CHAPTER 6

Discussion

6.1 Summary and Aims

Chapters 3 and 4 described the first steps in developing humanised monoclonal antibodies targeting the RH5-Basigin interaction, as potential novel anti-malarial therapeutics. In this Chapter, I describe a number of future experiments that could be done to improve these pilot therapeutics, including increasing the affinity of huMEM-M6/4, huMEM-M6/8 and huD9 antibodies for BSG, further characterisation of ch6D9 *in vitro* and *in vivo* and future work on the bi-specific monoclonal 2AC7-6D9 DVD-Ig.

In the second half of this Chapter I build on the AVEXIS screen described in Chapter 5 by discussing future experiments aiming to improve the recombinant expression of *Plasmodium* proteins. Finally, I discuss the future directions for the recombinant merozoite protein library, focusing specifically on the putative interaction between PF13_0125 and P4HB, and also on EBA-165.

6.2 The affinity of huMEM-M6/4 and huMEM-M6/8 for BSG can potentially be restored

To easily engineer and recombinantly express antibodies, I employed a plasmid vector system that allows the recombinant expression of rearranged antibody heavy and light chains in HEK293 cells. The plasmid system is based on the pTT3 expression vector and similar plasmid systems have been successfully used in the past for the recombinant expression of humanised or chimeric antibodies (Clarke *et al.*, 2010; Yu *et al.*, 2010).

By using this plasmid expression system, two anti-BSG monoclonals, MEM-M6/4 and MEM-M6/8, were successfully humanised by CDR grafting. Both huMEM-M6/4 and huMEM-M6/8 retained their specificity for BSG, but a biophysical analysis of the humanised antibodies demonstrated that the affinity for BSG decreased in comparison to the original mouse antibodies. While the decrease in affinity for huMEM-M6/8 was subtle, the affinity of huMEM-M6/4 decreased by at least two orders of the magnitude in comparison to MEM-M6/4. It is not an unusual phenomenon that the affinity of a humanised antibody is reduced in comparison to the parental mouse one (Lo, 2004; Almagro and Fransson, 2008). However, antibody affinity plays an important role in biological efficacy, and hence, antibodies with low affinity are unlikely to be used for therapeutic purposes (Ho and Pastan,

2009). An increased affinity may allow for a low dosage of a therapeutic antibody and toxic side effects may therefore be reduced (Ho and Pastan, 2009).

The decrease in affinity in humanised antibodies likely arises from interference between human FRs and murine CDRs. Such interference may involve CDR loop displacement or loss of CDR flexibility. Antibody affinity can potentially be restored by mutating certain amino acids in the FRs of the humanised antibody to match those found at the same positions in the murine antibody, but the identification of the residues to be mutated often requires three dimensional modelling (Lo, 2004; Almagro and Fransson, 2008). Because of the lack of such expertise in our laboratory, future directions may include the collaboration with other laboratories which specialise in structural biology and *in silico* modelling. If such an effort was to be undertaken, huMEM-M6/4, which appears to inhibit the RH5-BSG interaction through steric hindrance, is clearly not a high priority target. Collaborations with the aim of increasing the affinity of huMEM-M6/8 for BSG could however be fruitful.

Instead of mutating targeted candidate amino acids, which often requires detailed structural knowledge of the antibody-antigen interaction, high-throughput experimental approaches could be used to increase affinity of huMEM-M6/8 for BSG. Error prone PCR which introduces random mutations within the variable region is a common procedure for antibody affinity maturation, and is normally followed by large scale, display based screens, to isolate highly avid mutants (Hoogenboom, 2005; Sergeeva *et al.*, 2006; Geyer *et al.*, 2012).To reduce the number of mutants to be screened Chowdhury and colleagues developed another approach in which mutations are introduced only at germline hotspots which are naturally mutated during somatic hypermutation in antibody secreting B-cells (Chowdhury and Pastan, 1999). Nevertheless, all these approaches require display technologies which are not available in our laboratory, and therefore these experiments should also be performed in the terms of collaborations with other laboratories which are set up for this work.

6.3 The identification of structurally important amino acid residues within V region, may facilitate the restoration of hu6D9 affinity for BSG

The humanisation process for the novel anti-BSG monoclonal antibody developed in this thesis, m6D9, was less successful than the humanisation of MEM-

M6/4 and MEM-M6/8, although a chimeric 6D9 antibody was successfully developed. Several alternate approaches could be trialled to improve the humanisation of 6D9. In the present PhD thesis, the IMGT numbering system was used to identify CDR and FR sequences. The choice of this numbering system was simply because it is the most recent one and various useful bioinformatic tools are publicly available through the official IMGT website (www.imgt.org). However, other numbering systems do exist and could be applied. In particular, it would be interesting to repeat the humanisation process for the development of hu6D9 by engrafting the CDRs as defined by Kabat (Kabat *et al.*, 1991) which is likely the most widely used numbering system (Fig. 6.1).

Additionally, the amino acid residues at certain positions in the V region have been proposed to participate in the formation of a platform, known as the Vernier zone (Foote and Winter, 1992; Lo, 2004), which is necessary to support correct CDR conformation. Re-introduction of the murine residues at these positions could reinstate hu6D9 binding to BSG. Hence, the following mutations could be considered for introduction in hu6D9: M4L, Y49K in variable light chain and M48I, V67A, I69F in variable heavy chain (Kabat numbering). Other residues that have been proposed to be responsible for retaining the structure of CDRs should also be examined (Lo, 2004). Alternatively, *in silico* molecular modelling of hu6D9 will accurately indicate which amino acids interfere with binding to BSG.

6.4 Future directions for 2AC7-6D9 DVD-Ig

Side by side comparison between anti-BSG ch6D9 and the anti-RH5 monoclonal antibody ch2AC7, demonstrated that ch6D9 had a considerably lower IC_{50} in blocking erythrocyte invasion in parasite culture. The higher inhibitory capacity of ch6D9 in comparison to ch2AC7, is likely related to the exposure time of the BSG and RH5 to ch6D9 and ch2AC7, respectively, during an invasion assay. Whereas erythrocyte BSG is constantly exposed to ch6D9, there is only a short timing window (30-40s) following schizont rupture where RH5 is available for binding by ch2AC7.

Figure 6.1 Kabat and IMGT numbering systems have different definitions for CDRs and FRs. Alignment between hu6D9 and m6D9 variable heavy (top) and light (bottom) chains. Green, cyan and magenta arrows indicate the position of CDR1, CDR2 and CDR3 respectively as defined by Kabat and IMGT numbering systems.

Similarly, ch6D9 was more potent than the bi-specific antibody 2AC7-6D9 DVD-Ig in preventing erythrocyte invasion. However, these data should be interpreted with caution. Because there is a decrease in affinity for BSG in 2AC7-6D9 DVD-Ig, no conclusions can be drawn for the therapeutic potential of an anti-BSG/RH5 bi-specific agent. It will be interesting to test the effect of such an agent when the anti-BSG affinity is retained. Swapping the fusion orientation of the two variable domains in 2AC7-6D9 DVD-Ig is one approach that could restore anti-BSG affinity to that of its parent monoclonal, 6D9 (DiGiammarino *et al.*, 2012). It will also be important to test the efficiency of the anti-RH5/BSG bi-specific agent in blocking erythrocyte invasion in parallel with a combination of individual ch6D9 and ch2AC7 used in various ratios. These experiments are necessary to establish whether anti-RH5 and anti-BSG monoclonals have synergistic or additive effects in preventing erythrocyte invasion, which would greatly inform future development of the anti-BSG/RH5 bispecific agent.

6.5 Ch6D9 – Future directions

6.5.1 Epitope mapping refinement

To narrow down the BSG sequence recognised by ch6D9, future experiments may include the testing of ch6D9 binding to an array of overlapping synthetic peptides covering BSG domain 1. Although such screening experiments would be informative, the precise amino acids that are in contact between ch6D9 and BSG can only be elucidated by solving the crystal structure of the ch6D9-BSG complex. Some indications as to whether ch6D9 recognises a conformational epitope can be taken by testing the binding of ch6D9 to heat treated BSG.

6.5.2 Further investigation of the (in)ability of ch6D9 to stimulate antibody effector functions

Ch6D9 was demonstrated to have a reduced ability to bind FcγRIIA and C1q *in vitro*. Whilst important information about antibody effector functions can be obtained by *in vitro* experiments, it is very difficult to predict how an antibody will behave *in vivo*. Therefore, the capability of ch6D9 to elicit ADCC and CDC should be tested under conditions that are more representative of the *in vivo* environment.

The capacity of an antibody to induce ADCC and CDC, is usually assessed by using the chromium release assay (Brunner *et al.*, 1968). This assay is based on the

measurement of ^{51}Cr released from metabolically labelled target cells, following the incubation with the antibody under investigation in the presence of FcγR-bearing effector cells or serum (as a source of complement) in a given period of time (Brunner *et al.*, 1968; Otz, 2010). Europium (Eu^{3+}) and the naturally occurring cell marker lactate dehydrogenase (LDH) have also been used in the same way as safer alternatives to the radioactive ^{51}Cr (Korzeniewski and Callewaert, 1983; Blomberg *et al.*, 1986; von Zons *et al.*, 1997). In recent years, more sophisticated approaches have been developed, providing much higher accuracy. In the vast majority of them, the binding of the antibody of interest to an FcγR expressing cell line, as well as the ability of an antibody to trigger ADCC and CDC mediated cell lysis, is assessed by flow cytometric methods (Armour *et al.*, 1999, 2003; Lee-MacAry *et al.*, 2001; Kim *et al.*, 2007; Helguera *et al.*, 2011; Hernandez *et al.*, 2012).

The ability of ch6D9 to induce ADCC can also be examined *in vivo* in mouse models developed for such purposes (Bruhns, 2012). These mouse models have been genetically engineered to express a single or a combination of human FcγRs (Chapman *et al.*, 2007; Bruhns, 2012; Lux and Nimmerjahn, 2013). However, aberrant expression of some hFcγRs in cell types which are not normally expressed, has been reported (Bruhns, 2012). The latter observations suggest that data obtained from studies in animal models require careful interpretation to remain meaningful.

Besides the amino acids that have already been mutated in the Fc region of ch6D9 for the elimination of antibody effector functions, the presence of oligosaccharides attached to the asparagine at position 297 of hIgG1 Fc region has also been shown to be important for the stimulation of ADCC and CDC (Idusogie *et al.*, 2000; Jefferis, 2009). Therefore, future experiments may include the development and testing of an aglycosylated form of ch6D9. By using site directed mutagenesis, and exploiting the versatility of the plasmid system described in Chapter 3, the asparagine at position 297 of the hIgG1 Fc region can be mutated to another amino acid (normally alanine) and thereby destroying the N-linked glycosylation site.

6.5.3 Testing the safety, efficacy and pharmacokinetics of ch6D9 *in vivo*

Ch6D9 safety and pharmacokinetics are currently being assessed *in vivo,* in NOD-*scid IL2Rγ*^{null} mice which have been engrafted with human erythrocytes (Jiménez-Díaz *et al.*, 2009). The next step will be to challenge these mice with *P. falciparum* infected erythrocytes, and test the anti-malarial efficacy of ch6D9 at various doses. These experiments are being performed in collaboration with Prof. Peter Preiser group in NTU, Singapore.

We are still at the very early stages in testing ch6D9 in mouse (and *in vitro*), but If the preclinical studies provide satisfactory results, the next step may be to test ch6D9 in man, in a Phase I clinical trial. Administration of ch6D9 to volunteers will require the production of ch6D9 under European Union good manufacturing practice (GMP) conditions. The production process should be capable of producing product of consistent quality, with minimal impurities and free of pathogens. In a potential Phase I clinical trial, GMP-produced ch6D9 will be administered to a small group (normally 20–100) of healthy volunteers and will be assessed for its safety, tolerability and pharmacokinetics. Also, Phase I clinical trials usually include dose ranging and therefore, the maximum dose of ch6D9 which is safe to be administered can be identified.

Based on the results of Phase I clinical trial, ch6D9 dosing requirements and efficacy can be determined in a Phase II clinical trial. For this purpose, a range of ch6D9 doses could be administered to individuals who have been challenged with *P. falciparum* via mosquito bite. If ch6D9 passes successfully through a Phase II clinical trial, then the next step will probably be to test the efficacy of ch6D9 in a large scale, randomized Phase III clinical trial.

6.5.4 Possible complications of using ch6D9 *in vivo* in man

Monoclonal antibodies against erythrocyte cell surface proteins but also against cell surface proteins expressed on other cells have previously been shown to be effective and safe for administration (Armour *et al.*, 2006; Ghevaert *et al.*, 2008, 2013). For example, an anti-D antibody (Fog-1) which carries the same mutations in the Fc region as ch6D9, has been demonstrated to be able to coat circulation erythrocytes without however promoting clearance and haemolysis (Armour *et al.*, 2006)

Specifically for Basigin, an anti-Basigin monoclonal antibody has been used before in the clinic, for the treatment of steroid-refractory acute graft-versus-host disease (Heslop *et al.*, 1995; Deeg *et al.*, 2001; Macmillan *et al.*, 2007). Moreover, in 2005, the Chinese Food and Drug Administration licenced the use of an anti-BSG iodine-131-labeled $F(ab)_2$ fragment under the brand name Licartin (also known as metuximab), for the treatment of patients suffering from hepatocellular carcinoma (HCC) (Yu *et al.*, 2008; He *et al.*, 2013). The previous safe use of anti-Basigin monoclonal antibodies in the clinic (section 1.10.6.2.2 and 1.10.6.2.3) is encouraging that antibodies against Basigin would be safe and very effective in treating malaria infected individuals. If successful, this would be the first time that a host-directed anti-malarial has ever been developed.

It is important however, to make some critical considerations about the possible problems that may arise from the administration of ch6D9 to man as a putative anti-malarial. First, because ch6D9 targets Basigin exposed on the cell surface of erythrocytes, it should be administered intravenously and this could prove clinically impractical on a large scale. Moreover, the requirement of manufacturing and scaling up the product, as well as the cost and feasibility of delivering an antibody which should be refrigerated, could be major logistic challenges. Furthermore, ch6D9 would potentially treat malaria infected individuals, but it would only confer protection against malaria for a limited amount of time (several days) depending on ch6D9 pharmacokinetics. Therefore, ch6D9 is not a replacement of a highly efficient malaria vaccine, but it could potentially be used in combination with it.

From safety point of view, Basigin is widely expressed in human body, and ch6D9 could potentially bind to Basigin expressed in cell types other than erythrocytes, with unpredicted outcomes. Indeed, some side effects arising from administration of anti-Basigin antibodies to patients have been reported in previous studies (Deeg *et al.*, 2001; Macmillan *et al.*, 2007). Previous experiments in mice, where erythrocyte exposed Basigin was blocked by an anti-BSG $F(ab)_2$ fragment, resulted in the selective trapping of erythrocytes in the spleen (Coste, 2001).Therefore, the pre-clinical studies in humanised mice which are currently under way for ch6D9, should closely monitor for rapid erythrocyte clearance. Finally ch6D9 is a chimeric antibody and therefore, it may cause some human-anti-mouse

antibody (HAMA) responses. This problem can be minimized if ch6D9 is fully humanised.

6.6 Improving recombinant protein expression levels by molecular chaperones

While Chapters 3 and 4 focused on a known merozoite-erythrocyte protein-protein interaction, Chapter 5 detailed a screen carried out to identify novel interactions. That screen, and the RH5-BSG interaction itself, were facilitated by recent advances in the recombinant protein expression of *Plasmodium* proteins. Expressing *Plasmodium* proteins in heterologous systems has traditionally been a technically challenging problem and the general success rate of expressing *Plasmodium* spp. proteins in a biochemically active recombinant form has been very low (Birkholtz *et al.*, 2008; Fernández-Robledo and Vasta, 2010). For example, Mehlin and colleagues reported only a 6.3% success rate in recombinant expression of *Plasmodium* proteins in a soluble form and at levels sufficient to be purified (Mehlin *et al.*, 2006). In Chapter 5, I demonstrated that 18 out of 26 (~70%) merozoite proteins were expressed in a biochemically active recombinant form and at usable amounts.

While the overall success rate was high, in keeping with previous experience of the HEK293 system, and in stark contrast to the historical experience with expressing *P. falciparum* proteins in other systems, a few merozoite proteins still failed to express as a recombinant form or expressed at very low levels. Although the genes were codon-optimised for HEK293 cells, *P. falciparum* proteins still have relatively unusual amino acid distributions, with an enrichment for asparagine, glutamic acid and lysine, and frequent homopolymeric runs. Indeed, it has been recently demonstrated that the stability of several *Plasmodium* proteins depends upon their association with heat shock proteins which act as molecular chaperones, presumably specifically evolved to handle the unusual amino acid composition (Muralidharan *et al.*, 2012). It will be interesting to test if there is any improvement in protein expression in the presence of *Pf*Hsp110c which has been proposed to be a protein-stabilizing chaperone (Muralidharan *et al.*, 2012).

6.7 Future directions for the recombinant merozoite protein library

A previously developed protein library consisting of merozoite cell surface or secreted proteins was expanded by 21 proteins. The 21 new protein members were

systematically screened for interacting partners against a protein library consisting of erythrocyte cell surface receptors. Apart from the interaction between PF13_0125 and P4HB which was not confirmed, no other interaction was detected. However, the erythrocyte receptors included in the AVEXIS screen do not cover the complete repertoire of receptors exposed on the erythrocyte cell surface. For example, multipass receptors were largely excluded due to their low likelihood for recombinant expression in a soluble form. Therefore, it is likely that several of the 21 recombinantly expressed merozoite cell surface and secreted proteins bind to erythrocyte receptors that were not included in the screen. To test this possibility, future experiments may include the raising of antibodies against the 21 merozoite proteins which can then be used for immunofluorescence and erythrocyte invasion assays. Moreover, the 21 purified merozoite proteins can be tested in erythrocyte invasion assays and also for their ability to bind human erythrocytes.

6.8 Further investigation of the putative interaction between P4HB- PF13_0125 is required

Future experiments are required to validate the interaction between P4HB and PF13_0125. Although PF13_0125 has been shown to localise apically in merozoites (Hu *et al.*, 2010), it remains a largely uncharacterised protein. It will be interesting to test whether co-transfection of PF13_0125 with *Pf*Hsp110c prevents aggregation of the former (Muralidharan *et al.*, 2012) (see section 6.5). This will enable the reliable analysis of the putative interaction between P4HB and PF13_0125 by SPR. Other future experiments may include testing the ability of PF13_0125 to bind to human erythrocytes, and the inclusion of purified PF13_0125 and P4HB in erythrocyte invasion assays. Antibodies raised against PF13_0125 together with anti-P4HB can be used for immunofluorescence and invasion assays.

6.9 *Pf*EBA-165 – a merozoite cell surface ligand with possible implications in *Plasmodium* host specificity?

Experiments described in Chapter 5 demonstrated that *Pf*EBA-165 was capable of *in vitro* binding to Neu5Gc but not to Neu5Ac containing glycans. Neu5Ac and its hydroxylated derivative Neu5Gc are the most common sialic acid forms found in mammalian cells; the former is converted to the latter by an enzyme encoded by the CMP-N-acetylneuraminic acid hydroxylase (*CMAH*) gene (Chou *et al.*, 1998).

Early studies showed that Neu5Ac but not Neu5Gc was detectable in human tissues whereas body fluids and tissue samples from all four great apes (chimpanzee, bonobo, gorilla and orangutan) contained high levels of both sialic acid forms, with Neu5Gc being predominant (Muchmore *et al.*, 1998). It was later reported that the reason for the absence of Neu5Gc in human sialoglycoproteins, was the inactivation of *CMAH* gene due to a 92-bp deletion resulting in a frameshift mutation (Chou *et al.*, 1998). The inactivation of the *CMAH* gene must have occurred sometime after the separation of human from the common ancestor with bonobo/chimpanzee (Varki, 2001; Olson and Varki, 2003; Hayakawa *et al.*, 2006) which is estimated to happened five to seven million years ago (Olson and Varki, 2003).

Around the same time (~5 million years ago), *P. falciparum* and *P. reichenowi* diverged from their common ancestor (Escalante *et al.*, 1998). In the early days, because *P. reichenowi* was morphologically indistinguishable from *P. falciparum* which was already known, it was proposed that apes might be a natural reservoir for *P. falciparum* (Martin *et al.*, 2005). Evidence that *P. reichenowi* is a separate species was provided by *in vivo* experiments which demonstrated that *P. reichenowi* is unable to infect humans, and *P. falciparum* infection cannot be easily established in chimpanzees (Varki, 2001; Martin *et al.*, 2005; Rayner *et al.*, 2011).

Sialic acid residues on erythrocytes are important for erythrocyte binding and invasion by a number of *Plasmodium falciparum* strains (Stubbs *et al.*, 2005). Therefore, the strong preference of *P. falciparum* and *P. reichenowi* for humans and chimpanzees, respectively, was suggested to be due to the differences in Neu5Ac and Neu5Gc content between the two hosts (Muchmore *et al.*, 1998; Varki, 2001; Martin *et al.*, 2005; Rayner *et al.*, 2011). Direct evidence for the role of sialic acids in *Plasmodium* host specificity was provided by Martin and colleagues (Martin *et al.*, 2005). In that study it was demonstrated that *Pf*EBA175 prefers Neu5Ac than Neu5Gc, which could explain why *P. falciparum* infection was not easily established in chimpanzees, in previous studies. Conversely *Pr*EBA-175 strongly prefers Neu5Gc, perhaps explaining the previously reported inability of *P. reichenowi* to infect human subjects (Martin *et al.*, 2005). However, recent data in our lab suggests that *Pf*EBA-175 is less discriminatory between Neu5Ac and Neu5GC than previously thought, suggesting that more studies are needed (Wanaguru *et al.*, 2013).

My observation that a corrected *Pf*EBA-165 protein binds to Neu5Gc but not to Neu5Ac containing glycans suggests that EBA-165 might also be implicated in the molecular mechanisms mediating the strong host preference exhibited by *P. falciparum* and *P. reichenowi*. It is possible that the frameshifts in *Pfeba-165* may have been essential for the adaptation of *P. falciparum* to its human host, which carry Neu5Ac but not Neu5Gc containing sialoglycoproteins. Even if they were not required for the host transition event itself, the frameshifts observed in *Pfeba-165* could have played a role towards the isolation of *P. falciparum* from *P. reichenowi*.

To establish whether *Pf*EBA-165 mutations are indeed involved in host specificity, a number of experiments are required. First, in this study only a "corrected" *Pf*EBA-165 was expressed. It is therefore necessary to recombinantly express *Pr*EBA-165 and assess its binding to the glycan panel available in our laboratory, and also to human and chimpanzee erythrocytes. If the hypothesis is correct, recombinant *Pr*EBA-165 is expected to preferentially bind to Neu5Gc containing glycans and to chimpanzee erythrocytes. An alternative experimental angle would be to test the binding of *Pf*EBA-165 to human erythrocytes derived from stem cells which have been genetically engineered to express CMAH. Ideally, downstream experiments would include the generation and phenotypic analysis of a *Preba-165* knockout mutant. Such experimental designs are hampered by the fact that no *in vitro* culture system for *P. reichenowi* has been established, nor is it likely to be, given that chimpanzees are highly protected species. Finally, it will be critical to use the population genomic data being generated at the Sanger Institute to establish whether any clinical *P. falciparum* isolates possess a non-frameshifted *Pf*EBA-165. Several of those experiments are currently underway and are led by Dr William Proto in Julian Rayner's lab.

6.10 Concluding remarks

In the current PhD thesis I described the establishment of a versatile expression system which rapidly enables the recombinant expression of engineered antibodies. By using this system a high affinity anti-BSG chimeric antibody, ch6D9, was developed as a potential anti-malarial therapeutic. Ch6D9 demonstrated high efficacy in inhibiting erythrocyte invasion *in* vitro. Furthermore, ch6D9 displayed reduced binding to FcγRIIA and C1q *in vitro*, suggesting that this antibody may have reduced ability to trigger antibody effector functions. To the best of my knowledge

this is the first attempt to challenge malaria by targeting a host derived molecule. Further characterisation of ch6D9 is currently under way.

In a parallel project I expanded an existing *P. falciparum* merozoite recombinant protein library by 26 proteins, which were chosen based on transcription microarray data and information available in the literature. Twenty one out of 26 (>80%) merozoite proteins were expressed in a recombinant form and at usable amounts. These proteins will be a useful tool for the deeper understanding of erythrocyte invasion, and *Plasmodium falciparum* merozoite biology, in general.