]. Furthermore, MSP4 appears to be essential as attempts to delete the gene have failed [368].

EBA-175 is thought to be the most important invasion ligand in the sialic-acid dependent pathway as its disruption pushes parasites toward a sialic-acid independent invasion pathway [373]. Numerous groups have produced polyclonal antibodies of varying neutralising capacity against different regions of EBA-175 [134, 374, 375].

RAP1 is a rhoptry protein that can be genetically truncated [376], but nevertheless has been the subject of considerable vaccinological interest. RAP1 antigen purified from parasite extract has been shown to protect vaccinated non-human primates from *P. falciparum* malaria [377] and numerous growth-inhibitory monoclonal antibodies to RAP1 have been described [378–380].

4.2.2.2 Genomic, transcriptomic and polymorphism-based selection

The objective of the work described in this Chapter was to define a vaccine-inducible antibody specificity that was not only highly neutralising on its own, but that which worked synergistically with antibodies against PfRH5 to inhibit asexual-stage growth as measured by the *in vitro* assay of GIA. Anti-PfRH4 is the only antibody specificity that has been shown conclusively to function synergistically with anti-PfRH5 antibodies, although this effect is not strain-transcending [136]. I therefore designed an algorithm to find proteins of a profile similar to PfRH5 and PfRH4 in terms of stage-specific expression, trafficking and the level of polymorphism (summarised in Fig. 4.4).

I had budgeted for the purchase of 40 synthetic genes, meaning that I had to choose 40 proteins to screen from a pool of over 5000. I used a list of 435 genes whose products were implicated in merozoite invasion of RBCs by a published transcriptomic study [145]. Given that most proteins known to traffic to invasion-associated organelles such as micronemes and the rhoptries encode a signal peptide that can be predicted bioinformatically by the SignalP v. 4.0 server [381], I excluded genes that were not predicted to encode proteins with a signal peptide. This left a list of 104 gene products.

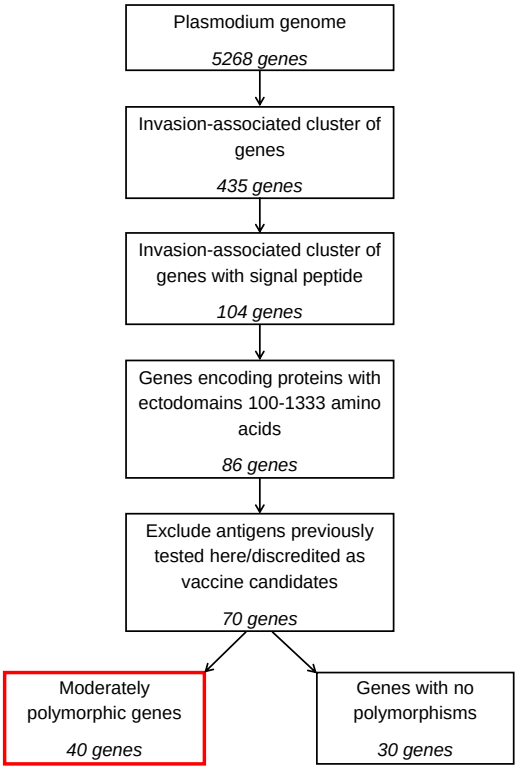

FIGURE 4.4: A strategy for prioritising 40 potential vaccine antigens from a potential pool of over 5000.

For reasons outlined in Section 3.1 I intended to purchase codon-optimised synthetic genes. Above 4000 base pairs, synthetic genes become significantly more challenging to produce, and thus more expensive. This put an upper limit of 1333 amino acids on my gene selection, excluding 18 more genes and leaving 86. Of these 86 genes, 16 had been previously tested in our laboratory and discarded as useful antigens to elicit antibodies functional in the *in vitro* assay of GIA (such as AMA1, PfRH4 or PfRH1). This left a set of 70 genes.

From this list of 70, I selected the last 40 genes by analysing polymorphism. This was facilitated by the use of plots designed by Jason P. Wendler that assembled data from a range of sources [219, 229–232] to allow straightforward visual discrimination of genes suitable for exploration as candidate vaccine antigens (Fig. 4.5 and Section 2.1.3.1). Sequencing coverage, calculated as the number of Illumina sequencing reads that align to a given position in the 3D7 genome, is a good proxy for vaccinological suitability. Drops in coverage can indicate: divergence of an allele from the 3D7 reference allele; A–T rich regions (i.e. low complexity); or the presence of indels. Spikes in coverage

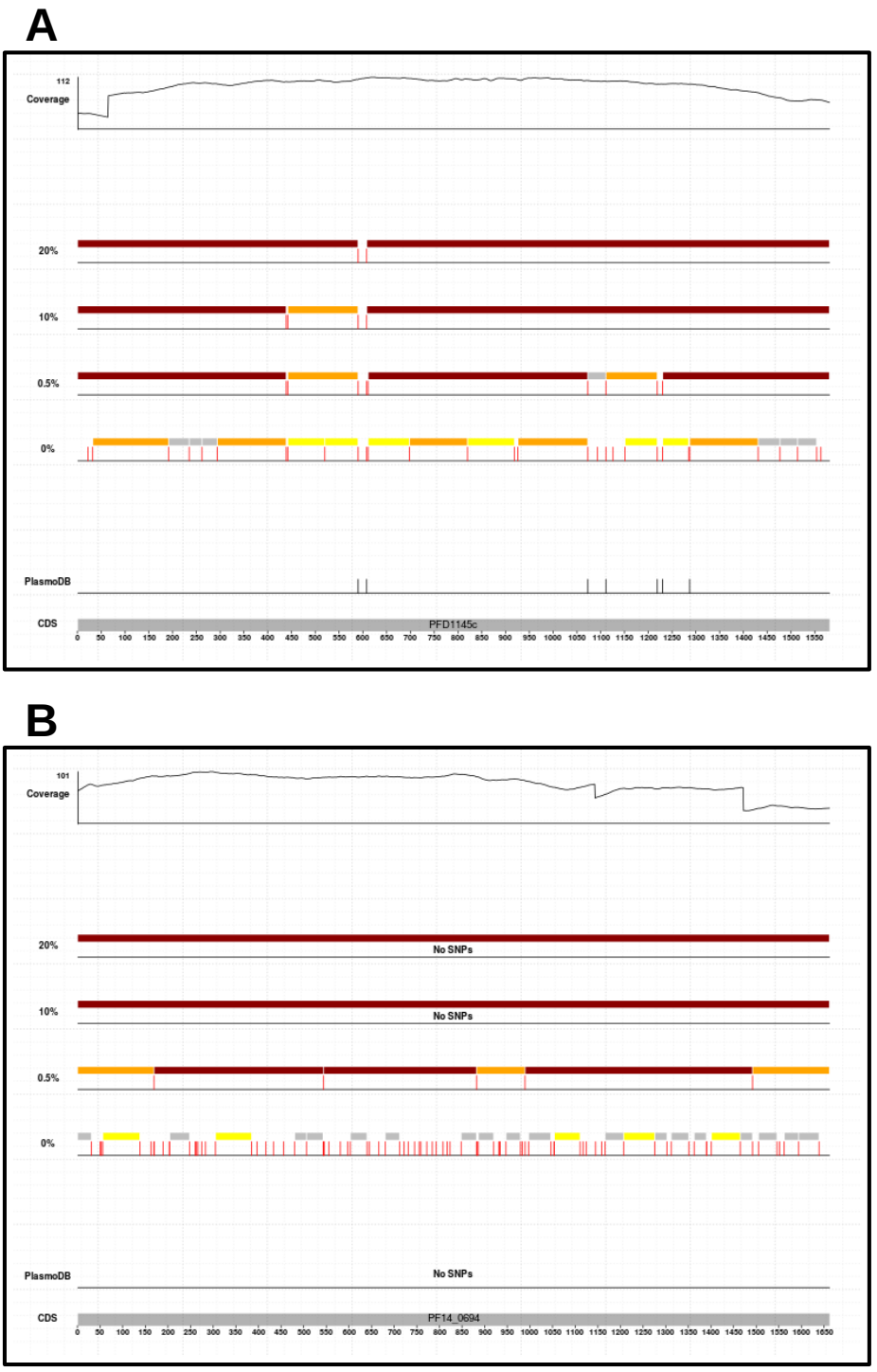

FIGURE 4.5: Visualisation of published polymorphism data [219]. For the 70 genes in the penultimate stage of the process outlined in Fig. 4.4, plots were generated by Jason P. Wendler and illustrate (from top to bottom): sequence coverage, defined as the number of sequencing reads that align to a given position in the 3D7 genome (top row); percentage prevalence and position of SNPs (middle 4 rows); the SNPs already defined in PlasmoDB (second from bottom); and the base pair positions in the spliced cDNA (bottow row). See Section 2.1.3.1 for construction details. (A) RH5, (B) a protein disulphide isomerase (gene accession code: PF3D7_1472600).

can indicate the presence of paralogous genes (leading to false alignment with a related allele) or repeats stacking on the same region of the reference genome. The presence of SNPs predicts not only amino-acid substitutions, but also the presence of additional aberrations such as indels or dimorphic genes. The code for the filtering algorithm was designed to include SNPs with a high false-positive rate, as these SNPs are indicative of this latter form of polymorphism (J. Wendler, personal communication).

Instead of assessing polymorphism by SNP density, I assessed polymorphism by counting the total number of SNPs along the length of genes. This was because antigenic escape mutations are often clustered around regions of a protein that are proximal in the three-dimensionally folded structure but are distant from one another in the primary amino acid sequence. To my surprise, I found that PfRH5 and PfRH4, respectively containing 4 and 6 non-synonymous SNPs of greater than 10% prevalence in circulating strains, were among the most polymorphic genes in my shortlist of 70 genes. Sequencing coverage was generally very consistent across the length of the genes, indicating that the genes were unlikely to show problematic levels of repetition or variation. In fact, of this set of 70 genes, there were 28 genes with uniform sequencing coverage for which I could not find a single non-synonymous SNP (as in Fig. 4.5, panel B). This contrasts with the paradigm that most invasion-associated proteins are highly polymorphic and under the influence of balancing selection. Along with two more genes that contained only one non-synonymous SNP each, these 28 genes were excluded, leaving a list of 40. These 40 protein-coding genes are listed in Table 4.1.

The 40 proteins selected by the strategy outlined above were combined with the 15 proteins selected based on literature reports, yielding a list of 55 invasion-associated proteins. These 55 proteins formed the basis of the antigen screen.

4.2.3 Pre-clinical vaccine production

In order to test the susceptibility of the 55 antigens described in Section 4.2.2 to neutralising antibody, it was necessary to produce pre-clinical vaccine to elicit the relevant antibody specificities. The results of this process are summarised in Table 4.1 (page

97). Both protein-in-adjuvant regimes and the 8-week adenovirus-MVA or heterologous adenovirus regimes have been shown to elicit high titres of functional antibodies against a range of antigens. However, where possible protein-in-adjuvant was preferred for a number of reasons: with well-expressing proteins, protein expression and purification is faster and cheaper than producing virus vectors; antigen can be administered at a precisely defined dose; and there are no problems arising due to anti-vector immunity when the same protein is administered repeatedly [1] [382].

[1] see Section 6.3.3 for more on anti-vector immunity, and Chapter 6, Table 6.1, for a comparison of the antigen-screening platforms considered in this Thesis.

TABLE 4.1: Constructs selected selected for inclusion in antigen screen
(footnotes and comments at end of table)

Gene	**Protein**	**Construct**[a]	**SNPs**[b]	**Vaccine**[c] Protein	Virus
Antigens already tested in our laboratory (with references)					
PF3D7_1133400	AMA1 (apical membrane antigen 1) [139]	Q25–K546 (522, Y622)	>40	✓[d]	✓
PF3D7_0423400	RH5 (reticulocyte-binding protein homologue 5) [133]	E26–Q526 (501, Q526)	4		✓[d]
PF3D7_0731500	EBA175_F2 (CAMP) [133, 374]	E447–D795 (348, K1502)	>20		✓[d]
Literature-selected antigens: full length ectodomains					
PF3D7_1218000	PTRAMP (thrombospondin-related apical membrane protein)	C28–K309 (281, D352)	0	✓	
PF3D7_0423400	AARPdTM (apical asparagine rich protein, ectodomain only)	K18–P191 (173, P217)	1	✓	
PF3D7_0323400	Ripr (RH5 interacting protein)	D21–N1086 (1065, N1086)	4	×	✓[e]
PF3D7_0828800	GAMA (GPI-anchored micronemal antigen)	R24–S716 (692, N738)	6	✓	
PF3D7_0207600	SERA5 (serine-rich repeat antigen 5)	T23–V997 (974, V997)	9	✓	
PF3D7_1033200	ETRAMP (Early transcribed membrane protein 10.2)	M77–E355 (278, E355)	3	×	✓[e]
PF3D7_1035200	S-antigen	K20–M585 (565, M585)	N/A[f]	✓	
PF3D7_0423800	CyRPA (cysteine-rich protective antigen)	D29–E362 (333, E362)	1	✓	
PF3D7_0707300	RAMA (rhoptry-associated membrane antigen)	L18–S841 (823, N861)	7	×	
PF3D7_0905400	RhopH3 (high molecular weight rhoptry protein 3)	G24–L897 (873, L897)	3	×	
PF3D7_1364100	Pf92	S16–D768 (752, I796)	6	✓	
PF3D7_1035400	MSP3 (merozoite surface protein 3)	K26–H354 (329, H354)	17		✓[d]

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Gene	Description	Construct[a]	SNPs[b]	Vaccine[c] Protein	Vaccine[c] Virus
PF3D7_0207000	MSP4 (merozoite surface protein 4)	Y29–S248 (220, S248)	5		✓[d]
Literature-selected antigens: designed fragments					
PF3D7_1410400	RAP1FL	I23–D782 (760, D782)	11		✓[d]
	RAP1p65	D191–D782 (592, D782)			✓[d]
	RAP1p52	D191–K635 (445, D782)			✓[d]
PF3D7_0731500	EBA175_F2 (3D7)	E447–S760 (311, K1502)			✓[d]
	EBA175_R3–R5 (3D7)	I753–H1272 (520, K1502)			✓[d]
	EBA175_F2–R5	E447–H1272 (826, K1502)			✓[d]
Transcriptomically selected antigens: full-length ectodomains					
PF3D7_1367900	Hypothetical protein, conserved	R26–I647 (621, I647)	0	✓	
PF3D7_0404700	DPAP3 (dipeptidyl peptidase 3)	D21–T939 (918, T939)	13	×	
PF3D7_1030200	Hypothetical protein (had TM)	M40–S138 (98, F450)	4	×	
PF3D7_0405900	ASP (apical sushi protein)	K21–K711 (690, A731)	2	✓	
PF3D7_1035700	DBLMSP (Duffy binding like Mz surface protein)	K24–N457 (433, K697)	25	✓	
PF3D7_1028700	MTRAP (merozoite-specific thrombospondin-related anonymous protein)	I23–K432 (409, E498)	2	✓	
PF3D7_0207500	SERA6 (serine repeat antigen 6)	K25–V1031 (1006, V1031)	1	×	
PF3D7_0207800	SERA3 (serine repeat antigen 3)	T23–I930 (907, I930)	1	×	
PF3D7_0208000	SERA1 (serine repeat antigen 1)	E25–V994 (969, V994)	1	×	
PF3D7_1035300	GLURP (glutamate-rich protein)	K24–I1233 (1209, I1233)	16	×	
PF3D7_1035100	Hypothetical protein	N26–K561 (535, K561)	23	×	

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Gene	Description	Construct[a]	SNPs[b]	Vaccine[c] Protein	Vaccine[c] Virus
PF3D7_1035900	Hypothetical protein	A21–P566 (545, P566)	5	×	
PF3D7_1102300	Hypothetical protein	P32–V415 (383, V415)	2	×	
PF3D7_1116000	RON4 (rhoptry neck protein 4)	F24–L1201 (1177, L1201)	1	×	
PF3D7_1136200	Hypothetical protein	I21–L679 (658, L679)	1	×	
PF3D7_1404700	Hypothetical protein	Q21–K290 (269, K290)	3	✓	
PF3D7_1463900	Hypothetical protein	E23–N445 (422, N445)	5	×	
PF3D7_1321900	Hypothetical protein, conserved	K21–S292 (271, S292)	2	✓	
PF3D7_1036000	MSP11 (AKA MSP3.7 AKA H103)	S25–Y405 (380, Y405)	1	✓	
PF3D7_0722200	RALP1 (rhoptry-associated leucine zipper-like protein 1)	S23–F749 (726, F749)	0	×	
PF3D7_0815300	FAD-dependent monooxygenase (putative)	K26–I1182 (1156, I1182)	7	×	
PF3D7_1143200	DnaJ protein (putative)	L22–M109 (87, D321)	1	×	
PF3D7_0404800	Hypothetical protein, conserved	L24–E567 (543, E567)	3	×	
PF3D7_0423300	Hypothetical protein, conserved	E42–R476 (434, R476)	2	×	
PF3D7_0507300	Hypothetical protein, conserved	Y19–R893 (874, R893)	4	×	
PF3D7_0932100	Hypothetical protein, conserved	K143–S1274 (1131, S1274)	5	×	
PF3D7_1216500	MDV1 (male development gene 1)	F20–D221 (201, D221)	0	×	
PF3D7_1229300	Hypothetical protein, conserved	L20–S890 (870, A990)	8	×	
PF3D7_1246000	Hypothetical protein, conserved	K25–L443 (418, L483)	2	×	
PF3D7_0214900	RON6 (rhoptry neck protein 6)	F16–G950 (934, G950)	3	✓	
PF3D7_0620400	MSP10 (merozoite surface protein 10)	H27–K506 (479, K506)	7	✓	

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Gene	Description	Construct[a]	SNPs[b]	Vaccine[c] Protein	Vaccine[c] Virus
PF3D7_0629300	Phosphatidylcholine-sterol acyltransferase (putative)	G21–N863 (842, N863)	13	×	
PF3D7_0507500	SUB1 (subtilisin-like protease 1)	E31–H688 (657, H688)	3	×	
PF3D7_1335100	MSP7 (merozoite surface protein 7)	N22–M351 (329, M351)	13	✓	
PF3D7_0935800	RhopH1 (high molecular weight rhoptry protein 1)	L268–F1340 (1072, F1340)	8	×	
PF3D7_0808200	Plasmepsin X	C26–N573 (547, N573)	4	✓	
PF3D7_0929400	Rhop H2 (high molecular weight rhoptry protein 2)	K25–S1378 (1353, S1378)	6	×	
PF3D7_0220800	RhopH1 (high molecular weight rhoptry protein 1)	S25–Q1440 (1415, Q1440)	16	×	
PF3D7_1322000	Adenosine-diphosphatase	Q31–L565 (534, L565)	5	×	
PF3D7_0831600	CLAG8 (Cytoadherence linked asexual protein 8)	S25–E1394 (1369, E1394)	>20	×	

[a]Given as: first–last residue (length of construct, last aa encoded in gene).

[b]Number of non-synonymous SNPs across entire length of gene with prevalence >10% in circulating strains [219, 229–232].

[c]✓ denotes successful production of preclinical vaccine in a given format (i.e. >300 µg purified protein, or two infectious recombinant virus vectors), × denotes failure, and no mark indicates that production of preclinical vaccine was not attempted with this format.

[d]Immunising regime regime was adenovirus-MVA (see Section 2.5.1).

[e]Immunising regime was based on two adenoviruses of heterologous serogroups (AdHu5 and ChAd63, see Section 2.5.1).

[f]Reliable SNP data for the full length of S-antigen is not available due to technical difficulties in assembling the data for this repetitive gene (J. Wendler, personal communication).

4.2.3.1 Construct design

Both the HEK293 protein-expression system (Chapter 3) and virus-vector based genetic immunisation involve the expression and secretion of protein by mammalian cells. While *Plasmodium* rarely attaches N-linked glycans to proteins [253, 254, 383], mammalian cells tend to attach large and often complex glycans. This may be problematic because glycans are on average 20 times larger than a standard amino acid side chain and may mask potentially neutralising epitopes on the antigen. N-glycan attachment sequons (Asn–X–Ser/Thr, where X is any amino acid but proline) can be modified in a number of ways so that they are no longer recognised by the glycosylation machinery. However, N-glycans are often essential to protein folding in the endoplasmic reticulum, and their removal does not always have a beneficial effect on recombinant *Plasmodium* immunogens. Occasionally, ablation of N-glycosylation can impinge on the ability of an antigen to elicit functional antibodies, as seen with Pfs48/45 when delivered by genetic immunisation (S. Biswas, personal communication). As various *Plasmodium* antigens have been demonstrated to elicit functional immune responses without any modification to the primary amino acid sequence [157, 384, 385], and in the absence of any empirical characterisation of the majority of the proteins in this screen, I designed constructs encoding amino acid sequences exactly as predicted from the 3D7 reference genome sequence [386].

Details for every construct considered in this antigen screen are shown in Table 4.1 (page 97). Almost every antigen was tested in the form of a construct corresponding to the full-length ectodomain of the parasite protein. This was to avoid producing small polypeptide fragments that do not fold correctly and thus fail to elicit a functional antibody response, as first occurred with PfRH5 [218, 220, 223]. In the case of proteins with no predicted transmembrane region, this meant that the fragment started from the first amino acid residue after the predicted signal-peptide cleavage site (predicted with signalP v. 4.0 [381]) and ended at the last amino acid before the predicted stop codon. For type I transmembrane proteins, this meant that the fragment started from the first amino acid residue after the predicted signal-peptide cleavage site [381] and ended at the last amino acid before the transmembrane region [387]. This accounted for

the vast majority of the proteins in this screen. For proteins predicted to have a type-II topology, such as ETRAMP, I designed a fragment stretching from the first amino acid after the transmembrane region to the last amino acid before the predicted stop codon. For proteins such as RhopH1 that are predicted to have multiple membrane-spanning regions, I selected the largest predicted external region. Two merozoite proteins were considered sufficiently interesting that multiple vaccines encoding smaller fragments of the proteins were designed by Alexander D. Douglas.

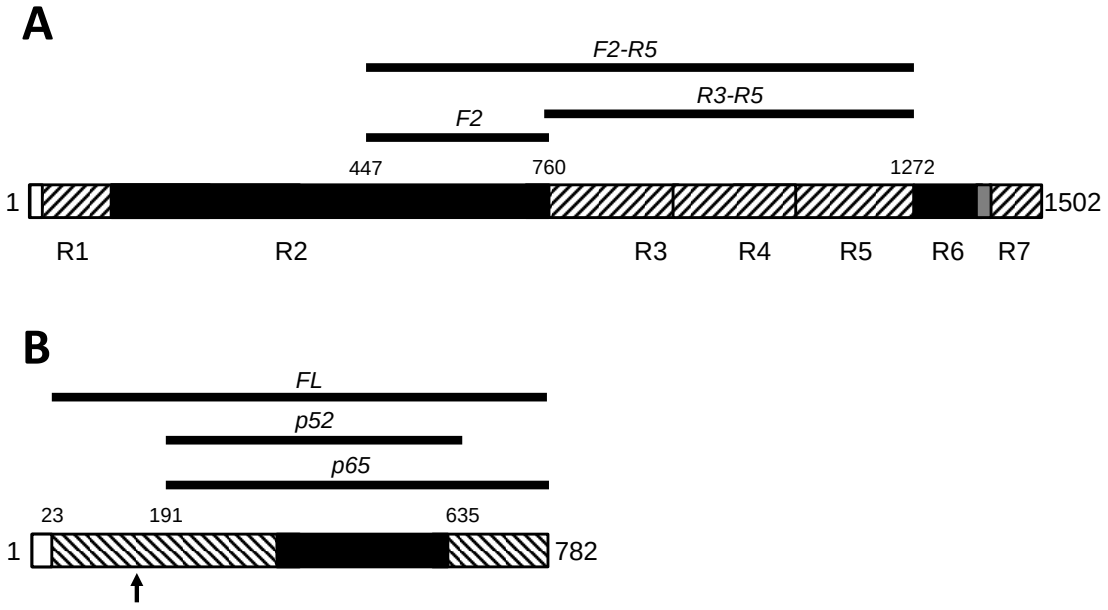


FIGURE 4.6: Literature-based construct design for EBA-175 (A) and RAP1 (B), designed and cloned by Alexander D. Douglas. Boxes represent the full-length predicted parasite protein with amino acid numbers shown at either end, black denoting cysteine-rich regions, white denoting signal peptides, and the grey box in (A) denoting transmembrane regions. Arrow represents proteolytic cleavage site. Horizontal black represent the regions corresponding to the vaccine constructs.

EBA-175 is encoded by a gene that is approximately 4500 bp, too long to be easily subcloned and stably integrated into a recombinant adenoviral genome. However, EBA-175 has been characterised by a number of research groups, facilitating the rational design of constructs (Fig. 4.6A). The region of EBA-175 that binds to its RBC ligand (GYPA) is homologous to the Duffy antigen binding region of *P. vivax* Duffy-binding protein, and is referred to as region II [321]. Region II is further subdivided into regions F1 and F2. The F2 domain from the CAMP

strain has been expressed in COS cells [388] and bacteria and shown to have RBC-binding activity [374]. A construct encoding the F2 region from the CAMP strain has already been assessed in our laboratory [133], but for this screen a construct encoding the F2 region from the assay-homologous 3D7 clone was also produced. Additionally, a construct was produced based on findings that a recombinant protein corresponding to regions R3–R5 from the 3D7 allele of EBA-175 is capable of eliciting functional antibodies [134, 375]. Finally, a novel construct encompassing both the previous fragments F2 and R3-R5 was produced and called F2-R5.

RAP1 has historically proven extremely difficult to express recombinantly and researchers have often resorted to purifying recombinant RAP1 from parasite lysate [389]. Given that virus vector (i.e. genetic) immunisation negates the requirement for purifying recombinant protein, we hoped to overcome these difficulties. A construct corresponding to full-length RAP1 (minus signal peptide) was accordingly produced. In addition, two fragments were designed, both beginning at the proteolytic cleavage site at D191 (Fig. 4.6B) [390]. p65 corresponds to the entire region downstream of this cleavage site, continuing to the final predicted amino acid before the stop codon. p52 begins at the same position as p65, but is truncated so as to end 20 residues after the last cysteine in the cysteine-rich region.

4.2.3.2 Protein production

With the exception of the EBA-175, RAP1, MSP3 and MSP4 constructs, I attempted to express recombinant protein for every construct listed in Table 4.1 by transfecting 200 mL of HEK293 suspension cell culture (Chapter 3). Proteins were expressed as fusion polypeptides C-terminally tagged with BAP and either Strep or 6His (see Section 3.1.5 for key to abbreviations) from the pENTR4 LP vector described in Section 3.2.5 and Fig. 3.6.

For the first batch of proteins that I attempted to express and purify, I used the top seven proteins listed in Table 4.1. This was considered a test batch and analysed more closely than the remaining proteins. This batch of proteins was tagged with the Strep

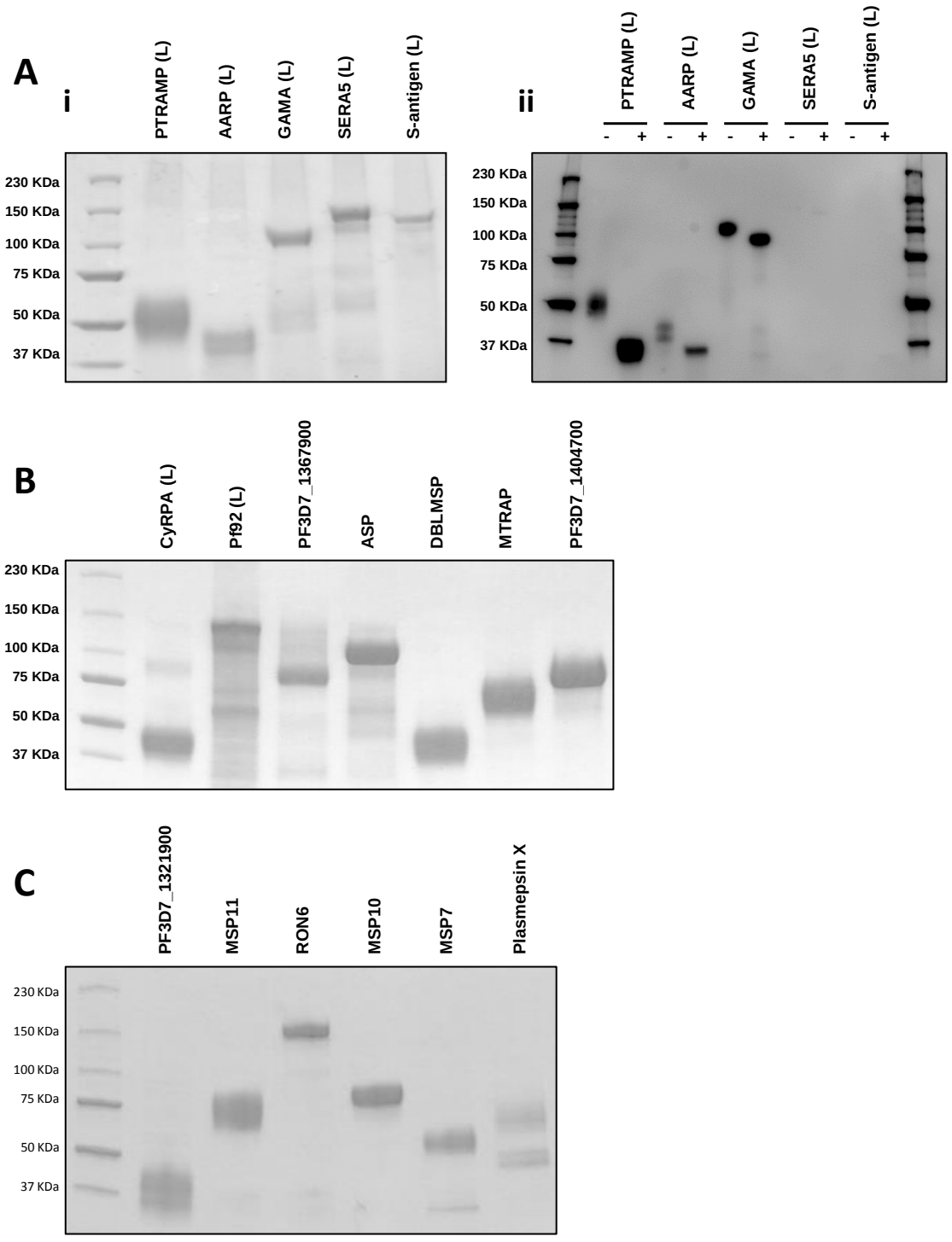


FIGURE 4.7: Successful expression and purification of 18 merozoite proteins. 51 plasmids encoding full-length ectodomains of the proteins described in Table 4.2.2 were transfected into HEK293E cells as described in Section 3.5. Supernatants were harvested, and proteins were purified by either streptactin-based purification (A) or nickel-ion affinity chromatography (B and C). Success was defined as obtaining at least 300 µg of pure recombinant protein. For these proteins, the concentration of purified protein was adjusted to 0.4 mg mL^{-1}, and proteins were frozen at −80°C until the day of immunisation, at which point aliquots were defrosted. The gels in this Figure show SDS-PAGE analysis of 5 µL of protein solution (i.e. 2 µg), just prior to formulation of the remainder of the protein aliquot with adjuvant. Proteins from the 'literature-selected' panel are labeled (L). Molecular weight marker is BioRad Precision Plus^{TM} Unstained Standard, with 5 µL loaded per lane. Panel (Aii), Western blot with (+) and without (-) PNGase F treatment (40 ng/lane), detection with streptactin–HRP.

tag and purified on a streptactin® sepharose™ column (see Materials and Methods, Section 2.3.4). Although the Strep system yielded protein of reasonable purity (Fig. 4.7, panel Ai), the necessity of dialysing HEK293 supernatant (to remove free biotin) prior to application to the streptactin-sepharose columns severely hampered throughput. For this reason, all subsequent constructs were C-terminally tagged with BAP.6His and purified on HisTrap Excel columns (Fig. 4.7, B and C), onto which HEK293 culture supernatant can be directly loaded without dialysis (Section 2.3.4). This batch of proteins was also analysed by PNGase treatment and Western blotting to gain an impression of the level of N-linked glycosylation (Fig. 4.7, panel Aii). Two proteins, S-antigen and SERA5, could not be detected with streptactin-HRP, suggesting that these proteins were not stable during the PNGase F treatment process (samples untreated with PNGase F still underwent the process of boiling and 37°C incubation—see Section 2.4). The remaining three proteins, PTRAMP, AARP and GAMA, all showed evidence of an electrophoretic mobility shift when PNGase treated, suggesting the presence of at least one glycan (Fig. 4.7, panel Aii). AARP appeared to be present in at least two glycoforms. In all three cases, the band seen upon PNGase treatment was not visible in the untreated lane, suggesting that the unglycosylated form of the protein was either a minor component of the untreated sample or absent entirely.

The success rate for expression and purification of a usable amount of protein (i.e. >300 µg purified material) was higher among the literature-selected panel (7/11) than among the panel selected based on transcriptomic and polymorphism data (11/40, see Table 4.1), probably reflecting publication bias towards proteins that can be recombinantly expressed. Of the 18 proteins expressed in total, three were completely unannotated hypothetical proteins, and the majority had never been expressed and purified as constructs covering their full-length ectodomains (MTRAP, PTRAMP and GAMA being notable exceptions [356, 362]).

4.2.3.3 Production of virus vectors

Protocols for virus vector production are well established. In the first batch of expression and purification trials, when ETRAMP and RIPr did not express detectably from

HEK293 cells, I decided to produce virus vector vaccines encoding these proteins. To simplify the production process, I used an immunisation regime that involves priming and boosting with recombinant adenoviruses of two different serogroups which has previously been shown to elicit high titres of functional antibodies [235, 391]. Two pairs of recombinant adenovirus were successfully produced and purified. However, adenovirus production was found to be time-consuming and costly and this approach was not extended to the remaining proteins that could not be expressed in HEK293 cells. For the EBA-175 and RAP1 constructs, an adenovirus-MVA strategy was employed. Recombinant AdHu5 and MVA vectors were produced by the Jenner Institute virus vector core production team at the request of Alexander D. Douglas.

Altogether, 28 novel vaccines covering 24 different proteins were produced. Of the 28 vaccines, 18 were protein-in-adjuvant and 10 were virus vectors (summarised in Table 4.1). Groups of four female BALC/c mice were immunised with the 28 pre-clinical vaccines over six independent experiments. The serum from groups of immunised mice was pooled, and IgG purified and prepared for the GIA assay (see Methods Chapter 2, Sections 2.5.1, 2.6.1 and 2.7.4).

4.2.4 Immunogenicity of pre-clinical vaccines

When screening new antigens, a negative result for a given antigen may reflect a genuine result—the antigen simply might not be susceptible to neutralising antibody. However, a negative result can also arise for a number of artefactual reasons. First, the pre-clinical vaccine may not be immunogenic, thus failing to elicit antibodies against the recombinant protein. Second, the pre-clinical vaccine may elicit antibodies that are reactive to the recombinant protein, but not to the protein as it appears in the parasite (or the protein may even not be expressed in the parasite). Third, the pre-clinical vaccine may elicit high titres of antibodies that are reactive to the native protein as it appears in the parasite, but against non-neutralising epitopes. While the third explanation is difficult to exclude, the plausibility of the first and second options can be assessed by ELISA and IFA respectively.

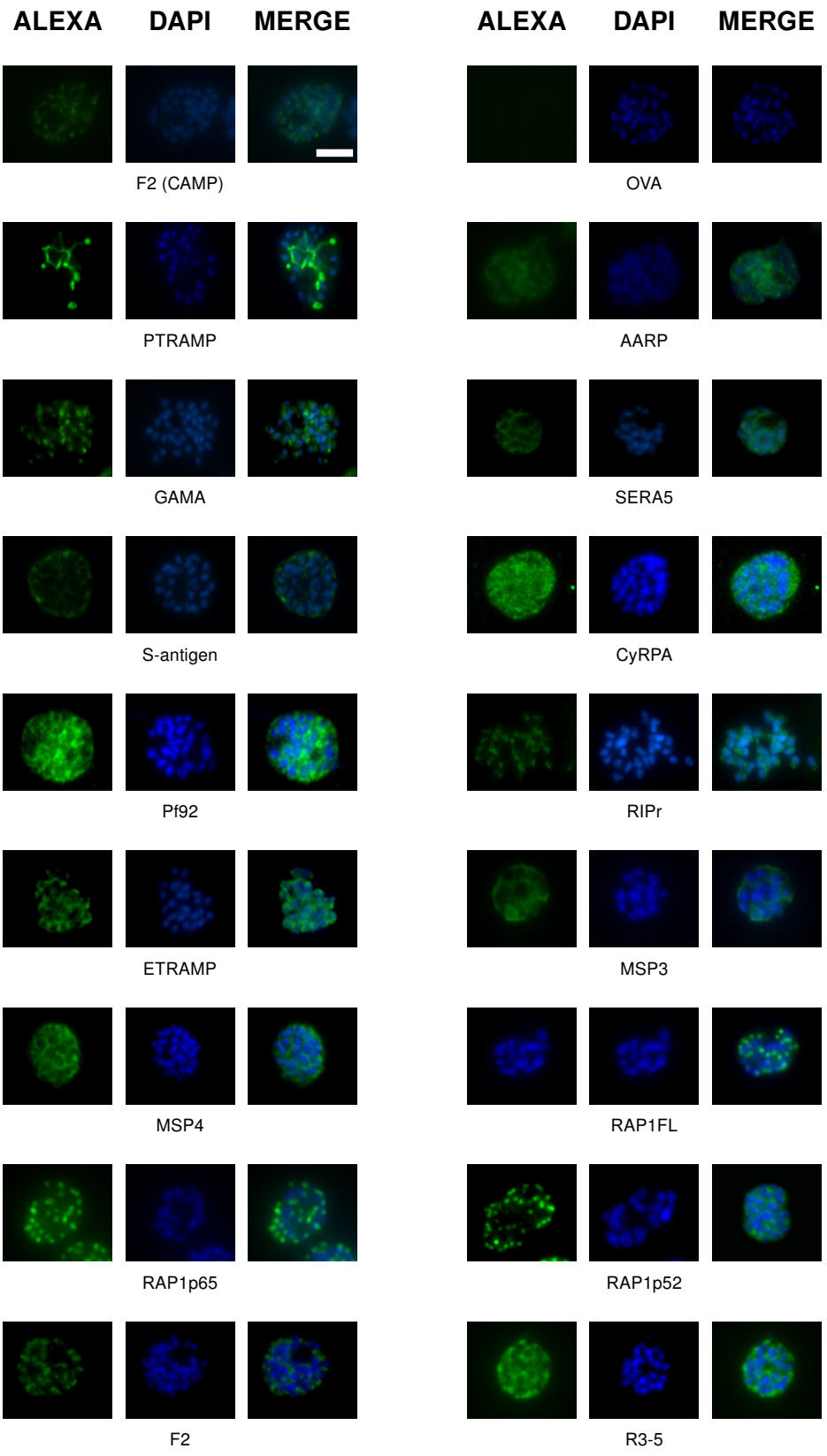
4.2.4.1 Reactivity to fixed schizonts

IgG purified from the pooled sera of immunised BALB/c mice (4 mice per pool) was applied to fixed, permeabilised schizonts at 20 µg mL^{-1} total IgG. 27 of the 28 the novel vaccines described in Section 4.2.3 elicited antibodies that were reactive to schizonts (Fig. 4.8), suggesting that all but the vaccine to MSP11 had activated a strong humoral immune response.

4.2.4.2 Reactivity to recombinant protein

Only the IgG from the 4 mice immunised with MSP11.BAP.6His protein-in-addavax pre-clinical vaccine did not react detectably with schizonts (Section 4.2.3). This suggested three possibilities. First, the MSP11 vaccine may have failed to elicit a strong humoral response in the BALB/c mice. Second, the MSP11 vaccine may have elicited antibodies, but MSP11 is not expressed in schizonts of the 3D7 clone. Third, the MSP11 vaccine did elicit antibodies, MSP11 is expressed in schizonts of the 3D7 clone, but the antibodies raised against recombinant MSP11 are not cross-reactive to native MSP11 as it appears in fixed schizonts. To test this, I measured the antibody response against MSP11.BAP.6His recombinant protein by ELISA (Fig. 4.9F, left-hand column). Only one of the four mice immunised had an anti-MSP11.BAP.6His titre greater than 1000, and one mouse had no detectable antibodies. This suggests the failure of the anti-MSP11 antibodies to react to fixed schizonts seen in Section 4.2.3 is at least partially due to a low concentration of antigen-specific antibody (although it does not formally exclude the second and third possibilities).

In addition to MSP11.BAP.His, I also measured the antibody titres elicited against the first batch of proteins that I produced (Fig. 4.7A and Fig. 4.9, left-hand column). The endpoint titres of the 2 mg mL^{-1} IgG samples to be used in the GIA assay were generally above 10,000. I observed striking cross-reactivity between different recombinant antigens (Fig. 4.9, right-hand column), which I hypothesised was due to reactivity to the purification tags on the protein immunogens. These constitute a 23 amino acid invariant C-terminal epitope on every antigen. I tested this hypothesis by pre-mixing serum samples



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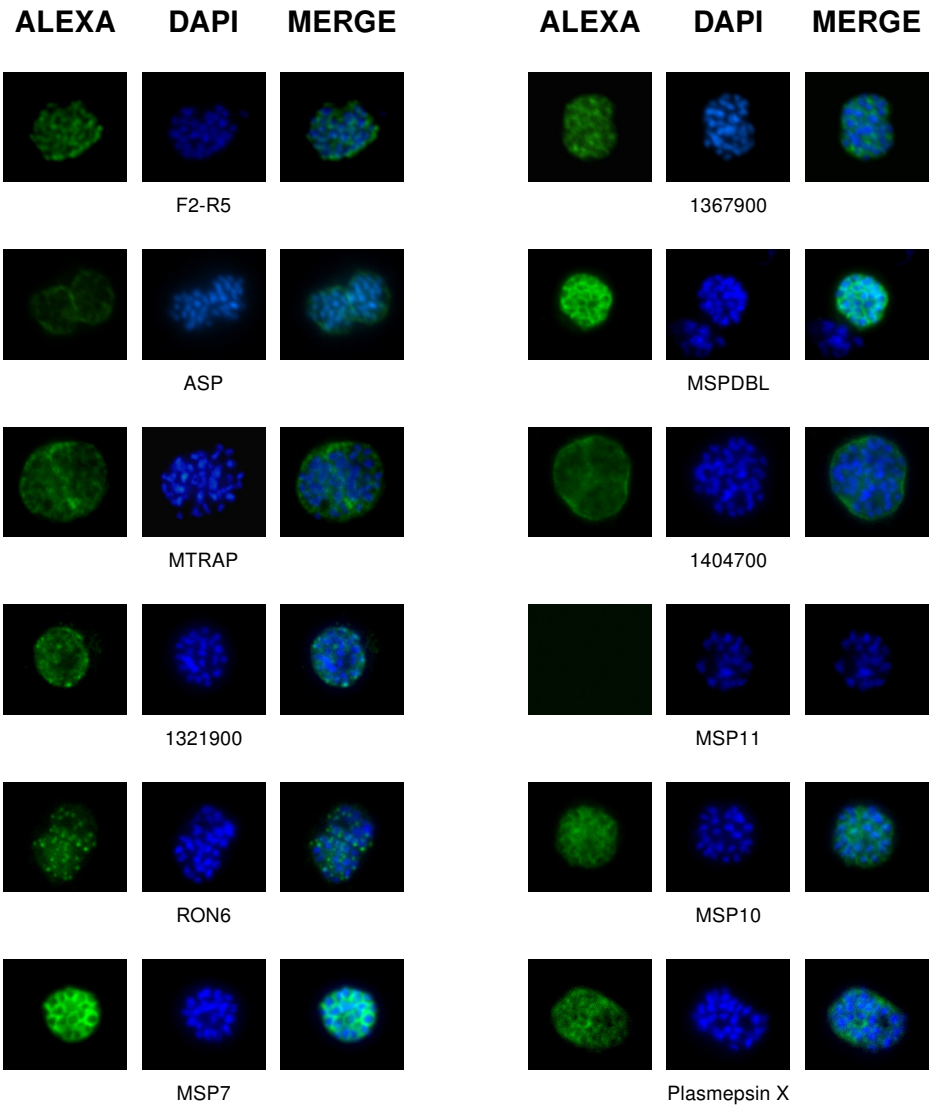

FIGURE 4.8: IgG from mice immunised with *P. falciparum* merozoite proteins reacts with antigen in fixed schizonts. Total IgG purified from the pooled serum of from mice immunised with parasite antigen (n=4) was applied to PFA-fixed schizonts at 20 $\mu g\,mL^{-1}$ in PBS and tested for reactivity by indirect IFA (green, alexa-488). DNA was visualised with 4,6-diamidino-2-phenylindole (blue). The first row shows staining with IgG from mice immunised with control vaccines (postive control: EBA-175 F2 fragment, CAMP strain, see [133]; negative control, chicken egg ovalbumin, see [349]). EBA-175 test vaccines are labelled with their construct names (see Table 4.1). Proteins that have not yet been named and annotated are given by their gene accession number (omitting the prefix PF3D7_). Scale bar, 5µm.

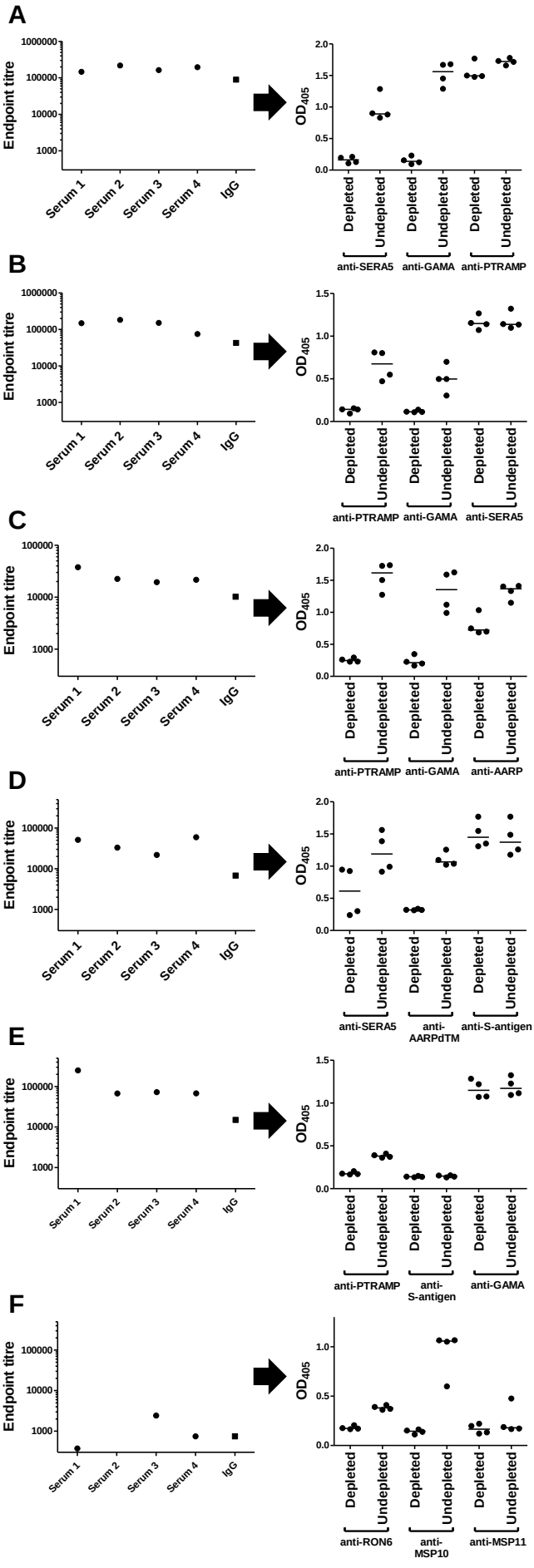

FIGURE 4.9: High-titre antibodies against both the antigen of interest and the C-terminally-fused affinity purification tags. Coating antigens were:
(A) PTRAMP.BAP.Strep,
(B) SERA5.BAP.Strep,
(C) AARP.BAP.Strep,
(D) S-antigen.BAP.Strep
(E) GAMA.BAP.Strep
(F) MSP11.BAP.6His
Left-hand column, recombinant proteins with C-terminal .BAP.Strep tags (see Section 3.1.5) were coated onto ELISA plates, and endpoint IgG titres were determined for both individual mouse sera and the 2 mg ml^{-1} total IgG sample to be used in the GIA assay (see Section 2.6.2 and 2.7.4). Right-hand column, each coating antigen was assayed for reactivity with serum (dilution 1:100) from mice immunised with both cognate and non-cognate antigen, with and without incubation with a depleting peptide (Section 2.6.2). Data points, individual mice; lines, median.

with a peptide of equivalent sequence to the purification tags (see Section 2.6.2). Pre-depletion with peptide ablated the signal arising from cross-reactivity but did not affect the signal from samples assayed against their cognate antigen. Thus, the BAP.Strep and BAP.6His tags found at the C-terminus of every recombinant protein immunogen are consistently immunogenic, but the antibodies raised against it can be depleted with a linear peptide.

4.2.5 Functional analysis of antibody specificities

The IgG produced in the six mouse immunisation experiments was assayed for functionality in the *in vitro* assay of GIA. To verify that the IgG purification and preparation had been performed correctly, each mouse experiment included a GIA-positive control group immunised with PfRH5 and a GIA-negative control group immunised with OVA, and the whole set of sera from each mouse experiment was purified in one batch. Results for antibodies raised against recombinant protein immunogens are shown in Fig. 4.10. The most striking GIA was seen for antibodies raised against AARP and S-antigen, at approximately 100% for AARP and approximately 50% for S-antigen at 1 $\mathrm{mg\,mL^{-1}}$ of total IgG. The very moderate GIA seen for antibodies against Plasmepsin X was not reproducible in subsequent assays and likely arose from noise in this biological assay. The only novel virus vector vaccines that reproducibly elicited anti-parasitic antibodies were two EBA-175 vaccines: R3-R5 and F2-R5 (Fig. 4.11). However, at less than 20% GIA this was considered a low level of functionality. In addition, each antibody specificity was assayed in combination with rabbit antibodies raised against PfRH5, in order to test whether there was an antibody specificity that had low or undetectable anti-parasitic activity when used alone, but which interacted synergistically with antibodies against PfRH5. However, none of the antibodies that were negative for GIA when assayed alone showed any positive interaction with anti-PfRH5 (data not shown).

These data support the consideration of S-antigen and AARP as candidate antigens for a blood-stage malaria vaccine. Thus, from a pool of 24 proteins, we identified two that should be investigated further.

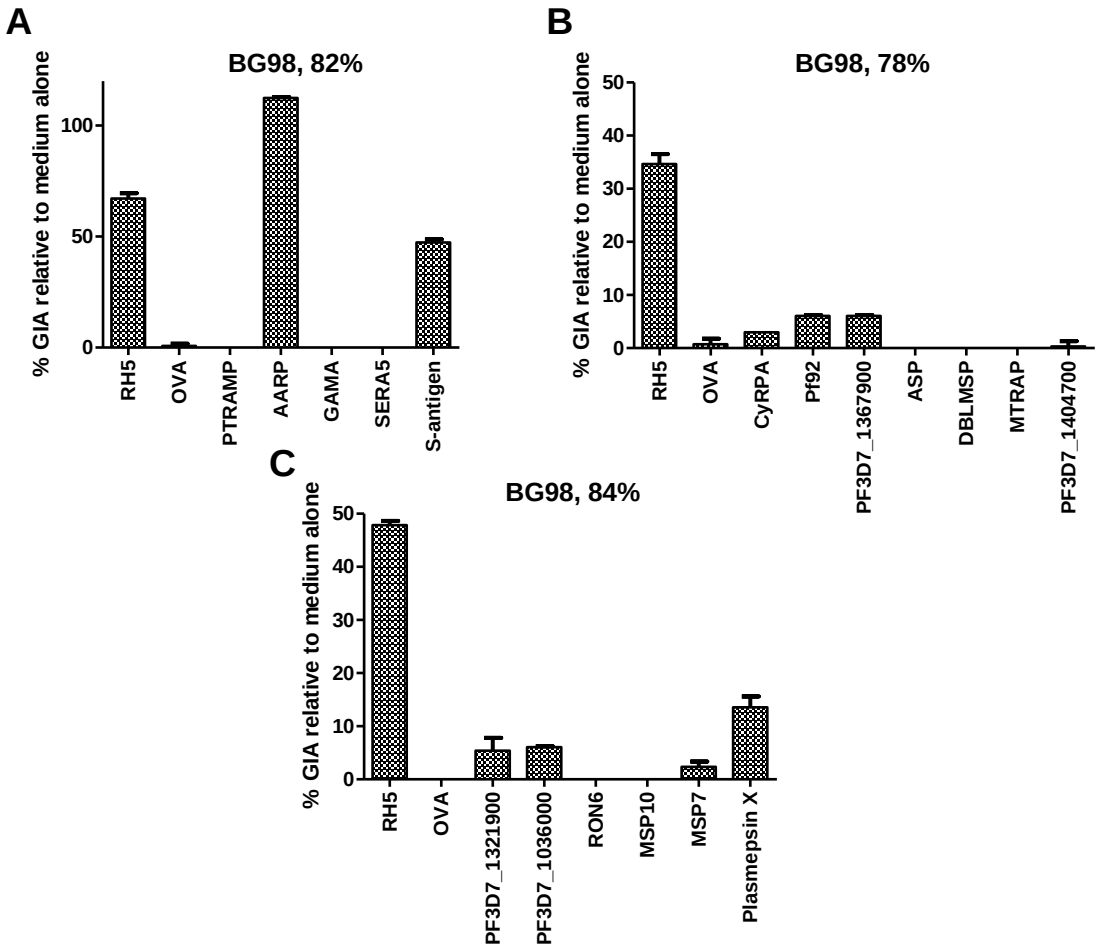

FIGURE 4.10: Anti-parasitic activity of a range of antibody specificities raised by protein-in-adjuvant immunisation. The 18 recombinant merozoite proteins shown in Fig. 4.7 were formulated with Addavax^{TM} adjuvant and immunised into groups of BALB/c mice (n=4). At the end of the immunisation schedule, serum was harvested, pooled from each dose group, and IgG purified and tested in the *in vitro* assay of GIA. All samples were tested at 1 mg mL^{-1} total IgG. Panels A–C each show the outcome of a separate mouse experiment corresponding to the protein gels in Fig. 4.7. All experiments contained groups of mice immunised by recombinant virus vectors encoding PfRH5 as a positive control, and OVA as a negative control (Section 2.5.1). Results are representative of two assay repeats. Measured GIA for the BG98 standard at 6 mg mL^{-1} is shown above each graph. Heights of bars, means; error bars, range of two replicate wells.

Characterisation of anti-AARP antibodies is described in Chapter 5.

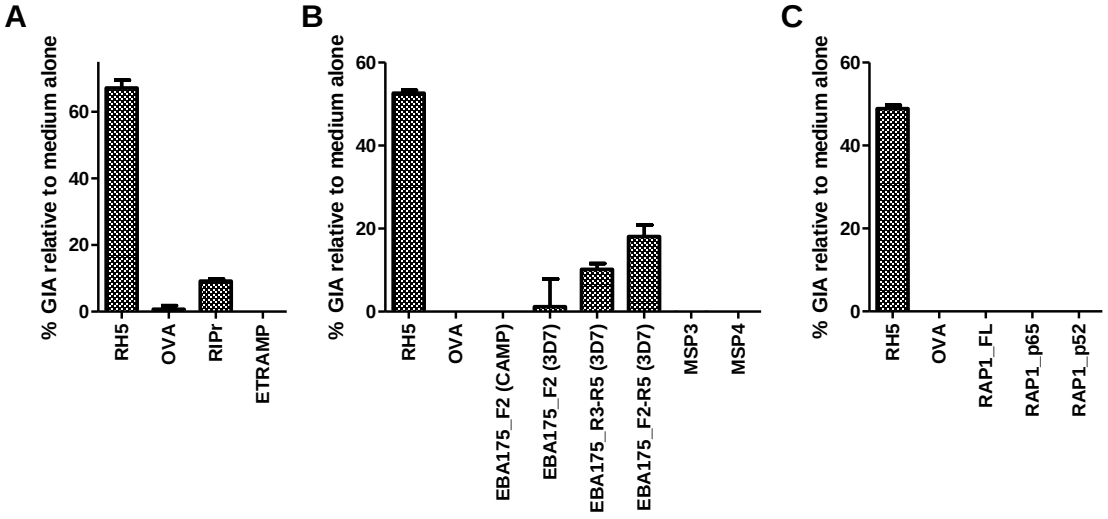

FIGURE 4.11: Anti-parasitic activity of a range of antibody specificities raised by virus vector immunisation. Virus vectors encoding novel full-length antigens (A) or novel fragments of antigen (B and C) were immunised into groups of BALB/c mice (n=4). At the end of the immunisation schedule, serum was harvested, pooled from each dose group, and IgG purified and tested in the *in vitro* assay of GIA. All samples were tested at 1 mg mL^{-1} total IgG. All experiments contained groups of mice immunised with recombinant virus vectors encoding PfRH5 as a positive control, and OVA as a negative control (Section 2.5.1). Results are representative of two assay repeats. Bars, means; error bars, range of two replicate wells. BG98 was not available at the time these assays were performed.

4.2.6 Antibodies against S-antigen

The antibody sample used to generate Fig. 4.10 was titrated against both the 3D7 clone and the FVO strain and found to give comparable GIA against both lines (Fig. 4.12). As it was an objective to find an antibody specificity that interacts synergistically with PfRH5 antibodies in the GIA assay, the S-antigen antibodies were titrated both on their own and in a mixture with rabbit anti-PfRH5 antibodies raised using a previously described pair of recombinant virus vectors [133]. The level of GIA that would be expected if the antibodies interacted additively (calculated as in [136]) was compared to the observed GIA when the antibodies were combined. Against 3D7, the interaction was almost exactly what would be predicted if the interaction was additive (Fig. 4.12A). Against FVO, the interaction seemed super-additive (i.e. synergistic), but because the sample was exhausted by this point it was not possible to perform a replicate assay (Fig. 4.12B).

To verify the ability of recombinant S-antigen protein to elicit functional antibodies, I had two rabbits immunised with S-antigen in Freund's adjuvant (see Section 2.5.2). Antibodies purified from the serum of these rabbits were compared to antibodies from rabbits immunised with PfRH5 recombinant protein (Hjerrild, Jin and Draper *et al.*, in preparation). Up to 2.5 $\mathrm{mg\,mL^{-1}}$, the anti-PfRH5 antibodies clearly outperformed the anti-S-antigen antibodies (Fig. 4.12C). However, at concentrations of 5 $\mathrm{mg\,mL^{-1}}$ and above, anti-S-antigen antibodies began to perform comparably to the anti-PfRH5 antibodies. Put another way, anti-S-antigen antibodies behave comparably to anti-PfRH5 antibodies at higher IgG concentrations, but their activity titrates out more rapidly than that of anti-PfRH5 antibodies.

These data demonstrate that S-antigen robustly elicits antibodies capable of inhibiting parasite growth, and thus support the further characterisation of S-antigen as a blood-stage candidate vaccine antigen.

4.3 Discussion

There is no approach to screening antigens that is free from caveats. However, this screen attempted to reduce the likelihood of false-negative results (i.e. overlooking a susceptible antigen because the antibodies elicited against the vaccine antigen did not have anti-parasitic activity for artefactual reasons). Where possible, a recombinant protein-in-adjuvant immunisation approach was used that I demonstrated to be highly immunogenic (Fig. 4.3). In almost all cases, these recombinant proteins corresponded to the entire length of the predicted ectodomains of the proteins of interest. This was to minimise the possibility of producing a fragment that either does not contain the neutralising epitopes, or does not adopt the correct tertiary structure, as first occurred with PfRH5 prior to its expression as a recombinant polypeptide covering the full length of the protein [218, 220, 223]. Furthermore, the proteins in this screen were produced by expression through the secretory pathway of mammalian cells, resulting in an increased likelihood of proper folding and disulphide bridge formation, meaning that conformational epitopes are more likely to be present (Chapter 3). Rather than

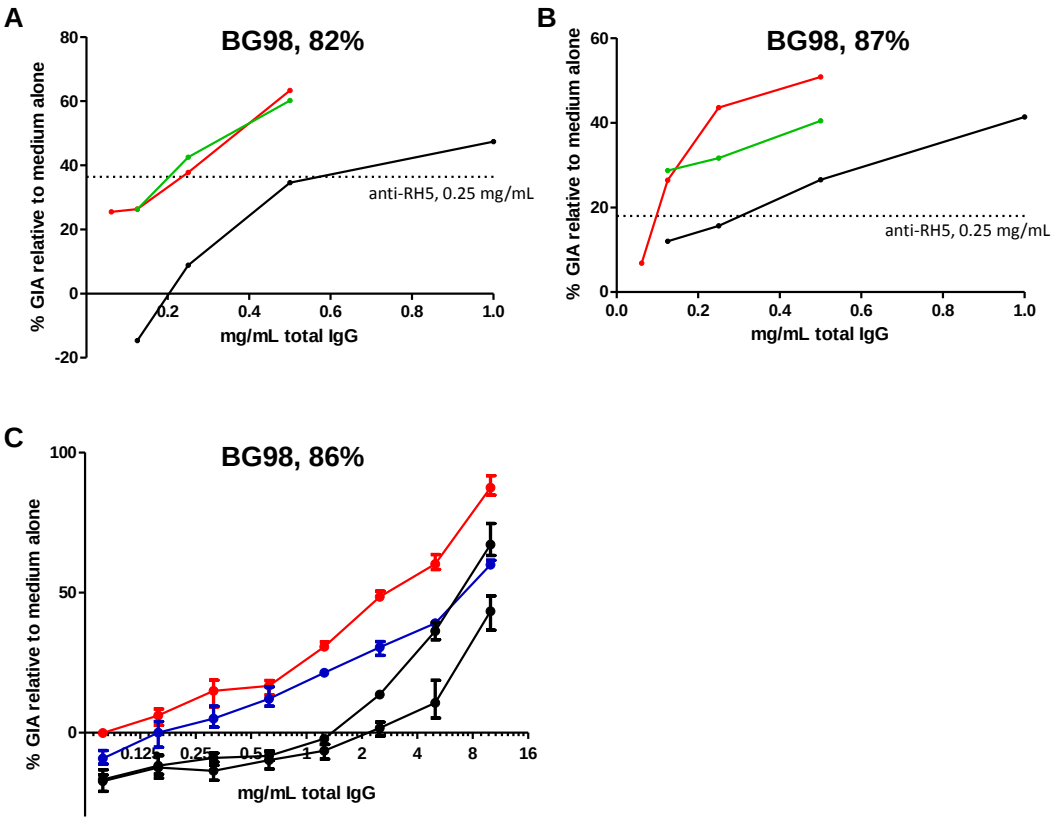

FIGURE 4.12: Antibodies raised against S-antigen have anti-parasitic activity. Mouse IgG against S-antigen (the same sample as in Fig. 4.10) either alone or in a mixture with 0.25 mg mL^{-1} rabbit antibodies against anti-PfRH5 (raised using a previously published pair of recombinant virus vectors [133]), assayed against the 3D7 clone (A) or the FVO strain (B). Dashed line, GIA of anti-RH5 rabbit IgG at 0.25 mg/mL (fixed concentration); solid black line, titrated anti-S-antigen mouse IgG; green line, predicted GIA of the titrated mouse anti-S-antigen and 0.25 mg/mL rabbit anti-RH5 mixture if the combinatorial effect was strictly additive (calculated as in [136]); red line, observed GIA of the titrated mouse anti-S-antigen and 0.25 mg/mL rabbit anti-RH5 mixture. Data points, individual wells on the GIA assay plate; lines connect means. (C) Black lines, GIA of IgG samples separately purified from two rabbits immunised with S-antigen.BAP.6His in Freund's adjuvant; blue line, GIA of IgG pooled from rabbits (n=4) immunised with recombinant RH5 protein in Freund's adjuvant; red line, GIA of IgG pooled from rabbits (n=2) immunised with virus vectors encoding RH5 (n=2) [133]. Results are representative of two replicate assays, except (B) where only one assay was performed as sample volume had become limiting. Data points, means; error bars, range of replicate wells on assay plate.

using Freund's adjuvant, a standard pre-clinical immunopotentiator that is known to disrupt the structure of even the most stably-folded proteins, I used Addavax^{TM}, a considerably less denaturing alternative. In some cases where it was not possible to produce recombinant protein I used virus vectors, a strategy known to elicit high titres of high-affinity functional antibodies that often recognise conformational epitopes [129, 157]. The use of mice as the antibody production organism (Fig. 4.2) considerably reduced costs and allowed modest throughput with regard to immunisation. In total, 28 novel antigens were screened, covering 24 separate proteins encoded in the *P. falciparum* genome. 27 of these 28 vaccines elicited antibodies that recognised antigen as it appears in fixed schizonts (Fig. 4.8). Of these 27 vaccines, two elicited antibodies that were capable of neutralising parasites (Fig. 4.10), thus identifying these two antigens as leads for further investigation.

4.3.1 Success rate for protein expression production

Overall, I was able to achieve yields >300 µg of 18 proteins from 51 attempted expressions and purifications. As the starting volume of culture was 250 mL, proteins therefore had to express at a minimum of 1.2 $\mu g\,mL^{-1}$. At a success rate of 18/51 or 35%, this is comparable with reported attempts by the laboratory of Gavin Wright to produce a library of *P. falciparum* secreted merozoite proteins. Crosnier *et al.* report that of 42 protein-coding genes transfected into suspension HEK293 cells, 21 yielded detectable protein in the supernatant at >0.5 $\mu g\,\mu L^{-1}$ [224]. It is diffficult to compare this success rate to my work. First, Crosnier *et al.* did not purify these proteins, but instead detected them in the supernatant by ELISA and thus did not suffer the loss of yield typically associated with affinity purification from a large volume of supernatant run over a small volume of resin. Second, in my system if a protein was detectable in the supernatant at >0.5 $\mu g\,\mu L^{-1}$ the total yield from a 250 mL flask would be only 125 µg which would be considered a failure. More readily comparable to my work is that of Zenonos *et al.*, also from the Wright laboratory [225]. This study describes the attempted expression of 25 proteins in 200 mL of suspension HEK293 culture, which were purified and eluted into 200 µL of elution buffer [225]. Eight of the 25 proteins examined were present at >30

µM in the eluate. Assuming an average MW of 50 kDa, 200 µL of protein at 30 µM equates to a yield of 300 µg, or 1.5 µg of protein per mL of supernatant. Zenonos *et al.* thus observed very similar yields of purified protein to me [225]. One piece of apparatus that facilitated the work of Zenonos *et al.* is a custom-made device to support parallel purification of up to 96 separate proteins (described in the supplementary material of [392]). However, it is questionable whether this would be a worthwhile acquisition for our group, given that the purification of libraries of recombinant protein for the purposes of antigen screening is now open to debate (see Chapter 6).

4.3.2 Hits and misses from the screen

In reality, the work in this Chapter encompasses two antigen screens. First, a panel of 15 proteins whose selection was based upon published literature (Section 4.2.2.1) and second, a panel of proteins selected using transcriptomics and polymorphism data (Section 4.2.2.2). Of the former, I produced pre-clinical vaccines covering 13 of the 15 selected proteins. Of the latter, I was able to produce pre-clinical vaccines covering 11 of the 40 selected proteins. Both of the 'hits' from the screen came from the literature-selected panel. If AARP had not been selected in the literature-based panel, it would have fallen into the second panel of genes. However, S-antigen would have been missed by the algorithm shown in Section 4.2.2.2—indeed, S-antigen was not identified as an invasion-associated gene by Hu *et al.* [145] and does not appear in their list of 435 invasion-associated gene products, nor in a previously published list of transciptomically defined invasion-associated candidate vaccine antigens (see Table S7 in [334]). It may well be that S-antigen is not involved in the process of RBC invasion. That S-antigen appears to be a target of neutralising antibody in the GIA assay is therefore surprising. Given the extreme variability of S-antigen between strains [353], the fact that antibodies raised against the 3D7 allele appear capable of neutralising parasites of the FVO strain (Fig. 4.12) was even more unexpected. S-antigen was selected because Alan Cowman has informally reported that knock-out attempts had been unsuccessful [35, 355], highlighting the importance of basic parasitology to the field of malaria vaccinology. The fact that S-antigen gave activity in the GIA assay also cautions against excessive

reliance upon transcriptomic studies when drawing up lists of candidate antigens for screening (see also Section 4.5, Future work).

For discussion and more detailed characterisation of antibodies against AARP see Chapter 5.

4.3.2.1 Unexpected negative results

Several of the proteins in this screen have been the subject of published reports describing their susceptibility to vaccine-induced antibody, yet did not appear to elicit growth-inhibitory antibodies in the present study. Most prominently, these included RAP1 [378–380], SERA5 [360], GAMA [356] and RIPr [361]. There are a number of possible reasons for these negative results, including reasons which do not contradict the previously published findings. RAP1, for example, has been the target of numerous growth-inhibitory monoclonal antibodies [378–380]. To my knowledge, however, there are no descriptions of the total IgG of animals vaccinated with RAP1 having measurable anti-parasitic activity at physiological concentrations in the GIA assay. This may be a quantitative issue—antigen-specific antibody generally constitutes no more than 5% of a sample of total IgG, whereas monoclonal antibodies are completely antigen specific. Thus, the RAP1-specific IgG assayed in Section 4.2.5 may have had detectable anti-parasitic activity if assayed at 1 $\mathrm{mg\,mL^{-1}}$ antigen-specific IgG, but not at the likely concentration of 10-50 $\mathrm{\mu g\,mL^{-1}}$ used in this experiment. Alternatively, it may be that the RAP1 constructs that I used did not contain the neutralising epitopes of RAP1. This seems unlikely, given that the full-length RAP1 vaccine that I tested here fully encompasses the sequence of a recombinant protein produced in *E. coli* by Collins *et al.* that proved capable of protecting *Saimiri boliviensis* from infection with *P. falciparum* when administered with Freund's adjuvant or Montanide ISA720 [389]. Other possibilities are that the virus vector regime that I used was not sufficiently immunogenic, or that the neutralising epitopes of RAP1 were somehow occluded, possibly by glycan masking (see Section 4.3.2.3). Alternatively, it may be that the mechanism of protection observed in the study by Collins *et al.* was not related to parasite neutralisation and thus was not measurable in the GIA assay.

These explanations can be extended to the other three antigens that were unexpectedly negative. SERA5, for example, is reported to be the target of antibodies that exert their activity via ADCI [141] as well as GIA. GAMA and RIPr both constitute long polypeptides, and previous reports of GIA by vaccine-induced antibody have used small fragments of the full predicted protein [356, 361], which may to some extent focus the antibody response to particular epitopes. By contrast, my strategy of immunising with full-length protein may have led to the evolution of antibodies against non-neutralising epitopes that nonetheless contribute to the homeostatically defined ceiling for antibody production in a given immune response.

4.3.2.2 Immunogenicity of vaccines

MSP11 was the only antigen that failed to elicit antibodies that were detectable by IFA against fixed schizonts (Fig. 4.8). I subsequently showed that this was due to poor immunogenicity of the MSP11/Addavax^{TM} preclinical vaccine (Fig. 4.9). Apart from MSP11, every other antigen elicited antibodies that were detectable by IFA at a total (non-antigen-specific) antibody concentration of 20 $\mu g\,mL^{-1}$ (Fig. 4.8). It should be noted, however, that detection of antibodies by IFA accompanied by a negative result in the GIA assay does not prove that the antigen in question is not susceptible to anti-parasitic antibody. First, the GIA assay only detects Fc-independent activity. Second, positivity by IFA is only a vague indicator of immunogenicity and does not give any information regarding the quality of the antibodies. For example, non-neutralising antibodies may still give a strong signal by IFA. Furthermore, the strength of the IFA signal is not solely dependent upon the concentration of the primary antibody, and is affected by the abundance of the antigen in the fixed schizont as well as the extent to which the protein has been distorted by the fixation procedure.

4.3.2.3 Glycosylation of proteins

When expressing proteins from mammalian cells, either *in situ* by the use of genetic immunisation, or *in vitro* from cultured cells, the glycosylation state of the recombinant

protein must be considered. This is particularly important for *Plasmodium* proteins, which are rarely glycosylated in the parasite [253, 254, 383]. N-linked glycans can be over 20 times the size of a standard amino acid side chain, and could potentially occlude large regions of the recombinant protein. This effect is used by viruses to evade humoral immunity [393] and can be exploited by vaccinologists to focus the antibody response to the most vulnerable epitopes on the surface of a protein, as demonstrated for *P. vivax* Duffy-binding protein [394]. However, in the absence of detailed structural characterisation, this kind of rational approach is not possible. With regard to antigen screening, one benefit of the protein-in-adjuvant immmunisation strategy is the possibility of analysing the composition and processing of the immunogen prior to immunisation. All 5 of the proteins that I analysed showed at least some level of glycosylation (Fig. 4.7Ai). My rationale for expressing polypeptides of identical primary structure to those encoded in the *P. falciparum* genome is set out in Section 4.2.3.1. However, there are alternative approaches to the issue of glycosylation that could be considered in future antigen screening studies. HEK293 cells can be grown in the presence of kifunensine or swainsonine [395], inhibitors that block the action of mannosidase enzymes. The trimming of mannose structures by these enzymes is a prerequisite for the formation of complex glycans (which cannot be cleaved by endoglycosidase H). Proteins expressed in the presence of kifunensine or swainsonine therefore lack complex glycans, meaning that the majority of N-linked glycans can be simply removed by treatment with endoglycosidase H [395]. Alternatively, a HEK293 line lacking the N-acetylglucosaminyltransferase I (HEK293S GnTI-deficient cells) has also been shown to produce glycans that are extremely sensitive to endoglycosidase H [396]. However, both of these strategies involve an extra protein treatment step, and as the endoglycosidase H represents a protein contaminant, further purification (e.g. by size exclusion chromatography) is necessary [395]. To mitigate this, the HEK293S GnTI-deficient cell line has been further engineered to contain a Golgi-targeted endo-β-N-acetylglucosaminidase, which means that proteins are allowed to correctly fold in the ER prior to N-glycan removal in the Golgi [397]. Unfortunately, the HEK293S GnTI-deficient line (and presumably its derivatives) has been shown to produce markedly less protein than standard HEK293 protein expression lines such as 293E or 293T cells.

4.3.3 Comparison to published reverse-vaccinology experiments

The antigen screen presented here was modest in scale by comparison to previously published reverse-vaccinology experiments [314, 327]. The decision to purchase synthetic genes, along with labour constraints in an academic laboratory, meant that it was only feasible to attempt production of vaccines against a set of 55 proteins, which comprised two distinct panels of proteins (see Sections 4.2.2 and 4.3.2). Table 4.2 compares my antigen screen, broken down into the two panels of proteins, with the two highest-profile reverse-vaccinology studies published to date.

TABLE 4.2: Comparison of the work in this this Chapter with published antigen screens

Pathogen [reference]	**Number of proteins[a]**	**Number of vaccines[b]**	**Candidate antigens identified**
Serogroup B *Neisseria meningitidis* [314]	570	350	7
Streptococcus agalactiae [327]	589	312	4
P. falciparum [this Chapter]	55	24	2
Breakdown of antigens in this Chapter			
Literature-selected proteins	15	13	2
Genomic-, transcriptomic-, and polymorphism-selected panel	40	11	0

[a]Total number of potential protein antigens shortlisted for screening.

[b]Total number of pre-clinical vaccines screened for the ability to elicit a functional immune response.

The failure to find any novel antigens amongst the 'Genomic-, transcriptomic-, and polymorphism-selected panel' is not surprising when one considers the rate of antigen discovery from the published screens—against Serogroup B *Neisseria meningitidis*, only

one antigen elicited functional antibodies for every 50 tested, and against *Streptococcus agalactiae* only one antigen elicited functional antibodies for every 147 tested. Given that my 'Genomic-, transcriptomic-, and polymorphism-selected panel' only contained 11 pre-clinical vaccines, the failure to identify any susceptible antigens is unsurprising. The statistical precedents set by the published studies also suggest that there is currently not enough evidence to determine whether my antigen screening strategy was in any way flawed.

More broadly, one aspect of the reverse-vaccinology pipeline that sets the published studies apart from the present work is the lack of an established assay that is predictive of protection against clinical malaria (discussed in Sections 1.5.3 and 1.5.5). Whereas the behaviour of a serum sample in the complement-mediated bactericidal assay strongly predicts protection against Serogroup B *Neisseria meningitidis* [314, 398], and the neonatal mouse model of *Streptococcus agalactiae* infection closely mimics the disease in human infants [327, 335], there is no clear correlate of protection for blood-stage malaria [31]. Thus, a clearer understanding of the mechanism of natural protection, better characterisation of the relationship between the course of disease in humans and *in vitro* GIA (as well as other measurable parameters of an antibody or serum sample, such as ADRB and ADCI), and the development of new assays that predict the *in vivo* functionality of antibodies are all urgently needed.

4.4 Conclusions

This screen identified two antigens for future investigation, one of which had never received any previous consideration as a vaccine candidate. Of the 55 proteins I selected for inclusion in the screen, I was able to produce 18 protein-in-adjuvant pre-clinical vaccines. While an expression rate of 35% compares favourably with the most successful published attempts at producing libraries of merozoite proteins [224, 225], this still leaves a large number of gene products unexamined. The virus vector platform was reliably immunogenic (Section 4.2.4.1) but remains too labour intensive to be a useful screening tool. The high ratio of 'misses' to 'hits' in previous antigen screens (Table 4.2)

highlights the importance of screening large numbers of antigens. Therefore, future blood-stage antigen screening programmes should aim to markedly expand the length of the candidate pre-clinical vaccine shortlist. However, the primary conceptual difficulty for malaria vaccinology would still stand: determining whether an antigen is susceptible to any kind of immune response remains challenging due to a lack of validated assays to measure correlates of protection [31].

4.5 Future work

The work in this Chapter uses the GIA of an antibody sample as the predictor of an antigen's protective value. However, as discussed in Sections 1.5.5 and 4.1.1, there are other assays of anti-parasitic activity that are mechanistically distinct from the assay of GIA. The activity of an IgG specificity in the ADRB assay has been shown to correlate with protection from clinical malaria in a single publication [108], but the protocol is not widely accepted in the field. However, the assay is now established in our laboratory [110], and it is a priority to screen all of the anti-serum samples generated as part of this study for ADRB activity.

The question of how to economically expand the antigen screen described in this Chapter is not trivial. Chapter 6 describes a novel approach that has the potential to reduce the labour and reagent cost associated with the generation of high-titre antibodies by comparison to protein-in-adjuvant and virus vector-based strategies, allowing more antigens to be included in the initial shortlist of candidate antigens. It also promises to increase the overall success rate for elicitation of high-titre functional antibody, as recombinant protein is not required. Alternatively, if protein-in-adjuvant immunisation is to be preferred, one approach is simply to screen more gene products. The *P. falciparum* encodes 1088 gene products with a predicted signal peptide, theoretically exposing them to antibody at some point in the parasite lifecycle. Assuming a success rate of over 25% for the expression of completely unannotated proteins, this means that approximate 250 recombinant proteins could be produced. Savings could be made on gene synthesis costs, a major expense of this programme, by attempting to express genes

as they appear in the parasite genome (although expression success rates may also fall). Alternatively, literature-based selection has proved fertile ground both in this screen and a previous study [133]. Although most merozoite proteins that cannot be readily knocked out have now been screened in our laboratory, a recent epidemiological study focuses attention on a set of generally poorly-characterised antigens [148].

Chapter 5

Characterisation of antibodies raised against recombinant AARP

Authorship statement

I designed and planned all of the experiments reported in this Chapter except the NMR spectrum shown in Fig. 5.8. Specific contributions by other laboratory workers were as follows. The AARP.BAP.His construct used to generate Fig. 5.3B and Fig. 5.3F was designed and cloned by me and then produced by Daniel G. Alanine according to protocols that I had established (Chapter 3). A bacterially expressed fragment of AARP ('N-half') was designed and expressed with ^{15}N labelling by Katherine E. Wright with no input from me. Analysis of 'N-half' by NMR spectroscopy (Fig. 5.8) was also performed by Katherine E. Wright. Some GIA assays were performed with parasites that had been maintained by Rebecca E. Brown. The IFAs shown in Fig. 5.6 were performed by Rebecca E. Brown under my supervision.

5.1 Introduction

Fig. 4.10 in Chapter 4 suggested that mouse total IgG raised against a full-length AARP construct (excluding signal peptide, see Fig. 5.1) can give total growth inhibition. This was observed with an IgG concentration of 1 $\mathrm{mg\,mL^{-1}}$, only 40% of the physiological concentration found in mouse serum. In light of this unprecendented GIA, I sought to characterise the anti-parasitic activity of these antibodies.

5.1.1 Previous descriptions of AARP

To my knowledge, no publication has reported an attempt to knock out the gene for AARP for any laboratory strain. It is therefore not known if this protein is essential to parasite growth. It was initially described in a 2007 paper as a merozoite protein predicted to contain 217 amino-acid residues [358]. There are a number of findings from this paper that are highly relevant to the work described in this Chapter. The domain structure of AARP was described, identifying the asparagine-rich region, a polyproline region and a transmembrane region (see Fig. 5.1, although the transmembrane region is not wholly convincing: whilst the TopPred server identifies a transmembrane region [399], the TMHMM server does not [400]). It was also demonstrated that AARP is transcribed almost exclusively in late schizonts (the period when merozoites are maturing) and appears to localise to the apical end of merozoites. It did not co-localise with AMA1, EBA-175 or RhopH1, leading the authors to suggest a positioning in the rhoptry neck. Expressing full-length AARP, or an N-terminal fragment (I20–D107), on the surface of COS cells caused RBCs to rosette around them, which did not occur when the RBCs were treated with trypsin or neuraminidase. When the N-terminal fragment was expressed as a soluble recombinant protein in *E. coli*, it was capable of binding to RBCs. This led the authors to conclude that AARP functions as an adhesin (see Section 1.7.2), binding to a neuraminidase- and trypsin-sensitive RBC receptor through its N-terminal region. Total rabbit IgG raised against the N-terminal fragment and purified with protein G was reported to give 70% inhibition of invasion against the 3D7 strain at 0.5 $\mathrm{mg\,mL^{-1}}$.

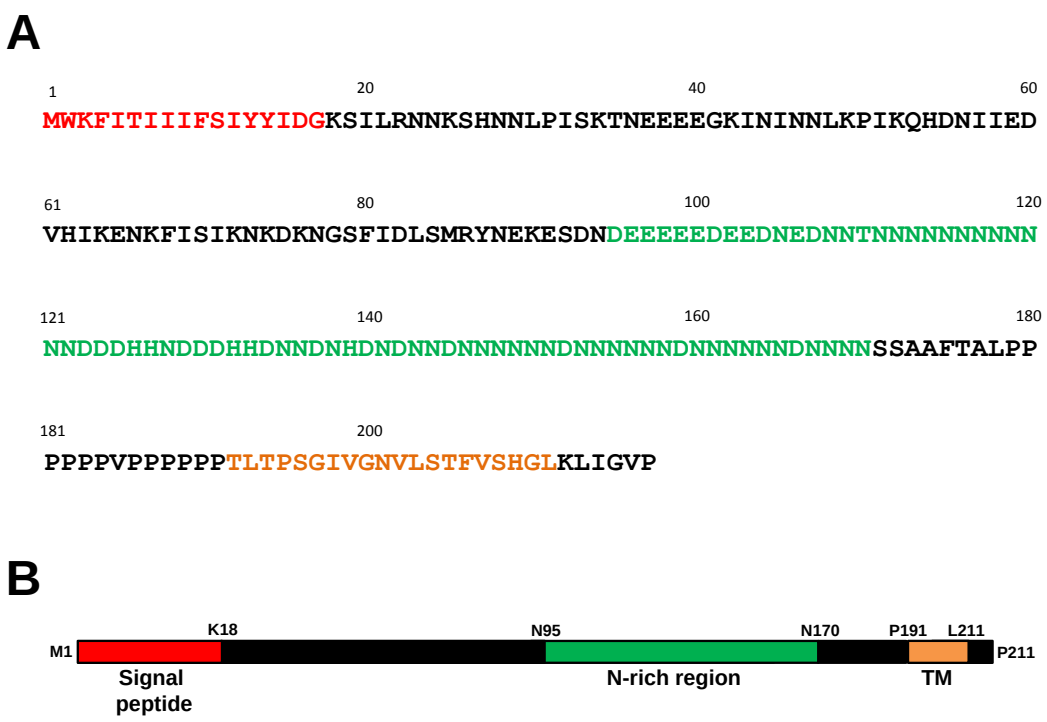

FIGURE 5.1: Sequence (A) and schematic (B) of the 3D7 allele of AARP. Colours of residues in (A) correspond to regions in (B). TM, transmembrane domain.

This would have constituted an unprecedented level of neutralisation, but it comes with two caveats. First, in these assays, the anti-AARP antibodies were outperformed by positive control antibodies to MSP-1_{42}, which is only moderately susceptibile to invasion-inhibitory antibodies by comparison to AMA1 and RH5. Second, the level of neutralisation reported in this paper was not reproduced in subsequent papers published by groups from the same research institute. In one such study, anti-AARP rabbit antibodies at 3.3 $\mathrm{mg\,mL^{-1}}$ only gave around 30% invasion inhibition, although AARP seemed to contribute to strain-transcending neutralisation when used in combination with other anti-adhesin specificities [135]. In another study, anti-AARP rabbit antibodies gave around 50% invasion inhibition when used at 10 $\mathrm{mg\,mL^{-1}}$, whereas an equivalent concentration of anti-RH5 rabbit antibodies gave over 80% GIA [401]. However, given that the antibodies raised against my longer AARP construct (K18–P191) had outperformed anti-RH5 antibodies (Fig. 4.10), I had firm grounds to believe that my longer construct represented a considerable improvement over the bacterially expressed construct produced by Wickramarachchi *et al.* [358].

5.1.2 Neutralisation of parasites by antibodies

A number of targets of vaccine-inducible growth-inhibitory antibodies have now been identified. As described in Chapter 1, Section 1.5.5.2, immune cell-independent neutralisation generally requires that antibodies bind to parasite protein via specific epitopes, thus hindering the protein from carrying out its normal function. Dissecting the molecular mechanisms of blockade tends to rely on mAbs which are assumed to recapitulate the activity of polyclonal serum, and is greatly facilitated by access to recombinant parasite protein. However, even where a parasite antigen is thought to have just a single well-defined function, there can be multiple aspects to the mechanism of neutralisation. Some of the best-characterised neutralising targets are described below.

- Antibodies raised against AMA1 probably inhibit invasion by blocking the essential AMA1–RON2 interaction (see Section 1.7.1.3) [402], with many growth-inhibitory mAbs binding to a loop of AMA1 involved in RON2 binding [131, 403, 404]. These insights do not, however, explain the observation that non-AMA1-specific antibody from patients exposed to *P. falciparum* appears to be able to block the function of AMA1-specific antibody [112].

- At least some inhibitory mAbs to EBA-175 block its interaction with the RBC [405], generally by binding to the F2 region which is thought to interact with GYPA [406] (see also Section 1.7.2 and Section 4.2.3.1). However, this cannot be the whole picture, as antibodies against F2 can block invasion of RBCs that have been treated to remove the sialic acid moieties thought to be essential for the GYPA–EBA-175 interaction [407]. Moreover, antibodies raised against constructs of EBA-175 that do not include the F2 region can also block invasion [134, 375], although this makes sense in light of recent work indicating that F2 is not the only domain of EBA-175 with a role in binding GYPA [408].

- The interaction between RH5 and its RBC receptor basigin is essential to parasite survival [128], and some growth-inhibitory mAbs to RH5 have been found to block this *in vitro* [129]. However, the most potent growth-inhibitory mAb against RH5 does not appear to inhibit binding to basigin [129], and indeed, the epitope of

this mAb does not appear to overlap with the basigin-binding site in the crystal structure of RH5 [220]. It has been suggested that antibodies that do not appear not to block the interaction between purified, recombinant RH5 and basigin *in vitro* may have subtly different effects *in vivo*, where RH5 is closely apposed to the parasite plasma membrane [220].

The examples above represent the most straightforward cases of antibody-mediated neutralisation. Where the function of the antigenic target is not clear, or when the target has multiple functions, there may be multiple mechanisms of immune cell-independent *in vitro* growth inhibition. Antibodies to MSP1 that interfere with its processing on the surface of the merozoite have strong GIA, implicating inhibition of processing as a mechanism of growth inhibition [409–411]. Anti-MSP1 antibodies can also be transported into the RBC along with the merozoite during invasion, resulting in reduced intra-erythrocytic growth [411, 412]. Both of these mechanisms are in addition to agglutination of merozoites, which also appears to contribute to *in vitro* growth inhibition [413].

Given the previously published data on AARP that suggested it was an adhesin protein whose role was to bind a receptor on the RBC, I confidently anticipated that the antibodies that I had raised would fall into the mechanistically straightforward former category. In this I was to be disappointed.

5.2 Results

5.2.1 GIA of anti-AARP antibodies

Initial experiments were performed using the same batch of mouse IgG as shown in Fig. 4.10, raised against AARP.BAP.Strep (containing residues K18-P191 of AARP from the 3D7 strain). Against both the 3D7 clone and the FVO strain, anti-AARP.BAP.Strep antibodies outperformed mouse anti-RH5 antibodies raised using virus vectors [133] (Fig. 5.2). Furthermore, mouse anti-AARP.BAP.Strep IgG was found to interact at

least additively with rabbit anti-RH5 antibodies against both parasite lines, although limiting quantities of this IgG sample meant that the result against FVO, which was not fully conclusive, could not be repeated.

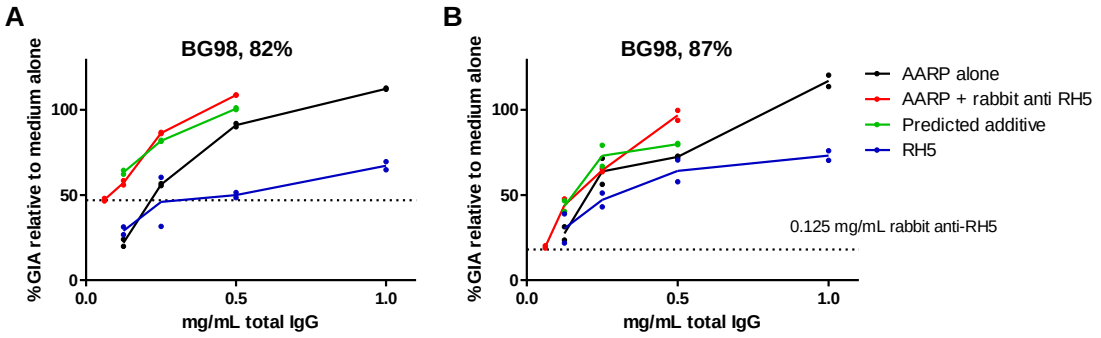

FIGURE 5.2: Antibodies raised against AARP.BAP.Strep recombinant protein outperform anti-RH5 antibodies raised with virus vectors. Mouse IgG against AARP (the same sample as in Fig. 4.10) assayed against the 3D7 clone (A) or the FVO strain (B). Dashed line, GIA of 0.25 mg mL^{-1} anti-RH5 rabbit IgG raised with virus vectors [133]; solid black line, anti-AARP mouse IgG; green line, predicted GIA if the mouse anti-AARP/rabbit anti-RH5 mixture interacted additively (calculated as in [136]); red line, observed GIA when the mouse anti-AARP/rabbit anti-RH5 mixture was assayed; blue line, mouse anti-RH5 IgG raised with virus vectors. Results in (A) are representative of two replicate assays, but for (B) only one assay was performed as sample volume had become limiting. Measured GIA for the BG98 standard at 6 mg mL^{-1} is shown above each graph. Data points, results from individual wells on the assay plate; lines connect means.

Disappointingly, in subsequent mouse immunisations performed to identical protocols, I found that AARP.BAP.Strep elicited potent antibodies only erratically (Fig. 5.3, A–E). A fragment of AARP (I20–D95 with 6His and a TEV site fused to the N-terminus, hereafter referred to as 'N-half') that was very similar to the construct used in previous published studies (Section 5.1.1) was expressed in *E. coli* by Katherine E. Wright. This also failed to elicit antibodies with any measurable GIA against the 3D7 strain. The effect seemed to be specific to the batch of protein used for immunisation, as IgG raised against the same batch of protein had comparable anti-parasitic activity (see especially Fig. 5.3, C–E). This inconsistency complicated the characterisation of the anti-parasitic activity of these antibodies. First, it meant that before I could dissect the mechanism of parasite-killing activity by the antibodies, I had to dissect the reasons for the inconsistency of the antibody function. Second, it means that sample volume was heavily constrained, as I was unable to routinely produce antibodies with anti-parasitic activity. The remainder of the experiments in this Chapter were therefore designed to

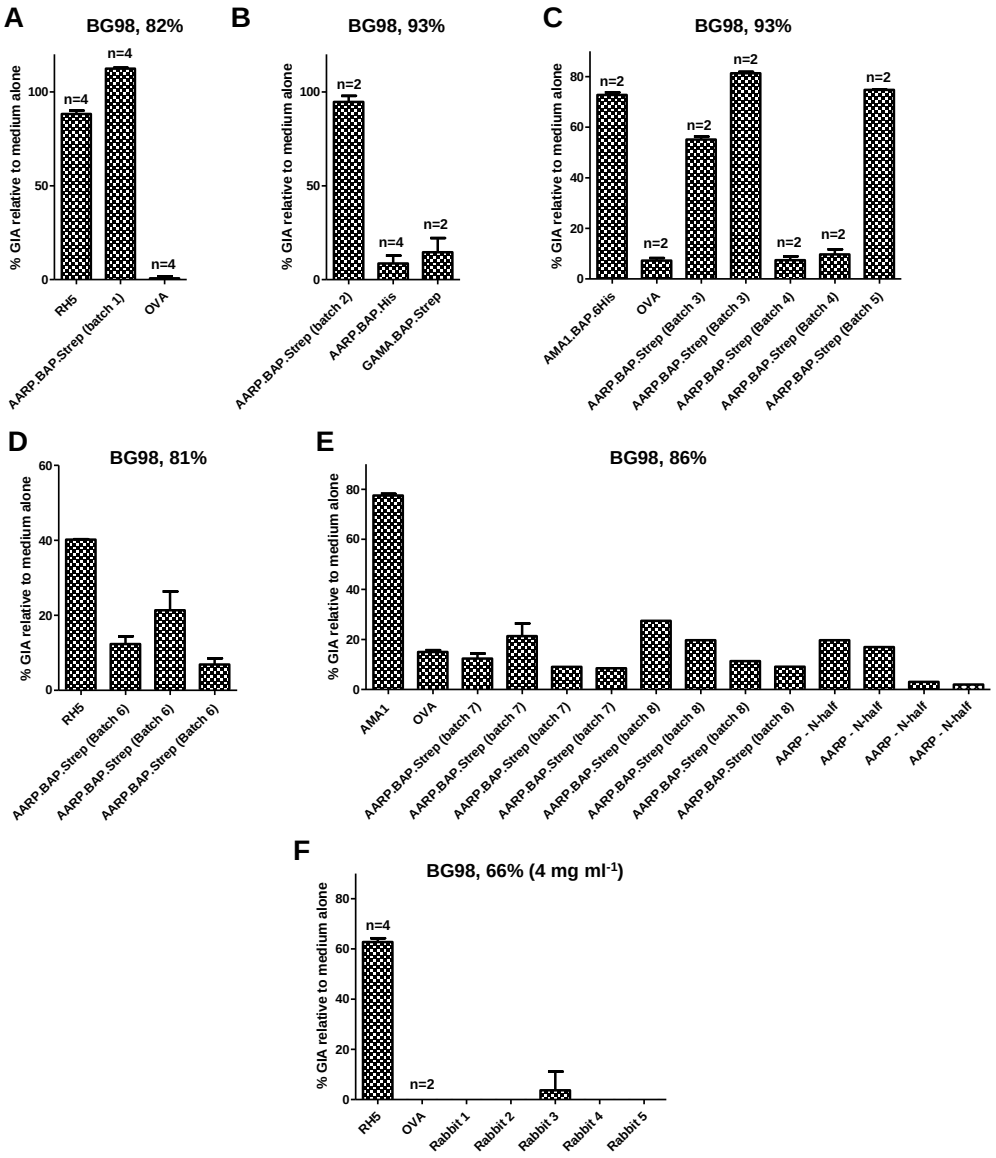

FIGURE 5.3: Inconsistent function of antibodies against AARP. (A–E) Results of five independent mouse experiments. Groups of BALB/c mice were immunised with the stated batch of antigen using a protein-in-adjuvant regime in all cases except RH5, where virus vectors were used [133]. IgG from pooled serum was assayed for GIA at 1 mg mL^{-1}. Eight batches of AARP.BAP.Strep protein were used in all. (F) IgG was purified from pooled rabbit serum against RH5 or OVA, or from the serum of 5 individual rabbits immunised with AARP.BAP.His (see Section 2.5.2), and assayed for GIA at 6 mg mL^{-1}. Where IgG was purified from pooled mouse serum, the number of mice in the pool is indicated above the bar. Results are representative of two replicate GIA assays. Measured GIA for the BG98 standard at 6 mg mL^{-1} (except in (E)) is shown above each graph. Height of bars, mean of three replicate wells on the assay plate; error bars, range across three replicate wells on the assay plate.

shed light on the mechanism of anti-AARP activity while consuming a minimum of sample.

5.2.2 Comparable titres against recombinant protein

I wondered whether the immunogenicity of AARP.BAP.Strep may be variable in mice, thus explaining the differences in functionality of mouse total IgG. The antibody samples assayed for GIA in Fig. 5.3, C–E, were found to contain comparable concentrations of AARP-specific antibody by ELISA (Fig. 5.4Ai) regardless of their anti-parasitic activity. Wickramarachchi *et al.* have reported that a bacterially-expressed fragment covering the first half of AARP (I20–D107) can elicit rabbit antibodies that are inhibitory to invasion [358] and have claimed that this region contains the RBC-binding domain [135, 358]. To test whether comparable reactivity to my longer fragment was disguising differences in reactivity to this region, I performed ELISAs using the 'N-half' fragment as the coating antigen. Again, there was no difference in reactivity that corresponded to the GIA of the sample. In the absence of rabbit antibody sample that had any GIA, it was impossible to determine whether the lack of functionality in the GIA assay for these samples was simply due to poor immunogenicity of the AARP.BAP.His protein when immunised into rabbits. In mitigation of this, the protein-in-adjuvant formulation with Freund's adjuvant is known to be robustly immunogenic, and the antibody samples used in the GIA assay showed titres of anti-AARP antibody at least 100-fold higher than in pre-immune serum or in anti-RH5 (i.e. negative control) rabbit IgG (Fig. 5.4B).

These results led me to conclude that the differential anti-parasitic activity of anti-AARP antibodies did not arise from differences in the magnitude of the immune response to the recombinant antigen, but instead implicated the fine specificity of the polyclonal IgG.

5.2.3 Differential reactivity to parasite antigen

To characterise the fine specificity of the anti-AARP.BAP.Strep response, I used the five IgG samples from a single mouse experiment that showed differential GIA in Fig. 5.3C.

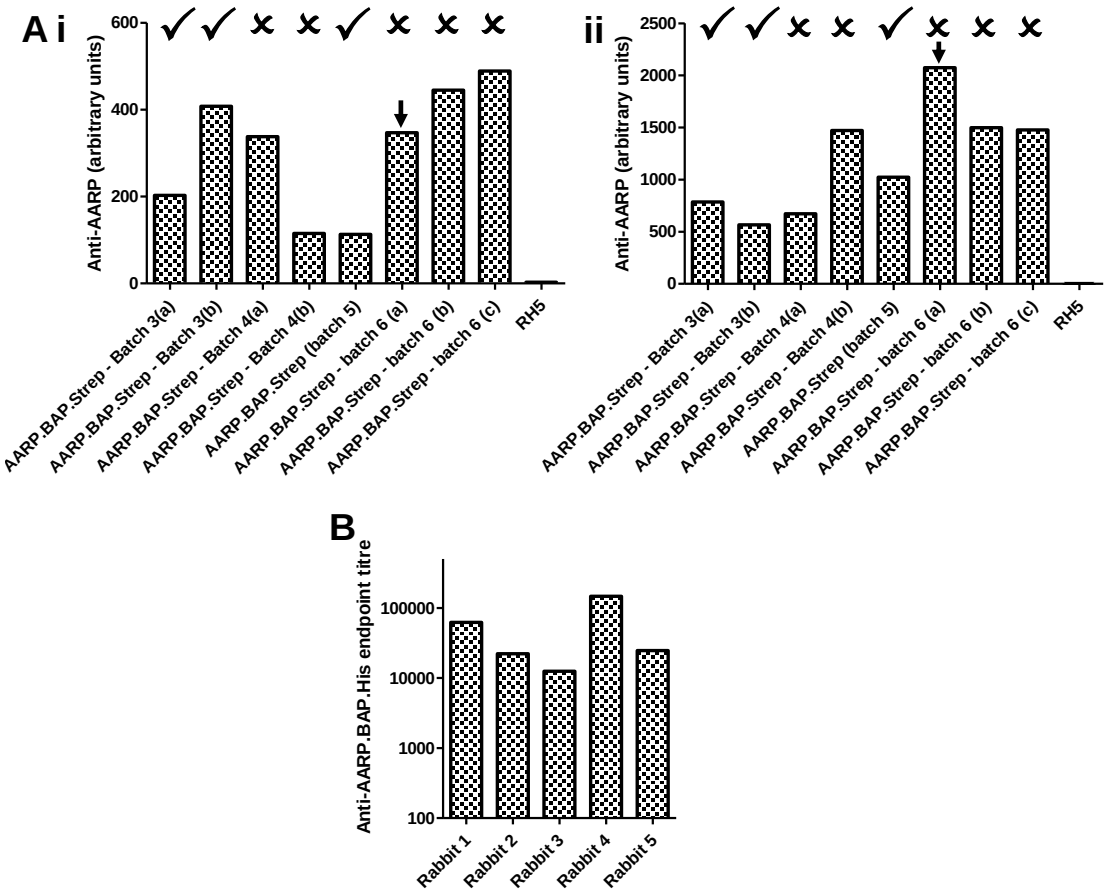

FIGURE 5.4: AARP.BAP.Strep and AARP.BAP.His are consistently immunogenic. (A) The mouse IgG assayed in Fig. 5.3, C and D, was analysed by direct standardised ELISA using AARP.BAP.Strep (Ai) and AARP 'N-half' (Aii) as the coating antigen. Purified IgG from a mouse immunised with the sixth batch of AARP.BAP.Strep (indicated by arrow) was used to construct the standard curve (see Section 2.6.2). Anti-RH5 antibody served as the negative control (Fig. 5.3, panel D). Ticks and crosses indicate if a sample demonstrated high functionality in the GIA assay (Fig. 5.3). (B) The rabbit IgG assayed in Fig. 5.3 panel F, was analysed by direct endpoint ELISA using AARP.BAP.Strep as the coating antigen. Pre-immune serum from each rabbit had a titre of less than 100. All ELISAs were performed with peptide depletion as described in Section 2.6.2 and Section 4.2.4.2. Height of bars, mean of two replicate wells.

5.2.3.1 Schizont lysate Western blot

The A–T rich genome of *P. falciparum* makes long runs of asparagine common [250, 414, 415]. This led me to speculate that antibodies against AARP are likely to cross-react with other proteins expressed at the asexual stage. I assessed this first by Western blot against lysed schizonts and RBCs. Here, cross-reactive staining to proteins in schizont lysate was negatively associated with function in the GIA assay. Against expectation, samples that gave strong GIA did not consistently react to any one band in the schizont lane, with Batch 3(a) and Batch 5 almost negative. Only Batch 3(b) showed

staining of a band whose position was approximately the same as that of recombinant AARP.BAP.Strep (see Fig. 4.7). All IgG samples reacted with a band at approximately 55 KDa in the RBC lysate, including antibodies raised against untagged OVA, suggesting that this was not a result of immunisation with AARP.BAP.Strep. Aside from this, Batch 4(a) and Batch 4(b) did not appear to react to any distinct bands in the RBC lysate, indicating that the high level of staining in the schizont lanes was predominantly against parasite-derived protein, as opposed to residual host proteins derived from the RBC. Although these blots did not reveal conclusively that reactivity to a specific parasite protein was associated with GIA, it did clearly implicate the fine-specificity and cross-reactivity of the antibodies in the inhibitory activity.

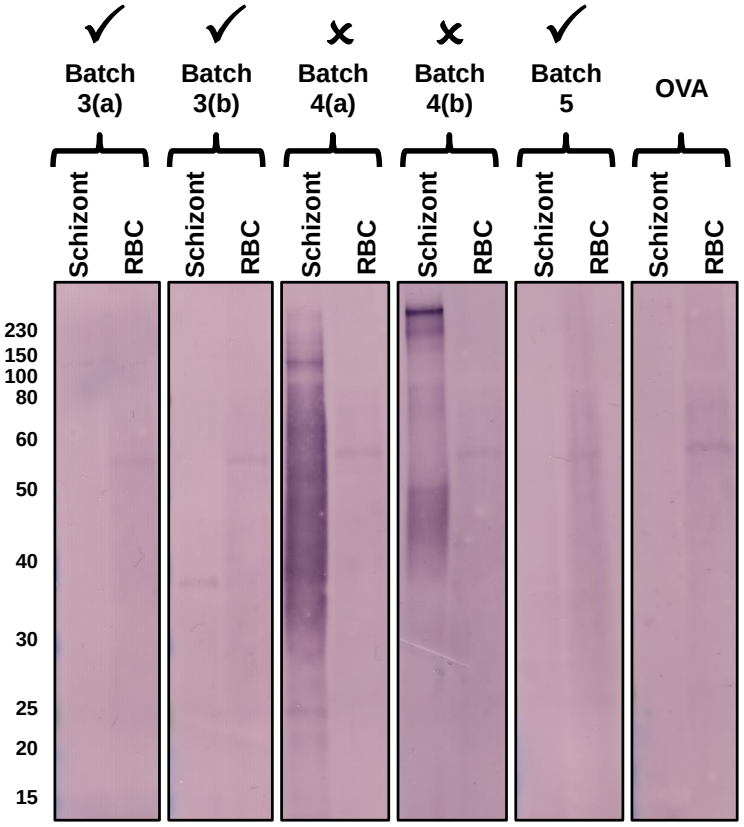

FIGURE 5.5: Differential cross-reactivity of AARP antibodies to schizont and RBC lysate. Schizont lysate and RBC lysate were separated by reducing SDS-PAGE, Western blotted, then probed with mouse IgG samples from Fig. 5.3C at 0.5 $\mu g\,\mu L^{-1}$. All strips were developed for the same length of time. Ticks and crosses indicate if a sample demonstrated high functionality in the GIA assay (Fig. 5.3C). Molecular weight marking based on NEB Colorplus ladder. Results representative of two experiments using separate batches of schizont extract.

5.2.3.2 IFA against fixed schizonts

The different bands recognised by IgG purified from mice immunised with a theoretically identical antigen suggested that the binding pattern of IgG against whole parasites was likely to vary as well. I used IFA to compare the staining of PFA-fixed schizonts by each of the five IgG samples assayed in Fig. 5.3C. The staining intensity and pattern was strikingly varied between samples in a manner not wholly consistent with an anti-schizont Western blot (Figs. 5.5 and 5.6). Batch 3(a) and 3(b), which barely reacted with schizont extract (Fig. 5.5), gave very intense staining of schizonts reminiscent of known merozoite proteins such as RAP1 and Pf92 (Figs. 4.8 and 5.6A). These samples also produced a high level of background staining of RBCs that was not seen in the Western blots. Batch 4(a) and Batch 4(b) showed considerably less background staining of RBCs, and also weaker staining of parasites (Fig. 5.6A). Batch 4(b), which seemed to be negative by IFA when analysed with the same exposure as the other samples, was found to be weakly positive when exposure time was increased (Fig. 5.6B). These observations seemed to implicate strength of staining by IFA with measured activity in the GIA assay. However, this cannot be the whole explanation because Batch 5, which also had strong anti-parasitic activity, was completely unreactive to fixed parasites, with no signal observed even with extended exposure periods (Fig. 5.6A). These data show that mice immunised with seemingly identical antigen can mount qualitatively different immune responses resulting in the production of IgG that varies markedly in its ability to recognise PFA-fixed whole parasite.

5.2.3.3 Mapping antibodies to AARP.BAP.Strep with linear peptides

Bioinformatic analysis indicated that AARP was highly likely to be a natively unstructured protein (Fig. 5.7). The DisEMBL server predicts disorder across almost the entire length of the protein with a high degree of confidence (Fig. 5.7A) [416]. Likewise, PSIPRED assigns the majority of the protein as random coil with a high level of probability, with two short regions (less than 10 amino acid residues) predicted with a much lower degree of confidence to have helical propensity (Fig. 5.7B) [417, 418].

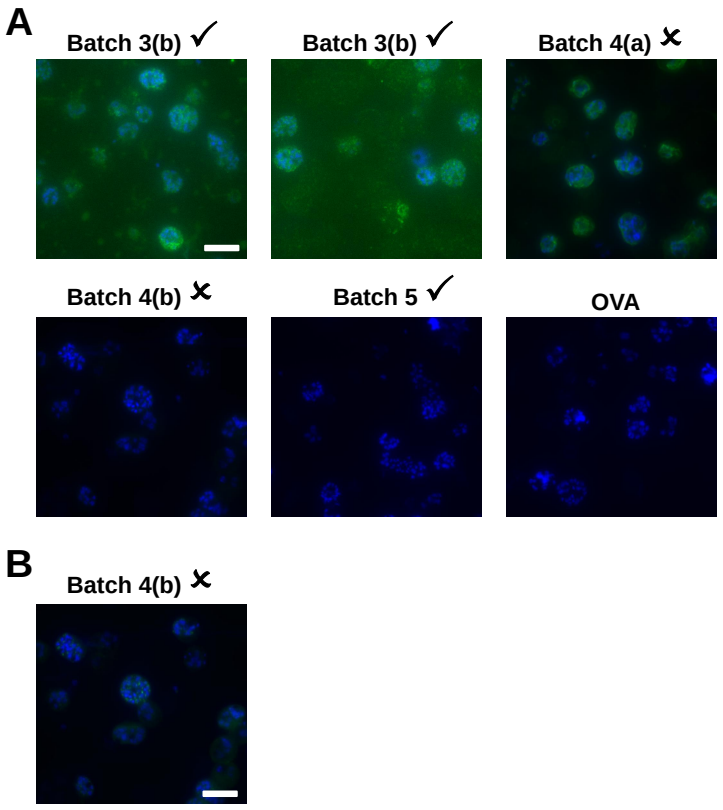

FIGURE 5.6: Differential reactivity of AARP antibodies to fixed schizonts, visualised by IFA. The mouse IgG assayed in Fig. 5.3 panel C, was applied to fixed schizonts at 20 µg mL^{-1} in PBS and tested for reactivity by indirect IFA (green, alexa-488). DNA was visualised with 4,6-diamidino-2-phenylindole (blue). (A) Representative fields, all images taken at the same exposure and no adjustment for brightness or contrast. (B) Signal from antibody Batch 4(b) can be detected by adjustment of exposure times, brightness and contrast (Batch 5 is blank regardless of exposure or image manipulation). Ticks and crosses indicate if a sample demonstrated high functionality in the GIA assay (Fig. 5.3). Scale bar, 10µm.

The 'AARP N-half' fragment was produced by Katherine E. Wright with ^{15}N labelling to facilitate NMR spectroscopy. In a two-dimensional heteronuclear single quantum coherence experiment, the fragment was shown have narrow ^{1}H chemical shift dispersal, characterisic of a protein without stable secondary and tertiary structure.

In light of this, I hypothesised that the antibody clones having parasite-killing activity in the assay of GIA were likely to bind to a linear epitope that could be recapitulated by a synthetic linear peptide. I therefore attempted to map the IgG response using biotinylated linear peptide as the bait antigen (for peptide details, see Section 2.6.3). These ELISAs yielded yet more confusing results. Batch 3(a) and Batch 4(a), having shown completely different parasite-binding activity in Figs. 5.5 and 5.6, showed similar

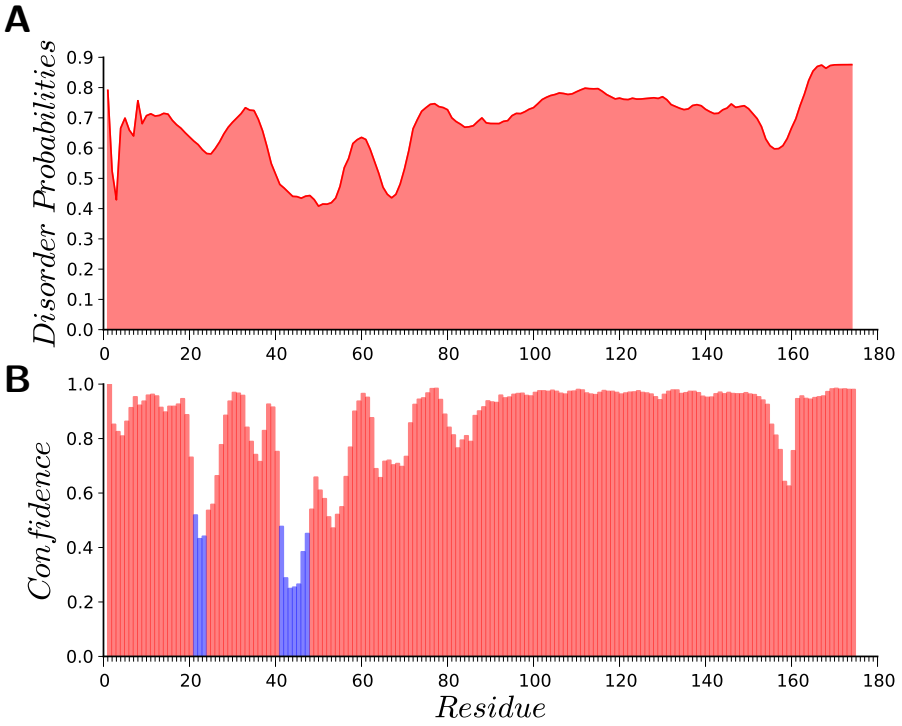

FIGURE 5.7: Bioinformatic analysis of AARP structure from K18–P191 (corresponding to residues 1–174 on the plots). (A) DisEMBL^{TM}1.5 [416] disorder prediction. (B) Secondary structure prediction using the PSIPRED method [417], obtained using the UCL PSIPRED server [418]. Pink, predicted random coil; blue, predicted helices. Plots produced with assistance from Jacob P. Brady.

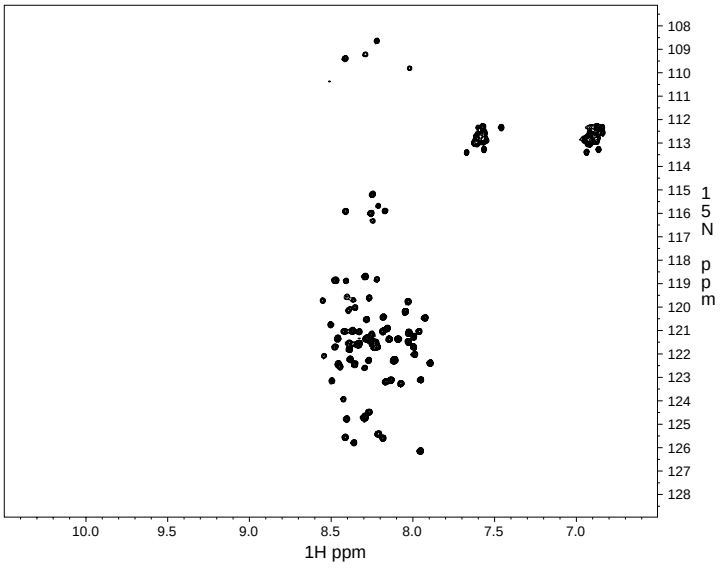

FIGURE 5.8: Heteronuclear single quantum coherence nitrogen–proton spectrum of ^{15}N-labelled 'AARP N-half' construct. Amino acids I20–D95 were N-terminally fused with a 6His tag followed by a Tobacco Etch virus cleavage site and expressed in *E .coli*. Design, expression, purification and NMR analysis were all performed by Katherine E. Wright.

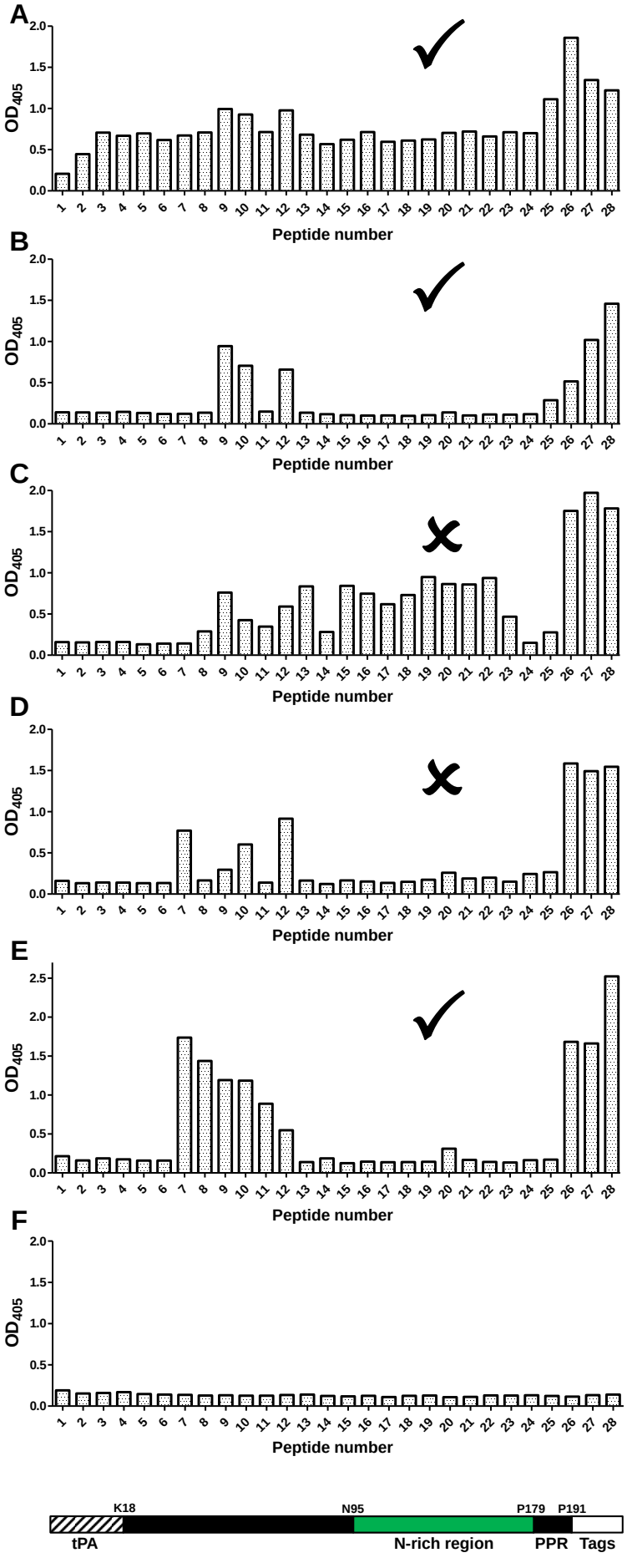

FIGURE 5.9: Linear peptide mapping by ELISA. The mouse IgG assayed in Fig. 5.3C was tested for reactivity to 28 20mer peptides offset by 8 amino acid residues covering the full length of tPA.AARP.BAP.Strep (see Section 2.6.3 for details). Peptide number is shown under each chart.
(A) Batch 3(a),
(B) Batch 3(b),
(C) Batch 4(a),
(D) Batch 4(b),
(E) Batch 5,
(F) OVA.
IgG was applied to immobilised 20mers at 3.3 $\mu g\,mL^{-1}$. For each given peptide, all IgGs were assayed on the same plate. Ticks and crosses indicate if a sample demonstrated high functionality in the GIA assay (Fig. 5.3). All results representative of two replicate assays. Height of bars, means of two replicate wells. Schematic of AARP is shown below. Residue numbers correspond to AARP as it is encoded in the 3D7 genome, not to the amino acid number in this recombinant construct. Tags are BAP.Strep (Section 3.1.5). PRR = proline-rich region.

degrees of reactivity to the N-rich region of AARP. Although most peptides varied in their reactivity to IgG, no peptide was consistently recognised only by the antibody samples that were functional in the GIA assay. Peptide 1 was the only peptide that was negative in all samples, and peptides 9, 10 and 12 were the only peptides that were immunogenic in all IgG samples. The fact that IgG in Batch 3(a) recognised peptide corresponding to the tPA signal peptide may indicate that the cleavage rate of tPA is less than 100% efficient for proteins expressed in the HEK293 system. Overall, these anti-peptide ELISAs demonstrated considerable variation in the fine specificity of the mouse IgG response to recombinant AARP.BAP.Strep.

5.3 Cross reactivity of functional antibodies with RBCs

The level of background staining of PFA-fixed RBCs seen with Batch 3(a) and Batch 3(b) in Fig. 5.6 led me to wonder whether any of the functional antibodies raised against recombinant AARP.BAP.Strep were reactive to unfixed RBCs. As the assay of GIA consumes large amounts of sample which was was by this point highly limited, I first performed a pilot experiment. I pooled IgG from Batch 3(a), Batch 3(b) and Batch 5, adjusted its concentration to 0.25 $\mathrm{mg\,mL^{-1}}$, and then mixed half of it with an equal volume of RBCs to deplete out the RBC-reactive antibodies (see Section 2.6.1). After removal of the RBCs, I compared the GIA of the RBC-depleted sample with that of the non-depleted sample against 3D7. Surprisingly, the GIA was totally abrogated by pre-incubation with RBCs, whereas the GIA of an anti-AMA1 sample was unaffected by the same treatment (Fig. 5.10A). I then sought to repeat this result against the FVO strain, using IgG from the individual batches at 1 $\mathrm{mg\,mL^{-1}}$ (Fig. 5.10B). This experiment yielded two surprises. First, Batch 4(a) and Batch 4(b), samples that showed limited or no GIA against the 3D7 clone at the same IgG concentration (Fig. 5.3), demonstrated moderate GIA against the FVO strain. Second, although RBC depletion decreased the GIA of all samples, it did not entirely eliminate the anti-parasitic activity. This may be a result of the higher IgG concentration compared to the pilot experiment, or the fact that this assay was performed against FVO instead of 3D7. Thus, pre-incubation

of anti-AARP antibody with RBC seems to reliably reduce its GIA, but this may be strain- and concentration-dependent, and requires additional assays to be confirmed.

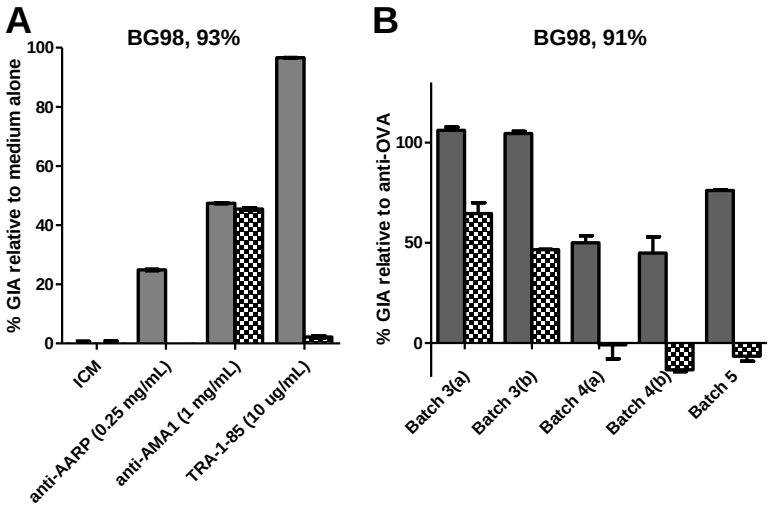

FIGURE 5.10: Depletion of anti-parasitic activity by pre-incubation with RBCs. (A) In a pilot experiment against the 3D7 clone, a range of antibody specificities was assayed for GIA activity with (hatched) and without (grey) 30 minutes pre-incubation and removal of an equivalent volume of 100% haematocrit RBCs (the same RBC donor was used for IgG pre-incubation and the GIA assay itself). Anti-AARP IgG was pooled from Batch 3(a), Batch 3(b) and Batch 5 (see Fig. 5.3C), and was assayed at 0.25 mg mL^{-1} total IgG to conserve sample. (B) The five IgG samples assayed in Fig. 5.3C were tested against the FVO strain at 1 mg mL^{-1} with and without RBC depletion as in (A). Results in (B) are reported as relative to anti-OVA antibodies concurrently purified with the test samples because of large variability between the ICM-only wells. (A) shows representative results of two assays with depletions using RBCs from two separate donors, (B) shows results of a single assay as sample was limiting. Measured GIA for the BG98 standard at 6 mg mL^{-1} is shown above each graph. Bars, mean; error bars, range of three replicate wells on the assay plate.

5.4 Discussion

Initial GIA results with the first batch of antibodies raised to AARP were exciting, as they outperformed anti-RH5 mouse antibodies and showed synergy with anti-RH5 rabbit antibodies against both the 3D7 and FVO strains (Fig. 5.2, although the differences in antibody function may be partially attributable to the different potencies of the protein-in-adjuvant regime used for AARP and the virus vector regime used for RH5). Unfortunately, it proved difficult to generate additional anti-AARP antibodies that showed comparable functionality. From a vaccinological standpoint this was disappointing, as it called into question the promise of the initial results. Of more immediate concern, it

also made progress technically challenging as sample volume came to limit the scope of the experiments that could be performed. Eventually, three more samples of growth-inhibitory anti-AARP antibodies were produced, and these were compared with two samples of anti-AARP antibodies from the same mouse experiment that did not appear to be inhibitory to growth (Fig. 5.3C). Given that each sample contained comparable titres of antibodies against both the immunising protein (Fig. 5.4Ai) and a construct corresponding to the N-terminal half ('N-half', Fig. 5.4Aii), I looked in more detail at the reactivity profiles of these samples. The results of this characterisation are summarised in Table 5.1.

5.4.1 Discordant reactivity to parasite lysate, fixed schizonts and synthetic peptides

When analysed by Western blot, the degree of IgG reactivity to schizont extract seemed to associate negatively with functionality. Whereas the inhibitory samples barely reacted to schizont extract, the non-inhibitory specificies showed a smeared pattern, indicating cross-reactivity to a range of parasite proteins (Fig. 5.5). My initial hypothesis was that the cross-reactive antibodies were likely to be directed against linear epitopes consisting of poly-asparagine. This was found not to be the case when the antibody samples were assayed for reactivity to an array of linear peptides corresponding to the sequence of the full-length recombinant immunogen (Fig. 5.9). Batch 3(a), a sample that barely reacted with schizont extract by Western blot, showed a high degree of reactivity to almost the full length of AARP.BAP.Strep, including peptides corresponding to the asparagine-rich region. The profiles of peptide recognition were also not reflected in IFA assays against PFA-fixed schizonts. Batch 3(a) and 3(b) showed completely different profiles of peptide recognition (Fig. 5.9, A and B), but stained schizonts with a similar pattern and staining intensity as assessed by IFA (Fig. 5.6). Batch 3(b) and Batch 4(b), which had showed a very similar peptide-reactivity profile (Fig. 5.9, B and D), showed completely different responses by IFA (Fig. 5.6, B and D).

TABLE 5.1: Summary of reactivity and functionality of 5 mouse IgG samples raised against AARP.BAP.Strep.

	Batch 3(a)	Batch 3(b)	Batch 4(a)	Batch 4(b)	Batch 5
GIA against 3D7[a] (Fig. 5.3C)	Med	High	Nil	Nil	High
GIA against FVO[a] (Fig. 5.10B)	High	High	Med	Med	Med-High
Reactivity to schizont extract (Fig. 5.5)	Low	Low	Many bands	Many bands	Low
Reactivity to PFA-fixed schizonts (Fig. 5.6)	Intense	Intense	Moderate	Weak	Nil
Peptide-binding profile (Fig. 5.9)	Whole immunogen	Narrow, residues 40–60	Broad, residues 70–170	Narrow, residues 30–60	Narrow, residues 30–60

[a]Whole IgG at 1 mg mL^{-1}

It is difficult to produce an explanation for these discordant results (summarised in Table 5.1). They presumably arise from subtle differences in the antigen treatment processes. For example, the epitopes in PFA-fixed schizont (as analysed by IFA, Fig. 5.6) will be chemically cross-linked and thus differ from those present after SDS treatment of schizont lysate (as analysed by Western blot, Fig. 5.5). The only explanation I can offer as to why two specificities with very similar peptide-reactivity profiles (Batch 3b and 4b) should react so differently to parasite-derived antigen is that AARP may be very extensively post-translationally modified in the parasite, so that the linear peptides bear virtually no resemblence to it. This seems unlikely, as Wickramarachchi *et al.* have reported that parasite-derived protein migrates as a single band [358], and *P. falciparum* is thought to glycosylate proteins only rarely [253, 254, 383]. Furthermore, although bioinformatic analysis (Fig. 5.7) and NMR spectroscopy indicated that AARP is likely to be predominantly randomly coiled (Fig. 5.8), it may be that full-length parasite protein has a propensity to adopt different structures to short 20mer peptides. Even with all these caveats, several published studies have successfully mapped polyclonal serum antibodies using similar protocols: protective responses to SERA5 have been

mapped to unstructured epitopes using synthetic peptides [141]; the serum antibody response to APN1, a potential transmission blocking vaccine antigen, was mapped to two immunodominant linear peptides [419]; and unglycosylated linear peptides were used to map the antibodies to HIV env [420], a protein which not only has a defined and reasonably stable three-dimensional conformation, but is also very highly glycosylated in its native form.

5.4.2 Antibody function and cross-reactivity

Strikingly, pre-incubating anti-AARP IgG samples with RBCs depleted out their ability to inhibit growth (Fig. 5.10). The extent to which this occurs is currently unclear. Against 3D7, low (around 25%) GIA was reproducibly depleted to no GIA (Fig. 5.10A). This is not a general effect of RBC depletion because anti-AMA1 antibodies were unaffected by RBC depletion. *A priori* of any other knowledge of the antibody specificity, two possible explanations present themselves:

1. The mechanism of antibody-mediated neutralisation is by binding to a protein on the RBC surface that the parasite must access in order to survive. Pre-incubation of IgG with RBCs depletes the antibodies that are reactive to this RBC protein, thus reducing the GIA of the sample.

2. The mechanism of antibody-mediated neutralisation is by binding to a parasite-derived protein (possibly AARP, possibly an unrelated protein), and these antibodies are cross-reactive to a protein or proteins on the RBC surface. Pre-incubation of IgG with the RBCs depletes out many or all of the antibodies that are reactive to the susceptible parasite protein, thus reducing the GIA of the sample.

In the RBC depletion experiment using the FVO strain, the baseline GIA of the undepleted samples was higher than against 3D7, and there was considerable residual GIA after pre-incubation with RBCs (Fig. 5.10B). This seems to argue against the first possibility. Given that an eight-fold reduction in antibody concentration is sufficient to almost totally eliminate anti-parasitic activity in an anti-FVO assay at 1%

haematocrit (Fig. 5.2C and Section 2.7.4), pre-incubation of IgG at 50% haematocrit (see Section 2.6.1) should be sufficient to totally remove all inhibitory activity if the neutralising epitope is found on the RBC surface. The fact that there was residual GIA therefore suggests that the neutralising specificity is directed to a protein found on the parasite, and that a percentage (but not all) of this neutralising specificity is cross-reactive to the RBC and can be depleted out. However, one should be wary of over-interpreting the results of this unreplicated single assay.

5.4.3 Varying susceptibilities of strains

Another surprise from the experiment shown in Fig. 5.10B was that antibody samples tested in Fig. 5.3C that were negative for GIA against 3D7 appeared to be inhibitory against FVO at the same IgG concentration. As already emphasised, this result is from one single assay replicate, but still calls for discussion. It is not clear if the difference between strains is due to varying epitope susceptibility (i.e. Batch 4(a) and Batch 4(b) do contain antibodies that can neutralise 3D7, but these are present at insufficient titre to exert an inhibitory effect; FVO is neutralised at the same epitopes as 3D7, but is more susceptible to these antibodies and hence can be neutralised at IgG concentrations that show no effect against 3D7), or an issue of antibody specificity (i.e. FVO and 3D7 are neutralised by a completely different set of antibody clones that recognise a different set of epitopes). This merits re-examination, because if the mechanism of GIA is identical against FVO and 3D7, the increased susceptibility of FVO would facilitate experiments to define the key neutralising epitopes—allowing the design of an immunogen that more consistently elicits antibodies inhibitory to the growth of 3D7. The sequence of the FVO allele of AARP has not been reported, but given that large-scale screening of field isolates found only a single non-synonymous SNP of >10% prevalence along the whole length of AARP (Table 4.1), it is unlikely that the 3D7 and FVO isoforms differ substantially. Taken more broadly, the results in Fig. 5.10 caution against simplistic classification of antibody samples as 'growth inhibitory' or 'non-inhibitory', as these properties are a function of specificity, titre and the particular parasite strain used.

5.5 Conclusions and Future work

The polyclonal mouse antibodies raised against three separate batches of AARP.BAP.Strep (Figs. 5.2 and 5.3) represent the most potent total IgG samples yet studied in our laboratory. The most immediate lines of enquiry involve defining the neutralising target of the antibodies. First, it would be worthwhile to replicate the result seen in Fig. 5.10B, where anti-AARP.BAP.Strep samples that had previously shown no inhibitory activity against 3D7 showed moderate neutralising capacity against FVO. If confirmed, this would mean that FVO could be used to define the target or targets of GIA using the abundant anti-AARP.BAP.Strep antibody samples that I have produced that do not appear to neutralise 3D7. Second, it is vital to determine whether parasite-derived AARP is indeed the neutralising target of antibody raised against recombinant AARP.BAP.Strep. A parasite line where AARP has been genetically disrupted is currently under development by Ellen Knuepfer at the National Institute of Medical Research. If this can be produced it will be interesting to determine whether antibody samples that are growth inhibitory against 3D7 also have GIA against the modified parasite line. It would also be useful to secure access to some of the rabbit serum generated during published studies of anti-AARP antibodies [135, 358, 401].

If it transpires that the neutralising target of these antibodies is parasite-derived AARP, there will be a strong vaccinological rationale for defining the host receptor responsible for the previously reported RBC-binding activity of AARP [358]. This could be performed in partnership with the laboratory of Gavin Wright, who have produced an extensive library of RBC proteins and developed an ELISA methodology for the detection of weak interactions between extracellular proteins [276, 421]. This has already been used to define the interaction between RH5 and basigin [128] and between MTRAP and semaphorin-7A [422]. An alternative possibility is the use of a virtually complete library of human proteins expressed on the surface of mammalian cells in microarray format, a platform owned by the UK company Retrogenix^{TM}. This technology enabled the discovery of the interaction between some EMP1 variants and EPCR [99], although it is not clear if the method is sufficiently sensitive to detect the significantly weaker interactions that occur between merozoite adhesins and their RBC receptors. When

expressed recombinantly, EMP1 binds to EPCR with a K_d below 30 nM, whereas full-length recombinant EBA-175 binds to GYPA with a K_d of approximately 260 nM, full-length recombinant RH5 binds to basigin with a K_d of 1 µM, and a recombinant fragment of RH4 interacts with the soluble domain of complement receptor 1 with a K_d of approximately 2.9 µM. Structural characterisation of an interaction between AARP and a putative RBC receptor would be particularly interesting in light of the bioinformatic data and NMR spectroscopy showing that AARP lacks stable secondary and tertiary structure (Figs. 5.7 and 5.8).

In parallel, it is important to not lose site of the first goal of this thesis, which was to define as many potential candidate antigens as possible. It is now a priority to validate the preliminary results against S-antigen, the other 'hit' from the screen reported in Chapter 4, and to explore other methodologies to screen the proteins that I was not able to recombinantly express and purify (see the next Chapter).

Chapter 6

High-titre, functional antibodies through a novel genetic immunisation platform

6.1 Introduction

Compared to previous work, the results described in Chapter 4 represent a considerable improvement in the rate of antibody elicitation against a range of *P. falciparum* proteins. However, even though production of a small amount of protein represents a considerable reduction in costs and labour compared to production of two recombinant virus vectors, performing expression and purification trials in suspension HEK293 cells for 52 proteins still took a considerable investment in media costs and labour, with the entire project taking over six months. I therefore sought a novel protocol to allow the rapid elicitation of high-titre humoral responses against novel antigens, aiming for a higher success rate.

6.1.1 DNA immunisation

DNA immunisation is an attractive proposition because plasmid DNA is straightforward to prepare, and in any case is a prerequisite for the production of both recombinant virus vectors and recombinant protein. The late 1980s and early 1990s saw rising interest in the use of DNA plasmids as vectors for gene therapy [423, 424]. The realisation that DNA plasmids could be taken up by mammalian cells *in vivo* and give rise to *in situ* protein expression quickly led to the use of plasmid DNA for the induction of antibody responses [10] and functional CTLs [11] by immunisation with DNA plasmids. Unfortunately, early clinical trials in humans [425, 426] showed only erratic elicitation of weak immune responses. The reasons for the failure to translate the promising pre-clinical results into human studies are unclear. There may be immmunological factors that prevent the translation of strong immune responses in small laboratory mammals into humans; it may be a matter of dosing, as pre-clinical experimenters often administer DNA in a very high dose:body weight ratio (typically in mice the dosing is 4 µg DNA per g of body mass, in human trials the dose is typically 1.5 ng DNA per g of body mass); or simply that the immune responses after DNA vaccination in pre-clinical studies are over-stated. Certainly it is rare to find studies comparing the antibody titre elicited after DNA vaccination with a well-validated approach for induction of high-titre antibodies (such as pure recombinant protein formulated with Freund's adjuvant), and attempts in our laboratory to replicate the high levels of antibody reported in other studies have failed [427]. For this reason, as a translational approach to eliciting an immune response, DNA vaccination has taken a position of reduced prominence as virus vector strategies have risen in profile [428, 429]. Despite these setbacks for DNA vaccination in humans, the potential advantages in speed and cost of manufacture led me to revisit this approach to immunising small mammals as part of an antigen-screening strategy.

6.1.2 Improving the immunogenicity of DNA vaccines

There are many complex immunological factors that influence the magnitude of a response to vaccination [430]. However with all other factors kept equal, one fairly clear

determinant of the response to genetic immunisation is the amount of protein expressed from the expression cassette *in situ* [431]. Many of the design elements of a plasmid to be used for DNA vaccination are the same as in any plasmid designed to drive protein expression from mammalian cells. These include: a strong promoter to drive transcription of the antigen-encoding gene [432]; inclusion of polyadenylation signal sequences [432]; strong signal peptide to derive expression into the secretory pathway [433]; and inclusion of a Kozak sequence [434]. The principles explored in Chapter 3 could therefore be used as a foundation for exploratory experiments with DNA vaccines. However, the essential lesson learned from the *in vitro* transfections described in Chapter 3 was that even the best-designed plasmid must be effectively delivered into cells before high yields of recombinant protein can be attained.

It is widely accepted that i.m. injection of naked DNA is an inefficient method of transporting DNA into cells [435]. To overcome this limitation, various groups have devised mechanistically distinct non-viral technologies for delivering genes into the cells of living organisms. The gene gun, or biolistic particle delivery system, was reported over 25 years ago [436] as a method to deliver DNA into plant cells. Delivery of genes into the cells of living mammals soon followed [437] and rapidly led to the first descriptions of genetic immunisation [10, 438]. *In vivo* electroporation to enhance uptake of exogenous DNA into the cells of live mice was first described in the late 1990s [439–441] and was quickly adopted by the DNA vaccine field [442–445]. Unfortunately both gene gun and electroporation have significant drawbacks: both require access to specialist equipment; gene guns require encapsulation of DNA into metal-based particles which are costly and time-consuming to prepare; and electroporation is extremely painful for the host organism (mouse, human or otherwise). Alternative DNA vaccine delivery methods include chemical carriers such as cationic lipids [446–448] and a range of polymers [449, 450], but these formulations are often expensive. Given that this work was concerned with establishing a protocol for immunising small mammals, with no view to translation, I hoped to use a non-proprietary carrier molecule such as PEI, that can be purchased off-the-shelf from a chemical company.

6.1.3 PEI for delivering genes to the cells of living mammals

The molecular structure of PEI is shown in Fig. 6.1. At neutral pH the nitrogen atom is protonable, meaning that linear PEI molecules can be considered as long chains of positive charge. This allows them to interact with DNA molecules, which they efficiently condense into microparticles called polyplexes [451]. Transfection-competent DNA/PEI polyplexes should have a net positive electrostatic charge, which allows them to interact with the negatively charged surface of most mammalian cells [452]. From the point of interaction with the cell surface, the precise mechanisms by which PEI aids nuclear delivery are not clear, and probably vary in importance depending upon the exact structure of the PEI and the cell type being transfected [453]. An early suggestion was that PEI acts as a proton sponge in the endosome, preventing acidification and leading to endosomal rupture [277], although other reports claim that DNA/PEI polyplexes do not pass via the endosome and that the main benefit of PEI is as a physical barrier to nucleases [454]. The interaction between DNA and PEI is strong enough that PEI is thought to remain associated with DNA even as PEI enters the nucleus [297], which can occur even in the absence of cell division [299].

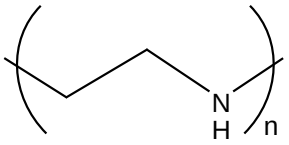

FIGURE 6.1: The molecular structure of a polyethylenimine monomer.

Although viewed primarily as an in *in vitro* transfection reagent (see Chapter 2), the utility of PEI for *in vivo* transfection is well described [277, 455]. However, as a carrier for DNA vaccine plasmids it is surprisingly poorly characterised, with only a few reports to date describing antibody elicitation [287, 456–458]. Kasturi *et al.* report the conjugation of PEI moieties onto poly(lactide-coglycolide) particles to facilitate loading of nucleotides [456]. The other three reports showed a marked improvement in immunogenicity when DNA/PEI polyplexes were delivered either i.n. [457, 458] or i.v. [287], although neither compared the humoral responses elicited with a 'gold-standard' regime, such as soluble recombinant protein-in-adjuvant. For the work in this chapter I focused upon i.v.

delivery of DNA/PEI polyplexes, as this is the more straightforward injection procedure. In particular, I was interested in performing comparative immunogenicity studies to assess whether DNA/PEI polyplexes could stand as an alternative to virus vector or protein-in-adjuvant platforms for screening novel antigens and constructs.

6.1.4 Nomenclature of immunisation regimes

This chapter describes results from a range of immunisation regimes that vary across multiple parameters: the nature of the inocula (virus vector, DNA/PEI); the route of injection; the interval between injections; and the number of injections. The regimes are represented in shorthand notation as follows:

Ad denotes i.m. injection of 10^{10} viral particles of a recombinant adenovirus (AdHu5 or ChAd63)

M denotes i.m. injection of 10^{7} plaque-forming units of a recombinant MVA

D denotes i.v. injection of 20 µg of DNA mixed with PEI (see Figure legends and Sections 2.3.3 and 6.2.1 for details of formulation)

The duration of the interval is given in numbers of weeks. For example, 'Ad-8-M' denotes the following regime:

- Day 0, an i.m. injection of 10^{10} viral particles of a recombinant adenovirus
- An interval of 8 weeks
- Day 56, an i.m. injection of 10^{7} plaque-forming units of a recombinant MVA

Likewise, 'D-4-D-4-D' denotes the following regime:

- Day 0, an i.v. injection of 20 µg of DNA mixed with PEI
- An interval of 4 weeks
- Day 28, an i.v. injection of 20 µg of DNA mixed with PEI
- An interval of 4 weeks
- Day 56, an i.v. injection of 20 µg of DNA mixed with PEI

This notation is used throughout the Chapter.

6.2 Materials and Methods

6.2.1 Vaccines and immunisations

6 week old female BALB/c mice were used for all experiments. The design and construction of the PfAMA1 and PfRH5 recombinant virus vectors has been described elsewhere [133, 139]. Inocula for vaccination were prepared and injected as described in Sections 2.2 and 2.5.1.

For immunisation with PEI, DNA was purified using a variety of kits (Plasmid *Plus* Midi Kit, EndoFree Plasmid Maxi Kit, EndoFree Plasmid Giga Kit, QIAGEN, UK), then phenol-chloroform extracted to remove residual salts and contaminants [233]. The DNA plasmids used for immunisation (pENTR4 tPA.PfRH5 and pENTR4 tPA.PfAMA1) were the same entry vector plasmids that were used to manufacture the adenovirus vectors used as comparators in the experiments. Thus, the recombinant adenovirus vectors and the DNA plasmids contained identical antigen expression cassettes.

Formulation with *in vivo*-JetPEI (Polyplus tranfection^{TM}, France) was performed according to the manufacturers instructions to achieve an N/P ratio of 8, except that the volume administered i.v. was halved to 100 µL (thus reducing the dose of DNA/PEI by half), as a dose of 200 µL was found to cause discomfort in the mice (Section 6.3.1). Briefly, for each dose, 50 µL of 5% glucose solution containing 20 µg DNA plasmid was mixed with 50 µL of 5% glucose solution containing 3.2 µL of *in vivo*-JetPEI. The solution was left at room temperature for 15–120 minutes to allow polyplexes to form before injection into the lateral tail veins of mice.

For L25, M40 and M160 (see Section 2.3.3), stock solutions were prepared as described in 2.3.3. In accordance with the protocol for *in vivo*-JetPEI each dose contained 20 ug of DNA, but the DNA was mixed with PEI in an N/P ratio of 15.5 in accordance with earlier work characterising the ability of PEI polyplexes to transfect suspension HEK293 cells (Sections 2.3.3, 3.2.4 and Fig. 3.5). Briefly, for each dose, 50 µL of 5% glucose solution containing 20 µg DNA plasmid was mixed with 50 µL of 5% glucose solution containing 40 µg of the relevant PEI. The solution was left at room temperature

for 15–120 minutes to allow polyplexes to form before i.v. injection into the lateral tail veins of mice.

6.2.2 Assessment of cellular immune responses in the blood by *ex vivo* IFN-ɣ ELISPOT

The protocol for the *ex vivo* IFN-ɣ blood ELISPOT is described in detail in Jenner Protocol J066. Briefly, blood cells from immunised mice were collected and red cells lysed with ACK buffer (150mM NH_4Cl, 1mM $KHCO_3$, 100mM EDTA). Surviving leukocytes were mixed with splenocytes from a naïve mouse to enhance antigen presentation, plated into a MAIP ELISPOT Plate (Millipore, UK, catalogue number MAIPS4510) coated with anti-mouse IFN-ɣ mAb AN18 (Mabtech, Sweden, catalogue number 3321-3-1000), and stimulated overnight with peptide. The following day the leukocytes were removed, and patches of mAb-captured IFN-ɣ were detected by biotinylated anti-mouse-IFN-ɣ mAbR4-6A2 (Mabtech, Sweden, 3321-6-1000) followed by streptavidin conjugated to alkaline phosphatase (Mabtech, Sweden, catalogue number 3310-10). Streptavidin conjugate was then detected with an alkaline-phosphatase conjugate substrate kit (Bio-Rad, USA, catalogue number 170-6432) and spots counted with an ELISPOT plate reader (AID, Germany).

For detection of cellular immune responses to PfRH5, a pool of 20mer peptides overlapping by 8 amino acids and representing the full length of the protein was used to stimulate PBMCs, with each individual peptide at 5 $\mu g\,mL^{-1}$. For detection of cellular responses to PfAMA1, PBMCs were stimulated with either the MHC class I-restricted peptide A42 or a pool of the MHC class II-restricted peptides A95 and A31, with all individual peptides at 5 $\mu g\,mL^{-1}$ [459].

6.2.3 Assessment of humoral immune responses by ELISA

ELISAs were performed as described in Section 6.2.2. Briefly, for detection of PfAMA1 I cloned the *PfAMA1* gene from the genetic vaccines into the standard pENTR4 LP

vector (Fig. 3.6 and Section 3.2.5) such that it was tagged at the C-terminus with BAP and 6x His. The plasmid was transfected into suspension HEK293 cells, and recombinant protein purified with HisTrap Excel resin as described in Section 2.3.4. For detection of PfRH5, I used a recombinant PfRH5 protein expressed from the standard pENTR4 LP vector with a C-terminal BAP tag. This plasmid was transfected into suspension HEK293 cells along with a plasmid encoding the *E. coli* BirA biotin ligase. This resulted in the production of biotinylated PfRH5. Supernatants were extensively dialysed with 10 kDa cut-off membranes to remove free biotin before application to streptavidin plates (Thermo Scientific, UK), resulting in the specific coating of plates with PfRH5 protein. A summary of the method for protein expression is described in Section 3.5.

6.3 Results

6.3.1 DNA delivered with PEI can elicit high titres of antibodies to PfRH5

A pilot experiment was designed with two objectives. First, to determine the appropriate dosing of DNA/PEI polyplexes, and second, to test whether mixing DNA with PEI yielded any enhancement in immunogenicity over immunisation with naked DNA. For these pilot experiments, I selected a proprietary PEI formulation, *in vivo*-JetPEI. I decided to inject complexes i.v. as work by Grant *et al.* [287] demonstrated superior immunogenicity when DNA/PEI polyplexes were administered by this route (although Grant *et al.* injected retro-orbitally, whereas I injected into the lateral tail-vein). Mice were immunised with a range of DNA doses mixed with *in vivo*-JetPEI at an N/P ratio of 8, starting at a maximum 40 µg of DNA, the dose recommended by the manufacturer [460]. Day 7 *ex vivo* IFN-ɣ blood ELISPOT (see Section 6.2.2) showed a steep dose-response curve, with immunogenicity peaking at 20 µg (Fig. 6.2A). At a 40 µg DNA dose, mice exhibited signs of discomfort within one hour of injection and these mice were culled. Mice immunised i.v. with equivalent doses of naked DNA failed to exhibit any responses detectable by ELISPOT on day 7 (data not shown), and previous work

in our lab has conclusively demonstrated the poor immunogenicity of naked DNA when injected intramuscularly [427]. i.v. administration of 20 µg of plasmid DNA mixed with *in vivo*-JetPEI at an N/P ratio of 8 therefore became the standard dose of DNA/PEI used for all other work described in this Chapter. The pair of mice that had received 20 µg of DNA was subsequently re-immunised on the lines of a D-4-D-8-D regime. Two weeks after the final injection mice were found to have anti-PfRH5 antibody titres comparable to those seen in mice immunised with an Ad-8-M prime-boost regime (Fig. 6.2B, for details of virus vector immunisation see Section 2.5.1, Chapter 2).

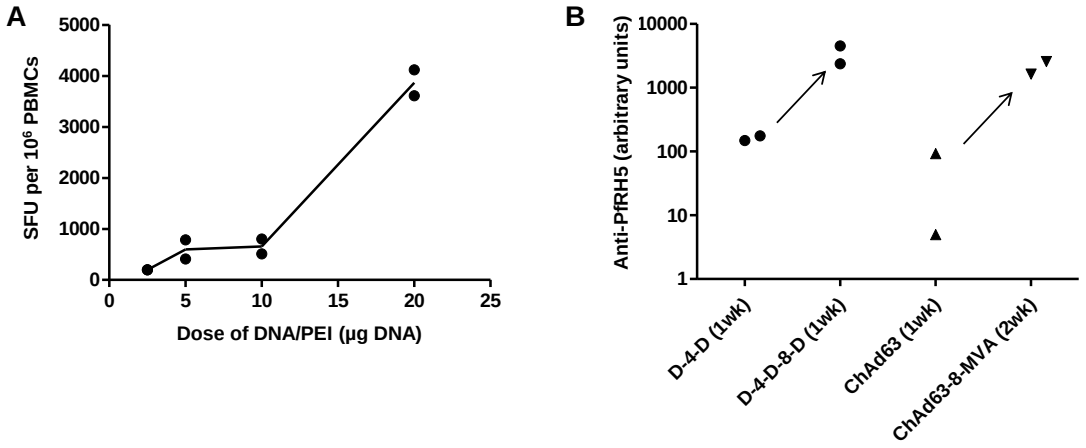

FIGURE 6.2: Pilot to assess immunogenicity of pENTR4 tPA.PfRH5 plasmid DNA and *in vivo*-JetPEI polyplexes. (A) 7 days after immunisation with the stated mass of DNA/PEI polyplexes, immune responses in the blood were assessed by IFN-ɣ ELISPOT (Section 6.2, chapter-specific methods). (B) The two mice from panel (A) that received 20 µg of DNA were immunised for a second time four weeks after the first immunisation, and then for a third time eight weeks after the second immunisation. After the second and third immunisations, serum was collected and the anti-PfRH5 antibody titre was measured by ELISA. For comparison, antibody responses after Ad-8-M vectors encoding tPA.PfRH5 are shown. The interval between the most recent immunisation and the time of serum collection is shown in parentheses. Each data point represents a single mouse. ELISA limit of detection = 1 arbitrary unit

The results presented in Fig. 6.2 encouraged me to compare DNA/PEI with virus vectors in an experiment with larger group sizes. The regimes used were Ad-8-M and D-8-D, and the DNA/PEI polyplexes were prepared in the same manner as in the pilot experiment. Three weeks after the priming immunisations, T cell responses were measured by *ex vivo* IFN-ɣ ELISPOT following stimulation of PBMCs with a peptide pool spanning the entire length of PfRH5 (Fig. 6.3A, and Section 6.2.2). Moderate responses were seen in mice receiving DNA/PEI (median, 1896; range: 1690–2296 SFU per 10^6 PBMCs;

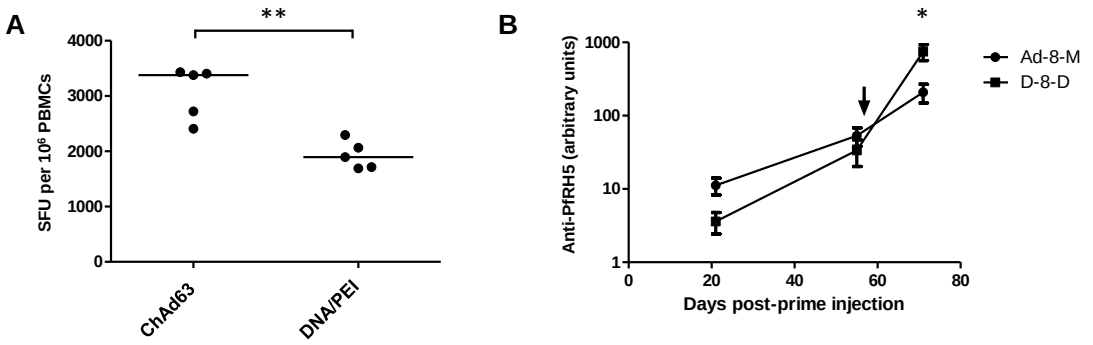

FIGURE 6.3: DNA/PEI administered in a D-8-D regime outperforms an Ad-8-M regime for antibody induction. (A) 6 week old BALB/c mice were immunised with either a standard dose of pENTR4 tPA.PfRH5 plasmid DNA mixed with *in vivo*-JetPEI, or 10^{10} viral particles of ChAd63 tPA.PfRH5, and cellular immune responses in the blood were assessed by IFN-ɣ ELISPOT 21 days later (Section 6.2, chapter-specific methods). Data points, results from a single mouse; lines, median. (B) Time course of antibody induction by D-8-D compared to Ad-8-M as measured by ELISA. The time of the second immunisation for both groups is indicated by an arrow. Data points, mean; error bars, standard error of the mean. ELISA limit of detection = 1 arbirary unit. $*p < 0.05$, $**p < 0.01$, by Mann-Whitney U test.

n=5), although these responses were not as high as those seen in mice vaccinated with adenovirus (median, 3378; range: 2407–3343 SFU per 10^6 PBMCs; n=5; Mann-Whitney $p < 0.01$). Likewise, day 21 antibody responses were higher in mice vaccinated with adenovirus (median, 15; range: 0–15 AU; n=5) than with DNA/PEI (median, 3; range: 0–7 AU; n=5), although in both groups the measured titres were well below the threshold for functionality in the *in vitro* assay of GIA. However, two weeks after the boosting immunisation, at day 70, this hierarchy was reversed. The D-8-D regime elicited antibody responses (median, 680; range: 246–1391 AU; n=5) significantly higher than those elicited by Ad-8-M (median, 167; range: 107–460; n=5; Mann-Whitney $p < 0.05$). To my knowledge this is the first description of a DNA-based immunisation regime that outperforms Ad-8-M [157] in a comparative immunogenicity study where both vaccines encode an identical immunogen.

6.3.2 Throughput can be improved with standard DNA purification protocols

The central design objective of this platform was to minimise the labour involved in vaccine preparation. Polyplus transfection^{TM} recommends using DNA prepared using protocols designed to yield material that contains minimal endotoxin contamination [460], such as with the QIAGEN EndoFree Plasmid Maxi Kit. Unfortunately this protocol is time-consuming (approximately 4 hours) and throughput is limited (a maximum of 6 preps can be run in parallel). Furthermore, each prep costs approximately £24. In my hands, the QIAGEN Plasmid *Plus* Midi Kit achieves similar yields, takes only 1 hour, and up to 20 preps can be run in parallel at a cost of approximately £7 per kit. However, before adopting a new DNA purification protocol it was necessary to verify that DNA purified with the Midi Kit performed comparably to DNA purified with the EndoFree Plasmid Maxi Kit. 500 ml of turbid liquid culture of *E. coli* carrying the pENTR4 tPA.PfRH5 plasmid was split in two, and DNA was purified using the two kits according to the manufacturer's instructions. With both kits, DNA was subsequently purified by phenol-chloroform extraction. DNA was then formulated with *in vivo*-JetPEI and immunised into mice at the standard dose. Day 7 *ex vivo* IFN-ɣ ELISPOT analysis of blood cells showed no differences in response (Fig. 6.4A). Both groups were re-immunised following a D-8-D regime. Two weeks after the final injection, anti-PfRH5 antibody responses were comparable (Fig. 6.4B). Based on these results, all future batches of DNA were purified using the QIAGEN Plasmid *Plus* Midi Kit followed by phenol-chloroform extraction.

6.3.3 Optimisation of the DNA/PEI dosing regime: PfAMA1

Vaccination with recombinant virus vectors results in immune responses directed against both the antigen of interest and the virus vector itself [461]. The antivector antibodies and cytotoxic $CD8^+$ T cells elicited as part of the priming immunisation limit viral transduction upon re-administration of homologous virus, resulting in reduced *in situ* transgene expression and a dampened secondary immune response [382]. Maximal

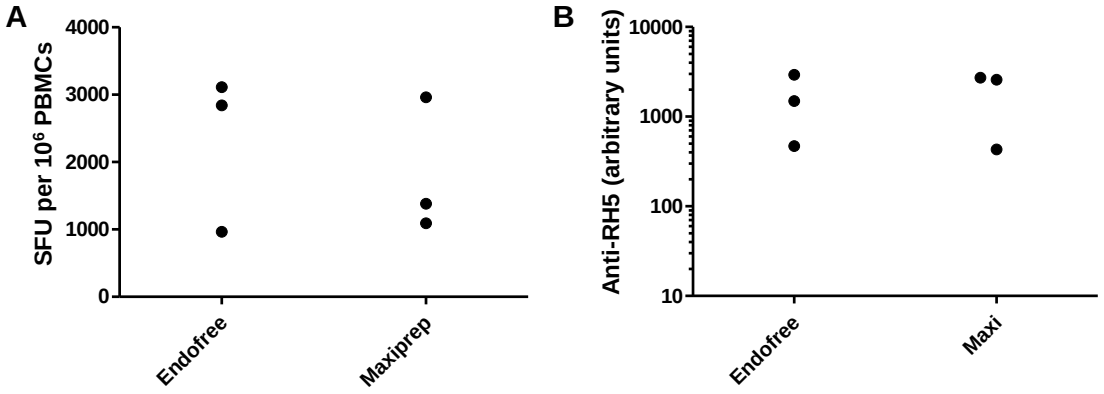

FIGURE 6.4: DNA purified with a QIAGEN Plasmid *Plus* Maxi Kit performs comparably with DNA purified with a QIAGEN EndoFree Plasmid Maxi Kit. pENTR4 tPA.PfRH5 plasmid DNA was purified according to the manufacturer's protocol, phenol-chloroform extracted, then administered i.v. to 6 week old BALB/c mice with *in vivo*-JetPEI at the standard dose. (A) *Ex vivo* IFN-ɣ ELISPOT was used to assess cellular responses in the blood 7 days post-immunisation. (B) The mice in panel A were re-immunised after an interval of 8 weeks, a further 2 weeks later serum was harvested and analysed by ELISA. ELISA limit of detection = 1 arbirary unit. Each data point represents a single mouse.

antibody induction can be achieved via heterologous immunisation regimes where one virus vector is used for priming, and boosting is accomplished with a heterologous virus vector [157, 382]. This means that for each successive immunisation a new virus vector of a different species or serogorup must be produced, with corresponding increases in cost and labour time. DNA immunisation does not result in anti-vector immunity, meaning that multiple boosting immunisations can be performed with no extra vaccine production costs. It was hypothesised that immune responses could be enhanced with the use of regimes incorporating multiple boosting injections, and to test this mice were immunised with vaccines encoding PfAMA1 according to a range of different regimes (Fig. 6.5, see box), and compared to an Ad-8-M regime. The evolution of $CD4^+$ and $CD8^+$ T cell responses in the blood was followed by *ex vivo* IFN-ɣ ELISPOT, stimulating with either an MHC class I-restricted peptide or a pair of MHC class II-restricted peptides (see Section 6.2.2). On day 7, the $CD8^+$ T cell response induced by the adenovirus against PfAMA1 (median, 889; range: 67–1130 SFU per 10^6 PBMCs; n=6) easily surpassed the response to a single shot of DNA/PEI (median 40, range: 0–276 SFU per 10^6 PBMCs; n=12). By day 74 the Ad-8-M regime had elicited a response to the MHC class I-restricted peptide (median, 3820; range: 1680–6350 SFU per 10^6

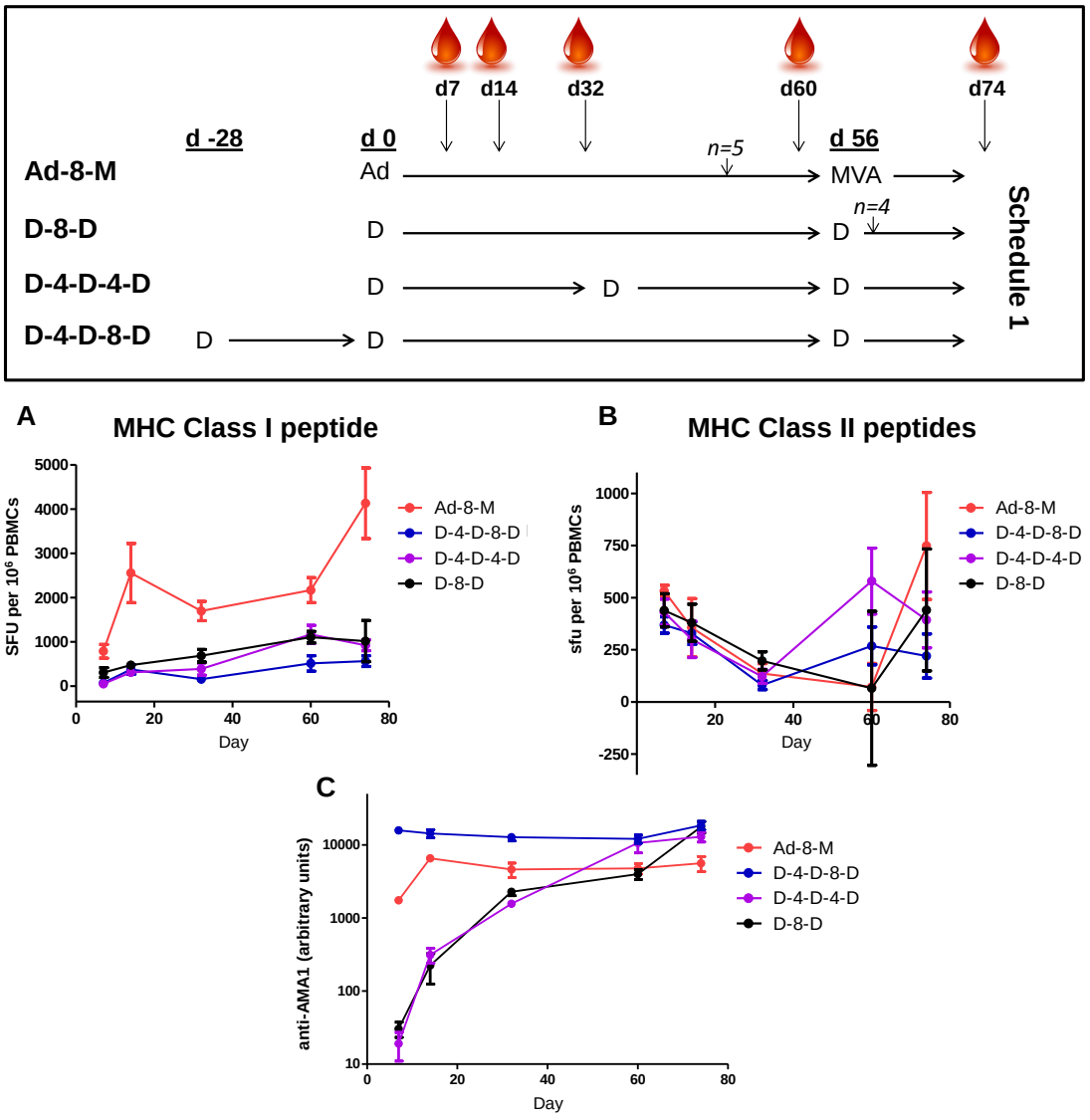

FIGURE 6.5: Optimisation of the DNA/PEI immunisation regime with a PfAMA1 vaccine. Mice were immunised either with virus vectors encoding tPA.AMA1 in an Ad-8-M regime, or with the standard dose of pENTR4 tPA.AMA1 plasmid DNA mixed with *in vivo*-JetPEI, in a variety of dosing regimes (immunisation schedule shown in box—serum collection times are denoted by red droplets of blood, and note that for group D-4-D-8-D it is the *second* immunisation that takes place on day zero). *Ex vivo* IFN-ɣ ELISPOTs were performed on blood samples using two separate batches of peptide, the first batch containing an MHC class I-restricted peptide (A), and the second batch containing a pool of two MHC class II-restricted peptides (B). (C) Anti-AMA1 antibody titre was measured by ELISA. n=6 in each group except when mice died due to unrelated causes, as shown in box. ELISA limit of detection = 10 arbitrary units. Data points, mean; error bars, standard error of the mean.

PBMCs; n=5) that vastly exceeded that elicited by the best DNA/PEI regime D-4-D-8-D (median, 857; range: 568–1360 SFU per 10^6 PBMCs; n=6; Mann-Whitney p <0.05). No clear hierarchy emerged in the responses to stimulation with the MHC class II-restricted peptides, and there were no significant differences between groups at any time points, even without correction for multiple comparisons. With regard to antibody titre, however, by day 74 all DNA/PEI regimes outperformed Ad-MVA, with D-8-D eliciting antibody titres (median, 16607; range: 11156–26455 AU; n=4) more than three times higher than the virus vector regime (median, 3899; range: 3458–10350 AU; n=5; Mann-Whitney p <0.05). I did not observe any significant differences between the different DNA/PEI regimes at day 74, although this result is worth investigating further with a panel of different antigens. Strikingly, I observed that, by day 7, mice in the D-4-D-8-D group had already evolved a maximal anti-PfAMA1 antibody response (median, 23478; range: 12733–35661 AU; n=6) which was equivalent to day 74 in the D-8-D group, raising the possibility of a five-week D-4-D immunisation regime (investigated in Section 6.3.4 and Fig. 6.6). Of note in this experiment, the adenovirus immunisation alone elicited high antibody titres that were not further boosted by the injection with MVA at day 54. This is in contrast to previous work in our lab using different batches of genetically identical virus vectors [139].

6.3.4 Optimisation of the DNA/PEI dosing regime: PfRH5

The experiment presented in Fig. 6.6 was designed to address a number of questions raised by the results described in section 6.3.3 and Fig. 6.5.

1. In the D-4-D-8-D immunisation schedule, antibody titre had peaked after the second immunisation, which came only 4 weeks after the priming immunisation. This raised the prospect of a D-4-D regime, and potentially even a D-2-D regime, which would have substantial advantages in time and costs associated with housing animals (Fig. 6.6B).
2. The D-8-D and the D-4-D-4-D regime performed very comparably (Fig. 6.5C). A regime with fewer injections has considerable advantages in terms of DNA preparation and minimising stress caused to animals by reducing the number of procedures. The relationship between the number of injections and final antibody titre was therefore investigated (Fig. 6.6C).

3. In the D-4-D-8-D immunisation schedule, antibody titre had peaked by day 7, suggesting that the widely adopted policy of waiting 14 days after an immunisation before harvesting serum equated to a wasted week (Fig. 6.6D).

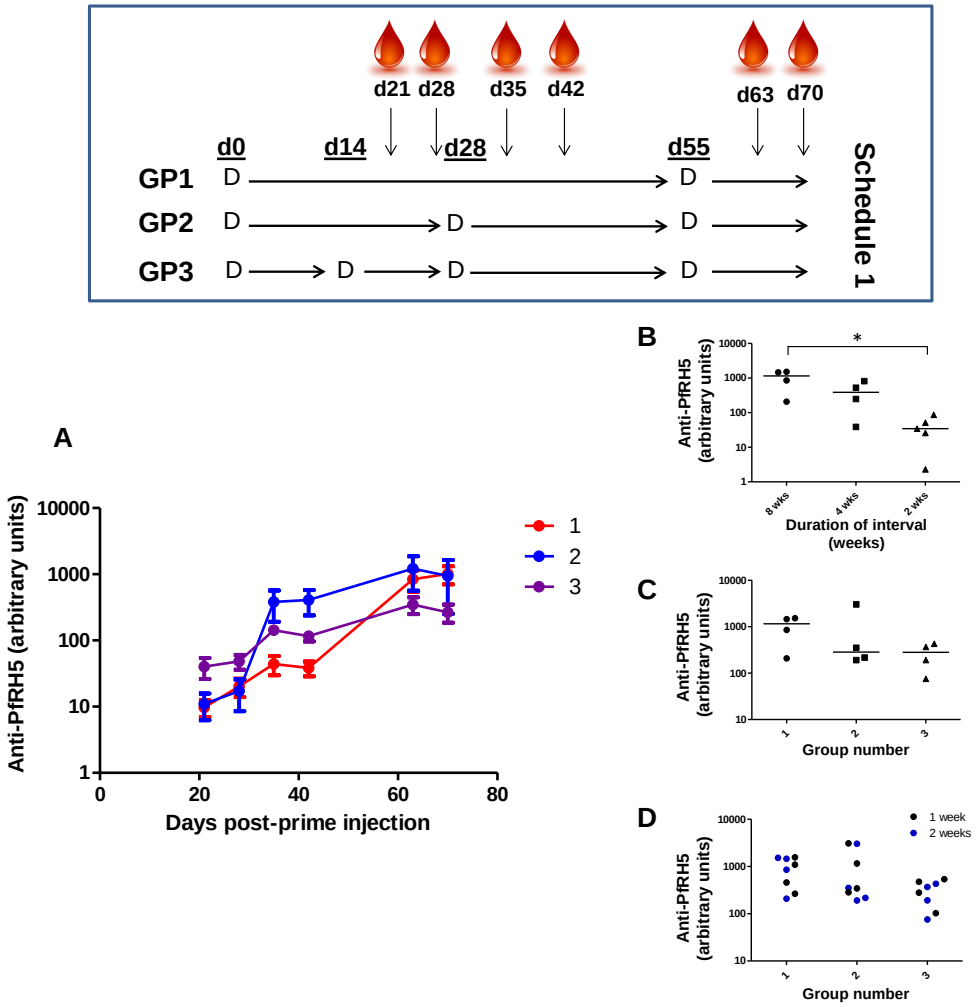

FIGURE 6.6: Optimisation of the DNA/PEI immunisation regime with a PfRH5 vaccine. Mice were immunised with the standard dose of pENTR4 tPA.PfRH5 plasmid DNA mixed with *in vivo*-JetPEI, with a variety of dosing regimes (shown in box—serum collection times are denoted by droplets of blood). (A) Time course of antibody evolution as measured by ELISA. Data points, mean; error bars, standard error of the mean. (B) From left to right: group 1, day 70; group 2, day 42; group 3, day 28. These time-points represent two-dose regimes with a range of interval lengths. (C) Day 70 antibody titres. (D) Antibody titres at day 63 (one week after the final injection) and day 70 (two weeks after the final injection). For panels B–C, Data points, individual mice; lines, median. n=4 in each group. ELISA limit of detection = 1 arbitrary unit. $^*p$ <0.05, by Mann-Whitney U test.

The experiment was designed to answer these questions using a minimum of groups (Fig. 6.6, see box). As expected, day 21 antibody titres were highest in group 3, these mice having received two injections of DNA/PEI (Fig. 6.6A). However, the antibody titres found in these mice 14 days after their second injection (i.e. day 28 of the experiment)

were lower than those seen in mice immunised twice but with a longer interval between injections (6.6B). Furthermore, the third and fourth immunisations received by group 3 did not substantially boost their antibody response (Fig. 6.6A), so that by day 70 the antibody responses in groups 1 and 2 had superceded that seen in group 3 (Fig. 6.6C) resulting largely from the large boost in antibody titre experienced by groups 1 and 2 after the second immunisation at day 56 for group 1, and day 28 for group 2 (Fig. 6.6A). The surge in antibody titre associated with boosting appeared to occur within 1 week of the injection, as no differences in titre were observed within any groups between antibody titre at day 63, 1 week post final immunisation, and day 70, 2 weeks post final immunisation (Fig. 6.6D). These experiments suggests that a two-shot regime with a four-week or an eight-week interval, with serum harvest one week after the second immunisation, gives the best balance between immunogenicity, time to antibody harvest, and reagent consumption.

6.3.5 Elicitation of anti-chicken egg ovalbumin antibodies

Chicken-egg ovalbumin (OVA) is a widely used model antigen. The only previous report of antibody induction using DNA mixed with PEI used a single-shot immunisation regime [287] and reported low antibody titres at day 21 and day 42 that rose substantially by day 70. I was intrigued to reproduce this result and to compare it to the D-8-D regime described in this Chapter. Day 70 antibody titres elicited by the D-8-D regime (median, 42; range: 29–62 AU; n=5) were substantially and significantly higher than those elicited by the single-shot regime (median, 12; range: 9–20 AU; n=5; Mann-Whitney p <0.01, see Fig. 6.7A). The regimes reported here that incorporate 2 or more injections therefore represent a substantial improvement over the previously-reported single-shot regime, although it should be noted that these results may not be directly comparable with the result reported by Grant *et al.* (discussed extensively in Section 6.4.1). Of note, in this experiment the D-8-D immunisation regime elicited a lower antibody response than the Ad-8-M regime (median, 69; range: 58–127 AU; n=4; Mann-Whitney p <0.05), the first time that this had occurred (compare Figures 6.3B and 6.5C with Fig. 6.7A). Because each experiment was designed to include an internal control

(pENTR4 tPA.PfRH5 plasmid DNA/PEI administered in a D-8-D regime) I was able to compare the results of this experiment to other experiments and found that the anti-PfRH5 response elicited by DNA/PEI was significantly lower by comparison to previous results. It is possible that this result was due to a technical factor such as incorrect formulation of the DNA/PEI polyplexes, or more likely improper storage of the *in vivo*-JetPEI resulting in multiple freeze-thaw cycles. As a consequence of this result all future batches of *in vivo*-JetPEI were frozen in aliquots to minimise freeze-thawing.

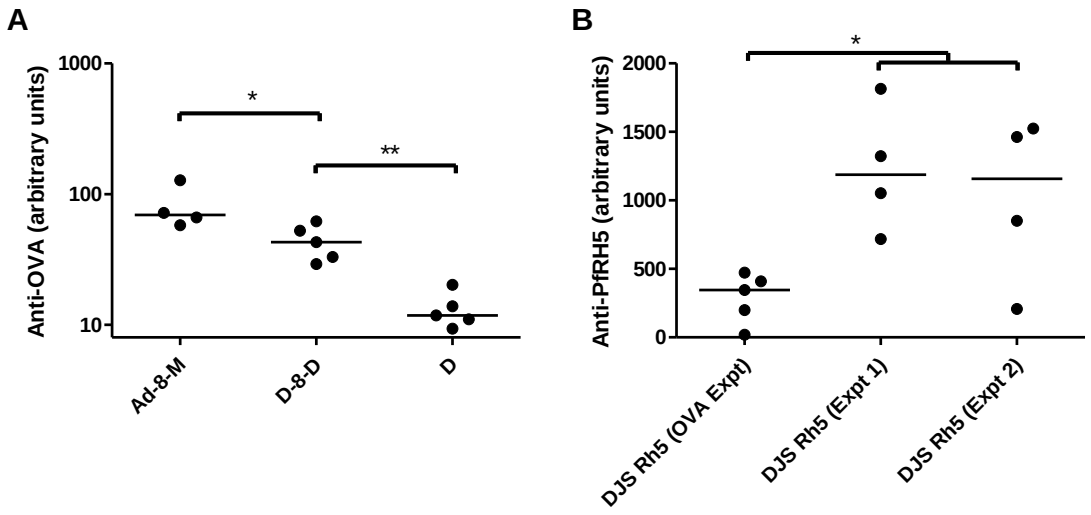

FIGURE 6.7: Underperformance of a chicken egg ovalbumin DNA/PEI vaccine may be due to improper storage of *in vivo*-JetPEI. (A) Day 70 serum anti-OVA antibody titres in mice immunised either with tPA.OVA virus vectors in an Ad-8-M regime (far left column), pENTR4 tPA.OVA plasmid DNA mixed with *in vivo*-JetPEI in a D-8-D regime (middle), or a single shot of pENTR4 tPA.OVA plasmid DNA mixed with *in vivo*-JetPEI (right). (B) Antibody responses elicited by the DNA/PEI tPA.PfRH5 internal control in the OVA experiment (left-hand column) plotted against anti-PfRH5 responses elicited with identical formulations and regimes used in two representative previous experiments, JI08 and DNA02. In all three experiments, mice were immunised with a standard dose of pENTR4 tPA.OVA plasmid DNA mixed with *in vivo*-JetPEI in a D-8-D regime. Data points, individual mice; lines, median. ELISA limit of detection = 2 arbitrary units. $*p < 0.05$, $**p < 0.01$, by Mann-Whitney U test.

6.3.6 Antibodies elicited with DNA/PEI are functional

Although the ability of DNA/PEI regimes to elicit high-titre antibody responses was now established, it was not clear that these antibodies would be functional in an *in vitro* assay of GIA. From the experiments depicted in Figures 6.5 and 6.6 the serum from groups of mice immunised with Ad-8-M regimes or D-8-D regimes was pooled to

give one serum sample per group. Antibodies from these serum pools were purified and assayed for GIA (see Sections 2.6 and 2.7). While antibodies raised against OVA using a D-8-D regime did not show any detectable GIA, antibodies raised against PfAMA1 (Fig. 6.8A) and PfRH5 (Fig. 6.8B) had substantial killing activity that exceeded the killing activity of antibodies raised against identical immunogens using the Ad-8-M regime.

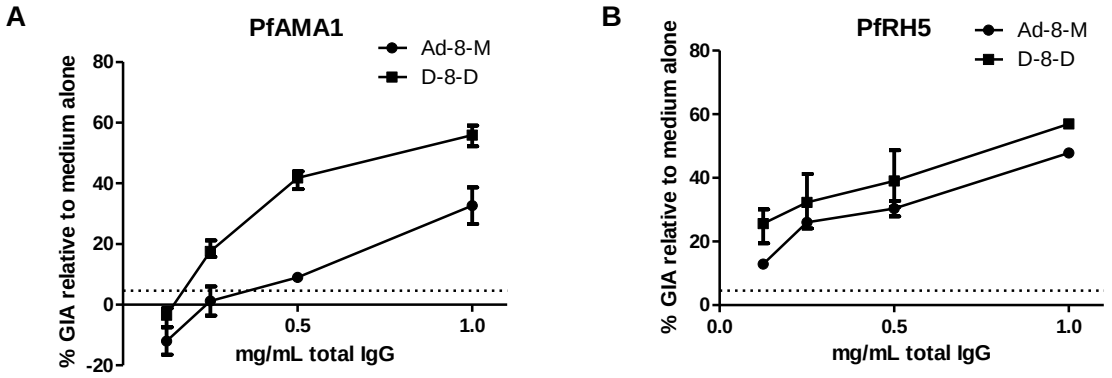

FIGURE 6.8: Antibodies elicited by immunisation with DNA/PEI are functional in an *in vitro* assay of GIA. Mice were immunised with either a virus vector-based Ad-8-M regime, or with plasmid DNA mixed with *in vivo*-JetPEI in a D-8-D regime. Serum from groups of 4 mice were pooled, and the antibodies purified and assayed for GIA. Panel (A) depicts the GIA of antibodies raised against PfAMA1, panel (B) depicts the GIA of antibodies raised against PfRH5. Dashed lines represent the GIA of antibodies elicited against chicken egg ovalbumin using DNA/PEI in a D-8-D regime. Results are representative of two independent GIA assays. Data points, mean; error bars, range across three replicate wells in the GIA assay plate.

6.3.7 Influence of PEI structure upon immunogenicity

This platform was initially conceived as an antibody-producing platform that had advantages over virus vectors in term of labour input, throughput and cost. As of 24th July 2014, 100 µL of *in vivo*-JetPEI cost £282 from the UK distributor VWR®. At 3.2 µL per dose, enough *in vivo*-JetPEI to immunise a group of 4 mice on a three-shot regime costs approximately £108, making antigen screening and construct optimisation costly. Grant *et al.* [287] made use of a range of PEIs produced by a collaborating group based at the Massachusetts Institute of Technology [236]. However, alternative high quality PEIs are commerically available. Chapter 3 describes the *in vitro* performance of a range of PEIs when used to tranfect DNA into suspension HEK293 cells (Section 3.2.4 and Fig. 3.5). When pENTR4 tPA.PfRH5 plasmid DNA was formulated with L25, M40 and

M160 PEIs and administered to mice (see Section 6.2.1), day 7 responses in the blood as measured by *ex vivo* IFN-ɣ ELISPOT were comparable between all groups (Fig. 6.9), suggesting that the moderate variability of transfection efficiencies seen *in vitro* does not substantially impact day 7 T cell responses after a single immunisation. However, over the next three weeks antibody responses were only seen in the groups that had received DNA formulated with *in vivo*-JetPEI and M160. This discrepancy become even more pronounced after the second immunisation (Fig. 6.9B, days 35 and 42). By day 70, A clear hierarchy had emerged with *in vivo*-JetPEI and M160 performing indistinguishably (medians, 284 vs. 475; ranges: 191–3031 vs. 179–2482 AU), L25 eliciting very low titres of antibody, and M40 eliciting only a moderate antibody titre. It is interesting to note that day 7 ELISPOT responses were a poor predictor of humoral responses, and also that the *in vitro* transfection efficiencies described in Section 3.2.4 and Fig. 3.5 correlated only loosely with antibody responses: L25 outperformed M40 *in vitro*, but was clearly outperformed by M40 with respect to *in vivo* antibody induction.

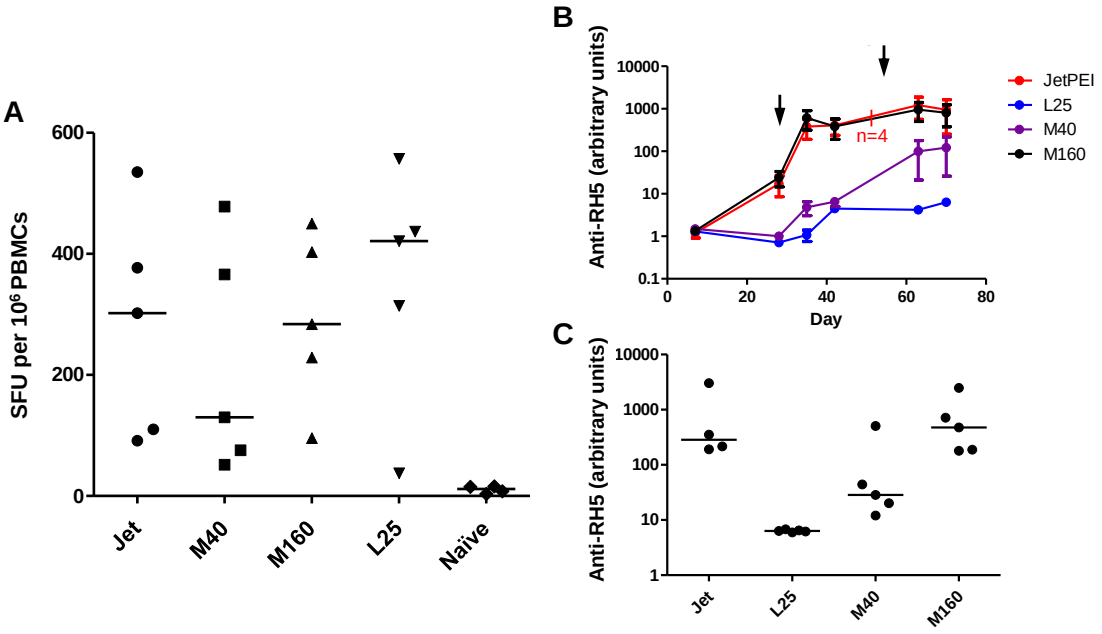

FIGURE 6.9: The impact of PEI structure upon vaccine immunogenicity. Mice were immunised with 20 µg of pENTR4 tPA.PfRH5 plasmid DNA mixed with a range of PEIs in a D-4-D-4-D regime. (A) Responses in the blood as measured by IFN-ɣ ELISPOT, measured 7 days after the first immunisation. (B) Time course of anti-PfRH5 antibody induction. Serum samples were taken on days 7, 28, 35, 42, 63 and 70. (C) Day 63 anti-PfRH5 antibody responses. For (A) and (C), each data point represents a single mouse and lines denote the median. For (B), data points represent means and error bars the standard error of the mean. ELISA limit of detection = 1 arbitrary unit.

6.4 Discussion

Chapter 3, Section 3.3 sets out a number of criteria to which an antigen-screening platform should conform, concluding that a recombinant protein-in-adjuvant system is ideal for a number of reasons. However, even with the best available platform for expressing secreted *Plasmodium* proteins (HEK293 cells) I was only able to obtain >300 µg pure, recombinant protein for 18 of 52 selected genes, in a full-time project spanning six months. Genetic immunisation by virus vectors negates the requirement for recombinant protein, but production of the recombinant viruses takes longer than protein expression and purification. Eliciting antibodies by vaccination with plasmid DNA would relieve much of the labour, time and cost associated with both of the aforementioned strategies. The exact sequence of events following plasmid immunisation, and the critical parameters determining the size and quality of the immune response, are complex and incompletely understood [462–464], but if all other factor are kept equal the magnitude (if not the quality) of an antibody response generally correlates with the size of the protein dose experienced by the host mammal [431], which in turn correlates with the amount of DNA delivered into the nuclei of the cells in the living organism. Having observed the importance of DNA delivery for protein production *in vitro*, I considered the possibility of using PEI for delivery of DNA vaccine plasmids. DNA/PEI polyplexes administered i.v. proved capable of eliciting antibody responses that outperformed the best virus vector regime in terms of both titre (Figures 6.3 and 6.5) and anti-parasitic growth-inhibitory activity (Fig. 6.8). This suggests that this platform could serve as an affordable and rapid alternative to protein-in-adjuvant or virus vector pre-clinical vaccination, particularly when screening new antigens and constructs.

6.4.1 Other reports of DNA/PEI immunisation

Grant *et al.* report a very high $CD8^+$ T cell response 7 days after i.v. injection of DNA polyplexes (17% of circulating $CD8^+$ showing antigen specificity), which contracts sharply to 6% by day 14 (see Fig. 3C in [287]). It is difficult to compare the FACS-based antigen-specific tetramer staining reported by Grant *et al.* [287] to the *ex vivo*

IFN-ɣ ELISPOTs reported in this chapter (Figures 6.3 and 6.5), because the FACS method detects all $CD8^+$ cells with a relevant TCR, whilst the ELISPOTs reported here only detect cells that express IFN-ɣ in response to stimulation. Furthermore, different immunogens can give very different responses, and whilst I used malaria antigens, Grant *et al.* used HIV envelope glycoprotein GP120. The nearest comparison to Fig. 3C in Grant *et al.* [287] to be found in my work is Fig. 6.5A, as these both looked at day seven responses to a single MHC class I-restricted peptide (see Section 6.2.2). Very roughly, the 17% antigen-specific $CD8^+$ T cells reported by Grant *et al.* corresponds to 3400–13600 reacting cells per 10^6 PBMCs ($CD8^+$ T cells represent 2-8% of all leukocytes). Even accounting for the fact that the IFN-$ɣ^+$ cells detected in my ELISPOTs may be only a subset of the total $CD8^+$ T cell response, there is no doubt that the responses reported by Grant *et al.* [287] are greater in magnitude than the responses I observed. There are a number of possible explanations for this.

Differences in the plasmid vector backbone could in part explain these differences, although the pVRC plasmid backbone used by Grant *et al.* (which is disclosed in overview in a 2010 patent application [465]) is very similar to the pENTR4 plasmid that I used: both contain a CMV promoter with an intron; both encode kanamycin-resistance genes (known to contain fewer immunostimulator motifs than the ampicillin-resistance gene [466]); and both use BGH polyadenylation sequence (which can impact upon transgene expression [467]). This said, a plasmid overview is of only limited utility because the mammalian DNA-sensing machinery detects specific CpG concensus motifs of around 6 nucleotides in length [466, 468] (although recently this doctrine has been challenged [469, 470]).

An alternative explanation invokes the site of immunisation: whereas Grant *et al.* injected mice retro-orbitally, I injected mice in the lateral tail-vein. Although i.v. injection is often thought to constitute systemic administration of a substance, the reality is more complex and the hydrodynamic force exerted during an i.v. injection is known to cause local uptake of substances [471, 472]. 100 µL constitutes around 7% of the blood volume of a 25g female mouse [473], and when injected into the tail-vein as a bolus this causes congestion in the inferior vena cava, leading to retrograde flow

down the hepatic vein [474, 475] and swelling of the liver [476]. The resultant pressure permeablises the cell membranes of hepatocytes, allowing molecules such as DNA to enter the cytoplasm [474, 477, 478]. The result is that injection of DNA through the tail-vein biases delivery to the liver [478, 479]. Injection of the retro-orbital sinus, as used by Grant *et al.* [287], is less well understood and has been compared to tail-vein as a route of administration for small molecules [480] but not for nucleic acids. Reports using retro-orbital injection tend to show preferential delivery to the lung [236, 287]. The site of injection can therefore markedly influence the site of DNA transfection, which in turn will impact the quality of the immune response. Although alone unlikely to account for the weak $CD8^+$ T cell responses that I observed, it may be worth exploring alternative sites of administering DNA/PEI polyplexes with a view to improving cellular immune responses.

6.4.2 Immunological comparison of DNA/PEI and virus vectors

The modest $CD8^+$ T cell induction that I observed meant that for both PfRH5 and PfAMA1, DNA/PEI regimes were outperformed by Ad-8-M (Figures 6.3A and 6.5A). This was also true for humoral responses, at least initially: mice that had received only a single shot of DNA/PEI always had lower antibody titres than those that had received a dose of adenovirus (Figures 6.3A and 6.5A). It was only when a second dose of DNA/PEI was administered that the antibody titres in the DNA/PEI groups exceeded that of the Ad-8-M groups. This suggests that the boosting effect of a second dose of DNA/PEI exceeds the boosting effect of an MVA administered to a mouse primed with adenovirus. Recombinant adenoviruses provide reliable and potent IgG priming responses [199], generally thought to be mediated by the large amount of antigen expressed *in situ* [481] and also by stimulation of the host virus-sensing machinery, thereby adjuvanting the response to the recombinant antigen [482]. The delivery of plasmid DNA with PEI may help close the gap between adenoviruses and DNA with regard to *in situ* antigen expression, but it is unlikely to lead to dramatic modulation of the innate immune antiviral response apart from increasing the 'dose' of DNA seen by the cytoplasmic DNA sensors. There is no clear explanation as to why DNA/PEI

outperformed A-8-M with regard to the humoral response. It is possible that the strong immune response elicited by the priming dose of adenovirus, particularly with regard to $CD8^+$ T cells and antibodies, leads to rapid clearance of the cells transduced by the boosting agent (i.e. MVA) and neutralisation of the antigen expressed [263], suppressing the effect of subsequent boosting. This effect may be less pronounced with intravenous DNA/PEI because the primary immune response is less severe.

6.4.3 Antibodies elicited with DNA/PEI are functional

Given that the purpose of this work was to establish a protocol for the rapid induction of functional antibodies, it was reassuring to see antibodies from mice immunised with DNA/PEI outperform antibodies produced with virus vectors in the *in vitro* assay of GIA (Fig. 6.8). It is tempting to attribute the high level of GIA seen with DNA/PEI to an increase in antibody quality, however, to verify this it is necessary to compare the antigen-specific antibody titres of the IgG samples that were entered into the GIA assay. Given the much higher antibody titres found in the serum of mice vaccinated with DNA/PEI, it is more likely that the higher levels of GIA can be simply attributed to the presence of a higher proportion of antigen-specific IgG in the GIA assay well.

6.4.4 Regime optimisation

Regime optimisation was guided by two competing considerations: first maximising the serum titre of the IgG response; second, minimising the duration of the immunisation protocol to save on mouse housing costs and time. Because the results in Fig. 6.5C appeared to show comparable antibody responses between a D-4-D regime and a D-8-D regime, I wondered whether a D-2-D regime would also perform well. I found that the D-2-D regime performed significantly worse than the D-8-D regime (Fig. 6.6B). Furthermore, the mice that had received D-2-D did not appear to be boosted by further administrations of DNA/PEI. With genetic immunisation, it is well established that a longer interval between doses improves immunogenicity [263, 427, 483–486], although

the exact mechanisms underlying this observation are not well-studied. Typically, explanations invoke contraction of the effector cells involved in the primary response into memory subsets [199] or apoptosis of T cells due to over-exposure to antigen [487–489]. Although the D-4-D and D-8-D regimes were not significantly different in their ability to elicit anti-PfRH5 antibodies (Fig. 6.6B), there is a trend towards the D-8-D regime eliciting higher responses. This is worth investigating further, as differences in antibody titre that are typically considered negligible by ELISA can have a large impact on subsequent functional assays: a 2-fold difference in antibody titre is considered small, but could have a large impact on the readout of a functional assay such as the *in vitro* assay of GIA (see for example Fig. 6.8A).

6.4.5 Adjuvant properties of PEIs

Grant *et al.* describe a detailed comparison of the ability of different PEIs to elicit cellular immune responses when mixed with DNA, and found that different PEIs can cause large variabilities in the magnitude of the immune response [287], with the strength of the immune response tending to correlate with the *in vivo* transfection efficiency of the PEI used. I did not measure *in vivo* tranfection efficiencies, but the ability of DNA/PEI polyplexes to transfect HEK293 cells *in vitro* (Fig. 3.5) did not correlate with strength of the cellular immune response (Fig. 6.9). This is not surprising in itself, as the evolution of a T cell response requires only a very low level of antigen, beyond which point there is only a weak a dose:response relationship [431]. There was also a discontinuity between the humoral response (Fig. 6.9, B and C) and the *in vitro* performance of the PEIs (Fig. 3.5, Section 3.2.4), with L25, one of the strongest *in vitro* transfection reagents, eliciting antibody responses more than 100 times lower than M160 and *in vivo*-JetPEI. Assuming that performance as a transfection reagent *in vitro* corresponds with performance *in vivo*, this result was unexpected because antibody response after genetic immunisation is thought to correlate well with the amount of antigen expressed [199, 490–493]. These results appear to suggest that PEI adjuvants the T cell response and the B cell response via separate, distinct, mechanisms. The only factor that appeared to predict the strength of the humoral immune response was the average length of the PEI molecules (although

the average molecular weight of *in vivo*-JetPEI is not disclosed). Interestingly, longer PEIs tend to be more cytotoxic [286, 494]. The mechanism of toxicity is ascribed to very acute plasma membrane peturbation, resembling necrosis [495], followed by a more ordered apoptosis phase approximately 24 hours later [495]. To my knowledge, the only study characterising the immunological influence of PEI was performed by Regnstrom *et al.* [496]. After intra-tracheal administration of PEI to mice, Regnstrom *et al.* used gene arrays to observe widespread changes in the gene expression profiles of splenocytes that had been cultured *ex vivo* by comparison to the splenocytes of naïve mice [496]. These genes included those for cytokines involved in Th1 and Th2 responses, T helper cell response markers, and markers of B cell activation [496]. It is possible that the properties of PEI that lead to cytotoxicity, which is more pronounced in larger PEI molecules, are also responsible for the stimulation of these immune markers seen by Regnstrom *et al.*.

In any case, the elicitation of equivalent antibody titres by M160 and *in vivo*-JetPEI was a pleasing result because the cost per dose of M160 is less than 1/2000 of the cost per dose of *in vivo*-JetPEI, removing the final expense associated with this procedure. On 30$^{\text{th}}$ July 2014, enough M160 for 100 doses costs £0.24, whereas 100 doses of *in vivo*-JetPEI costs £632.96, see Sections 2.3.3 and 6.2.1.

6.5 Conclusion: DNA/PEI for antigen-screening

The only other strategies in true contention with DNA/PEI immunisation for the production of high-titre antibodies are immunisation by virus vectors, and immunisation by protein-in-adjuvant. Given that the overarching objective of this work is to test antigens for their susceptibility to neutralising antibody, any high-affinity binding molecule could in theory replace antibodies. However, alternative technologies for producing high-affinity binding molecules such as phage display [336, 337] and nucleotide aptamer libraries [338, 339] remain time-consuming, and are more suited to discovery of highly specific monoclonal binding molecules for use in diagnostics and therapeutics. Table 6.1

highlights the relative advantages of the three immunisation strategies considered in this thesis.

TABLE 6.1: Relative advantages of immunisation strategies for eliciting of functional antibodies for research

	Protein-in-adjuvant	**Virus vectors (Ad-8-M)**	**DNA/PEI**
Success rate (for *Plasmodium*)	33%	High	High
Throughput (antigens per month, excluding mouse immunisation)	>10	<4	>20
Consumable cost per antigen	£50	>250	£10
Controlled dose of immunogen?	Yes	No	No
Assessment of final antibody titre?	Straightforward (ELISA)	Not straightforward (ELISA requires protein)	Not straightforward (ELISA requires protein)
Induction of cellular immunity	Poor	Strong	Not fully characterised

As stated in Chapter 3, protein-in-adjuvant is the ideal immunisation platform for antigen screening as it allows a controlled and known dose of antigen. However, it is not always possible to produce recombinant protein, and here genetic immunisation platforms become useful. As a platform for elicitation of mouse antibody for research use, DNA/PEI is superior to adenovirus-MVA regimes in every respect. A set of novel DNA plasmids can be cloned and prepared within a week. By contrast, to produce a virus vector requires multiple cloning steps, transfection of cell lines, passage of the virus-infected cells to encourage growth, lengthy purification procedures and time-consuming viral quantification assays. Aside from the considerable infrastructure required to produce adenoviruses (such as sterile laminar flow hoods for mammalian cell tissue culture, humidified incubator with CO_2 control), a single Adenopure® column costs $150, meaning that to raise antibodies against a single novel antigen using a heterologous adenovirus prime-boost regime [235, 391] would cost $300 in purification costs alone. DNA/PEI therefore represents a viable solution for screening of novel antigens and constructs for their ability to elicit antibody where recombinant protein is difficult to obtain. For cellular immunity, however, Ad-8-M clearly outperforms

DNA/PEI (Figures 6.5 and 6.3) and therefore this incarnation of DNA/PEI would not be an appropriate replacement for virus vectors.

6.6 Future work

The most pressing application of this technology is the re-screening of the library of antigens detailed in Chapter 4. Of the 52 genes available, I was only able to obtain recombinant protein from 18. This means that there are 34 genes in my library whose products have not been screened. Immunisation of animals with DNA plasmids encoding these antigen is now a priority. The laboratory of Gavin Wright also has a library of genes encoding *P. falciparum* merozoite proteins, many of which are already known to express *in vitro* [224, 225] and could also be tested.

DNA/PEI administered using a D-8-D regime is capable of eliciting serum antibodies of a titre sufficient to have functional activity in a GIA assay at physiological concentrations of total IgG (Fig. 6.8). However, it may yet be possible to further improve the strength of the humoral immune response. More useful still would be an effort to improve the ability of DNA/PEI to elicit cellular immune responses, as the only robust strategy established in our laboratory for achieving this, pre-clinically or otherwise, involves virus vector production. Although this thesis is primarily concerned with the discovery of antigen susceptible to neutralising antibody, a platform allowing the rapid induction of $CD8^+$ T cell responses would greatly facilitate antigen screening and design in areas such as liver-stage malaria, dengue fever, HIV and *Toxoplasma gondii*. As partially illustrated in Fig. 6.9, effective gene delivery and subsequent *in situ* protein expression level is not the only determinant of immunogenicity from plasmid DNA. The inclusion of immunostimulatory motifs such as unmethylated CpG [466, 497] has been shown to enhance responses. Likewise, co-administration of DNA encoding cytokines such as GM-CSF, IFN-ɣ and interleukins 2, 4, 10 and 12 has been shown to improve responses [498–501] when both administered as part of a separate plasmid, or included upon the antigen-expression plasmid. Formulation of DNA plasmids with conventional adjuvants such as alum [502] or LPS [503] has also been shown to modulate immunity [463].

Chapter 7

Conclusions

7.1 Summary of output

7.1.1 Rapid production of antigen-specific antibody

This Thesis describes the establishment of two platforms to facilitate the production of antigen-specific antibody. The first of these, described in Chapters 3 and 4, is a protocol for the production of recombinant protein. HEK293 cells are a well-established system for recombinant protein expression [504], but the utility of HEK293 cells for the production of recombinant full-length secreted merozoite proteins has only recently come to be recognised, largely thanks to the laboratory of Gavin Wright at the Sanger Institute [128, 224, 225]. To be used to generate antigen for animal immunisation, the system had to be somewhat adapted using RH5 as a model merozoite antigen. Virtually every construct produced in the Wright laboratory is C-terminally tagged with domains 3 and 4 of the rat CD4 molecule, which is used by them as an epitope tag [224, 225, 276, 505]. Unfortunately, the presence of a large tag with homology to a protein that is important in the humoral immune response in mice, rabbits and humans makes it an inappropriate appendage to a pre-clinical vaccine immunogen. Furthermore, even small epitope tags were found to complicate analysis of antibody responses (Fig. 4.9). Initial attempts to

express RH5 from a similar plasmid to that used in the Wright laboratory, but lacking the rat CD4 tag, saw a complete ablation of RH5 expression (Fig. 3.2). This suggested that CD4 actually played a role in improving the expression of RH5, possibly acting as a scaffold. It took some time to find a plasmid that was able to express RH5 in the absence of the CD4 tag (Fig. 3.6). It also took time to find a re-usable purification resin–affinity tag combination that allowed moderate-throughput protein purification (described in Section 4.2.3.2). This worked well, but with our existing laboratory infrastructure it was only possible to purify 4 proteins in each batch. For future projects, it may be worth investing in apparatus to facilitate parallel purification of many proteins in a single batch [392].

The second platform delivered in this Thesis is a very potent genetic immunisation process that allows high-titre antibodies to be generated with no requirement for purified protein (Chapter 6). It was found that when a mammalian protein-expression plasmid was formulated with PEI and injected intravenously into mice, it lead to the evolution of very strong antibody responses. The system was able to elicit antibodies against AMA1 and RH5 that outperformed those elicited with virus vectors in an *in vitro* assay of GIA (Fig. 6.8), demonstrating its utility for antigen screens such as the one described in Chapter 4. The immunisation schedule was optimised to minimise time from cloning of the antigen to obtaining antigen-specific antibody (Sections 6.3.3 and 6.3.4), and the use of affordable, off-the-shelf PEIs was investigated Fig. 6.9. The resultant platform is the most rapid and cost-effective method for eliciting antibodies that I am aware of. The use of PEI as a DNA carrier for genetic immunisation had been described in a previous publication, but humoral immunity was considered only as an afterthought [287]. The use of DNA/PEI for antibody elicitation therefore constitutes a novel and useful advance for vaccinologists and therapeutic immunologists.

The relative merits of the three platforms now available in our laboratory for antigen screening are discussed in Section 6.5 and summarised in Table 6.1. It should be noted, however, that these two platforms have implications for vaccinology and basic research beyond antigen discovery. For example, the protocols established in Chapter 3 have allowed, for the first time, members of our group to produce recombinant antigen for

use in clinical-trial ELISAs to assess the humoral immunogenicity of human vaccines (Hodgson *et al.* in press). The ability to rapidly produce antibody against folded antigen, as in Chapter 6, has also faciliated parasitological studies in our laboratory (Lyth *et al.* in preparation).

7.1.2 AARP and S-antigen: candidate blood-stage vaccine antigens

The antigen screen reported in Chapter 4 resulted in the identification of S-antigen, a protein that has hitherto received no serious consideration as a vaccine candidate, as a target of growth-inhibitory antibody. However, S-antigen had been identified as essential to parasite growth [35, 355]. Furthermore, my results validated findings from separate research groups [135, 358, 401] that AARP may be susceptible to antibody. The AARP construct that I used differed from that used in the previous studies, and in some cases was able to elicit whole IgG that outperformed those raised against RH5 (Figs. 5.2 and 5.3), which is thought to be the merozoite protein most susceptible to neutralising antibody [129, 136]. I attempted to further characterise the antibodies against AARP and found that antibodies of potent growth-inhibitory activity were elicited only erratically, and that these antibodies had differing reactivity profiles against parasite-derived, recombinant and synthetic antigen (Chapter 6). Antibodies against S-antigen, although less potent than the most effective anti-AARP antibodies (Fig. 4.10A) appear to be reliably elicited (Fig. 4.12C) and it is now a priority to define the point in the intra-erythrocytic growth cycle when parasites become susceptible to these antibodies.

7.2 Antigen selection in the vaccine-design pipeline

7.2.1 An ideal antigen-production pipeline

The question of how best to expand upon the antigen-screening work described in this Thesis, given the constraints of an academic budget and work-force, is addressed in Section 4.5. However, it is interesting to consider what might be attempted with unrestricted funds and manpower. As described in Section 3.3, where available, recombinant

protein-in-adjuvant would always be the preferred platform for animal immunisation. In the absence of any construct optimisation, transient transfection of HEK293 cells seems to provide useful yields of full-length recombinant protein [1] in around 35% of cases [224, 225]. If culture volumes were increased, with corresponding increases in reagent cost and incubator requirements, the work of Crosnier *et al.* suggests that 50% of proteins could be obtained as full-length constructs [224]. This would still leave 50% of proteins unexplored. Borrowing from the experience of protein scientists working with other expression systems, the remaining 50% could be examined using truncated fragments of antigen. The history of RH5 highlights the pitfalls surrounding this approach [133, 211, 220, 223], but equally, it has allowed the expression of functionally important regions of proteins that could not otherwise be produced in prokaryotic cells. $MSP1_{19}$ [506–508] and RH4.9 [130, 509] represent two vaccinologically relevant fragments of proteins that still cannot be produced in high yields as full-length recombinant polypeptides. Extending this approach to the large number (>1000) of merozoite proteins predicted to be surface-localised would involve drawing on advances from structural genomics, including improved algorithms for construct design, and technologies for high-throughput cloning and large-scale expression trials [510–512].

Transient transfection of HEK293 cells still represents a reasonably high-throughput method of producing recombinant antigen, as supernatants can be harvested within days of transfection and protein can be purified in a single step with a 6His affinity tag and HisTrap Excel resin. However, in our hands, *Drosophila* S2 cells have been able to express almost every construct tested, including RH5 [220], AARP (my personal unpublished observations) and *P. vivax* Duffy-binding protein. This expression system is low-throughput, requiring 2-3 weeks' worth of antibiotic pressure to select for stable cell lines, and then 10–12 days of culturing the resultant cell lines before supernatant can be harvested [513]. Furthermore, purification protocols may require extensive optimisation (Simon Draper, personal communication). With this said, in an ideal scenario where time, labour and money were not limiting, of all the available expression platforms the S2 system would probably yield the most comprehensive library of full-length merozoite proteins.

[1] i.e. >300 µg from a culture volume of 200-250 mL.

7.2.2 Feedback between antigen selection and immunogen optimisation

A model for pre-clinical vaccine development in the post-genomic era is shown in Fig. 7.1. Ideally, reverse vaccinology should comprehensively address the first stage in the pipeline: identification of target proteins. In reality, this is not possible. To begin with, in the case of blood-stage malaria, no assay has been comprehensively shown to predict protection. Thus, while in an ideal scenario the only screening assays to be used would be those that work through a mechanism with an established causal involvement in protection, in reality the selection of screening assays is based at least partially on practical considerations such as convenience and reproducibility.

Furthermore, the approach of defining target proteins by their susceptibility to antibody elicited with unoptimised recombinant immunogens relies a great deal on the fortuitous evolution of antibody responses against neutralising epitopes. As such it is vulnerable to false-negative 'misses'. It may well be the case that some highly susceptible proteins will only be recognised as suitable targets after a degree of immunogen optimisation. Thus, there must be feedback between the first and second stages of the pipeline shown in Fig. 7.1. Although immunisation with full-length RH5 dependably induces highly functional antibodies, this may not be the case for every protein. This is especially the case for multi-domain proteins with longer amino acid sequences, which may contain a lower ratio of neutralising:non-neutralising epitopes. This presumably explains why antigens previously reported to be susceptible to growth-inhibitory antibody (such as RIPr, SERA5, GAMA and sometimes AARP) failed to distinguish themselves as targets of neutralising antibody in my hands (see Section 4.3.2.1). Previous reports with these proteins have used shorter recombinant fragments—admittedly these are not the end-points of painstaking immunogen optimisation, but more likely the only recombinant proteins that could be produced with the expression tools available. RH4 provides a good example: antibodies against an initial fragment showed no inhibitory activity [514], then a second, larger fragment that entirely encompassed the first was produced and shown to elicit antibodies of moderate functionality [509]. Antibodies to this second fragment were then shown to work synergistically in combination with antibodies against

RH5 [136]. To my knowledge, no group has successfully produced antigen corresponding to the full-length ectodomain of RH4. This story illustrates how antigen optimisation may be prerequisite to the recognition of a protein as a target of neutralising antibody. It also illustrates the difficulty of ever ruling out a protein as a potential vaccine target, making it impossible to perform a truly 'conclusive' antigen screen.

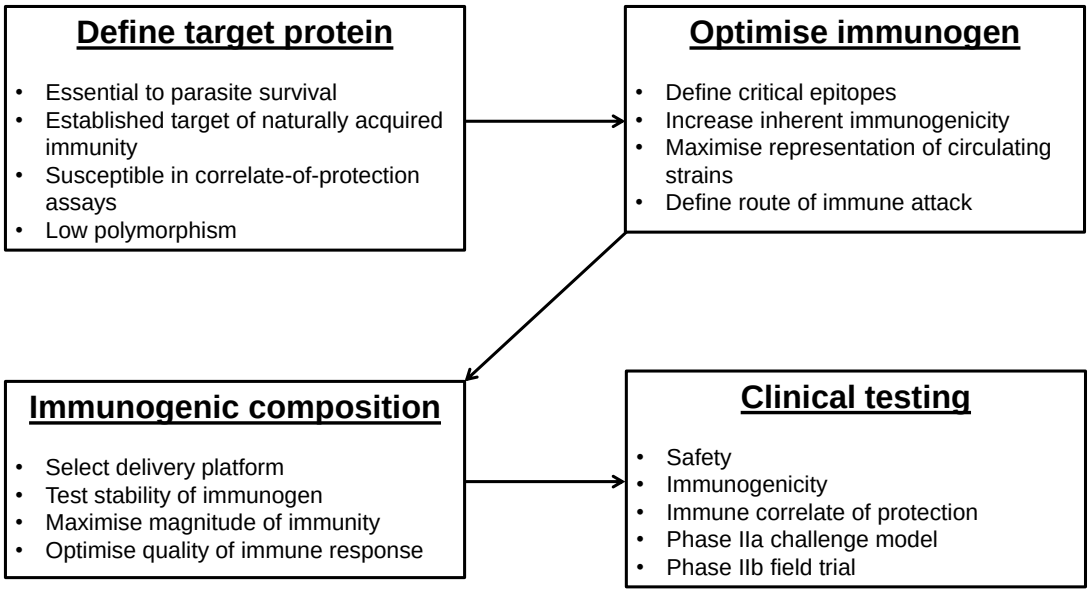

FIGURE 7.1: A ideal model of pre-clinical subunit vaccine development in the post-genomic era. The first stage is to define the antigenic targets of the vaccine. In theory this is a role that could be played by reverse vaccinology or antigen screening (i.e. susceptibility in assay that correlates with protection), but there are a number of other criteria that could be used. Once defined, these target proteins can undergo detailed structural, microbiological and epidemiological characterisation. This should lead to identification of key neutralising epitopes, consensus sequence design to maximise coverage of circulating strains, and rational mutation to increase stability, and result in an optimised immunogen. This can then be formulated with an appropriate delivery platfrom (virus vector, soluble protein, virus-like particle, DNA, etc.) to elicit an appropriate immune response. If the pre-clinical toxicology results are acceptable, the long (at least 10 years) process of clinical testing can begin.

7.2.3 Reverse genetics

Reverse genetics represents an alternative approach to defining target proteins in a comprehensive manner. This essentially involves genetic disruption of the target gene, and observing the phenotype of the parasite. Presumably if parasites are viable in the absence of a gene, the gene product is not a suitable vaccine antigen. This is because immune escape will require nothing more than down-regulation of the target antigen,

at limited cost to the parasite. Knock-out parasite lines exhibit a continuum of growth-impairment phenotypes, with attempts to knock out some genes failing altogether. This is presumably because the products of these genes are so important to parasite survival that it cannot grow without them. Such gene products immediately suggest themselves as candidate vaccine antigens.

From the perspective of vaccine development, where reverse-vaccinology is susceptible to false-negative results, reverse genetics is more likely to yield false-positives. Simply playing an essential role in parasite growth does not necessarily confer susceptibility to neutralising antibody. Pf92, PTRAMP and MTRAP are examples of proteins that are indispensable at the asexual stage [35, 363, 515] but which in my hands (and others) do not appear to be the target of neutralising antibodies (Chapter 4 and [422])—or at least, the neutralising epitopes are yet to be effectively targeted. This said, a high proportion of proteins that cannot be deleted during asexual growth have been reported as susceptible to neutralising antibody. These include RH5 [133, 218], MSP1 [516], S-antigen [35], RIPr [361] and (more controversially) AMA1 [313, 517, 518]. Generation of a gene-deleted parasite line is time consuming and labour intensive, but it has been achieved on a moderate scale before. This was performed most notably by Maier *et al.*, who attempted to knock out 83 genes in a single study [519].

In an argument against the use of reverse genetics for defining candidate antigens, previous research seemed to indicate that a protein need not be essential to be targeted by neutralising antibody. Putative vaccine candidate antigens such as RH4 [509], EBA-175 [374, 375], Pfs25 and Pfs28 [520] are all demonstrably sensitive to anti-parasitic antibody *in vitro*, but single knock-out lines for each individual gene are viable [134, 520]. Theoretically, vaccines targeting multiple proteins that are individually degenerate could overcome the issue. This has led to the description of multi-valent candidate vaccines containing a cocktail of functionally redundant antigens [134, 135]. However, I would contend that the emergence of RH5 as a highly susceptible target that cannot be knocked out in any tested line [218] has raised the bar for the field. Renewed focus should be directed to the discovery of antigens that are individually essential. For this reason, I would argue that the most comprehensive route to defining a list of potential

candidate antigens is to begin with a reverse-genetics screen of predicted surface proteins. This should be followed by a considered and thorough attempt to define which of these essential proteins constitute the best targets of neutralising antibody.

The conclusions of Sections 7.2.2 and 7.2.3 are well illustrated by the results of the antigen screen in this Thesis (Chapter 4, Fig. 4.10): a small fragment of AARP had been previously described as a target of growth-inhibitory antibody [135, 358, 401], and S-antigen had been reported as refractory to knock-out [35, 355].

7.3 Final remarks

The previous section finished by recommending a programme of reverse genetics and subsequent antigen characterisation that would take several years to complete. However, the contemporary literature abounds with reports of putative asexual-stage antigens, and short-term advances could be made with the proper assessment of these. A previous study from our laboratory attempted to do this by testing the ability of a range of previously-described antigens to elicit growth-inhibitory antibodies in rabbits when all were delivered with the same virus vector platform [133] . This study produced some surprises. For example, the F2 region of EBA-175, which had been reported to elicit antibodies with almost 100% GIA at 1 $\mathrm{mg\,mL^{-1}}$ whole rabbit IgG [374], in our hands yielded around 10% GIA at 10 $\mathrm{mg\,mL^{-1}}$ whole rabbit IgG. Since the potency of an immunogen is dependent upon the delivery platform, we could not exclude the possibility that our formulation was sub-optimal. However, we were not the only researchers to find such a discrepancy with this fragment of EBA-175 [521]. As commented by the authors of the third study, widespread confirmation of results in a centralised GIA assay reference laboratory, and a greater focus on the 3D7 clone, would engender confidence in the reproducibility of such work. PATH MVI had until recently funded the operations of a GIA assay reference lab in Bethesda, but have now withdrawn their support. This is disappointing. At the time of writing, RH5, RIPr, GAMA, SERA5, AARP, SEA-1 and RAP1 each contend to be targets of potent polyclonal or monoclonal antibodies, using assays conducted according to disparate methodologies in laboratories around the

world. There is no consensus on which of these proteins constitutes the most promising antigen, or combination of antigens, for future development. Transparency and sharing of reagents between competing groups is now paramount so that this question can be resolved.

Funding organisations are currently turning away from blood-stage merozoite malaria vaccines, and toward the proven ground of the pre-erythocytic stage, and the untested promise of the sexual stage. To reverse this trend it is vital that the blood-stage malaria community works in a co-ordinated manner in order to develop immunogens that will deliver a more convincing and coherent series of *in vivo* efficacy results.

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