CHAPTER 3

Establishment of a plasmid vector system for recombinant antibody expression

3.1 Summary and Aims

To develop an anti-malarial that targets a host protein, I sought to develop an anti-Basigin therapeutic monoclonal antibody that could prevent *P. falciparum* invasion of erythrocytes by blocking the RH5-Basigin interaction. To achieve this, it was first necessary to establish a rapid and cost effective method of expressing functional monoclonal antibodies in a recombinant form within the laboratory. In this Chapter, I describe the successful adoption and establishment of a versatile plasmid system which enables recombinant expression of engineered antibodies in HEK293 cells. I use two anti-Basigin monoclonal antibodies that will be described in detail in Chapter 4 (huMEM-6/4 and huMEM-M6/8), as exemplar recombinant antibodies to show that the recombinant antibodies are successfully expressed at high levels, and retain their ability to bind their target antigens. This antibody expression system may prove useful for the rapid cloning and expression of recombinant monoclonal antibodies for functional studies outside of malaria research.

3.2 Introduction

3.2.1 The recombinant antibody technology has revolutionised the application of antibodies

Traditionally, monoclonal antibodies have been produced by immunising animals and then establishing hybridoma clones which express the antibody of the desired specificity (Köhler and Milstein, 1975). Hybridoma technology, despite providing the advantage of infinite supply of the antibody of interests, is labour intensive and inflexible in terms of antibody engineering. The discovery of recombinant DNA technologies has enabled the engineering of antibodies, and has revolutionised their application extending them beyond research and diagnostics, into the clinic for the treatment of various diseases (Peterson, 2005). Based on these technologies, elegant approaches have been devised to reduce the antibody size to its functional minimum (Holliger and Hudson, 2005), make bi-specific agents (Marvin and Zhu, 2005; Morrison, 2007; Chames and Baty, 2009), increase antibody affinity by developing and screening large antibody libraries (Hoogenboom, 2005), or modulate antibody pharmacokinetics (Vaccaro *et al.*, 2005) and effector functions (Shields *et al.*, 2001; Carter, 2006; Yan *et al.*, 2011). Indeed, recombinant monoclonal antibodies have emerged as a very successful group of biological drugs,

used either alone or in combination with other therapies (Nelson *et al.*, 2010; Rasmussen *et al.*, 2012).

The widespread application of recombinant antibody technology (and recombinant protein technology in general) has led to the development of a variety of antibody expression systems. The choice of the expression system depends on the structure of the antibody variant to be expressed, the intended use of the antibody, as well as the antibody yield derived from each system (Chadd and Chamow, 2001). For example, bacterial systems are not generally good for expressing large proteins and lack glycosylation machinery, and are therefore more suitable for the expression of smaller antibody fragments like scFvs (single chain variable fragment) and F(ab)s (Fragment antigen binding; Holliger and Hudson, 2005) which are not naturally glycosylated. On the other hand, mammalian expression systems offer the advantage of expression of soluble fully-folded proteins which carry most of the naturally-occurring post translational modifications (e.g. glycosylation of antibody Fc region), and are therefore more appropriate for expressing full-length antibody molecules (Birch and Racher, 2006).

In this Chapter, a variant of the HEK293 (Human Embryonic Kidney cells) mammalian cell line that is grown at high densities in suspension, was used for recombinant antibody expression. The HEK293 line was first established in the late 70s (Graham *et al.*, 1977), and since then, because it is readily transfected by exogenous DNA, has emerged as a widely used expression system for recombinant protein and antibody production (Meissner *et al.*, 2001; Thomas and Smart, 2005; Clarke *et al.*, 2010; Yu *et al.*, 2010).

3.3 Results

3.3.1 Design of a vector system for recombinant antibody expression in HEK293 cells

To easily engineer and recombinantly express antibodies, I adopted and established a previously used two plasmid vector system (Clarke *et al.*, 2010; Yu *et al.*, 2010), that rapidly enables rearranged antibody light and heavy chains to be expressed recombinantly in the mammalian HEK293 expression system. For this purpose, I designed two plasmids, one for the light and one for the heavy chain that

could be co-transfected (Fig. 3.1), both based on a modified pTT3-plasmid backbone (Durocher *et al.*, 2002).

PTT3 is a plasmid used for transient transfection of mammalian cells and has several features that facilitate high-level protein expression. Because pTT3 harbours the OriP origin of replication (Fig. 3.1) that binds the Epstein-Barr nuclear antigen 1 (EBNA1) protein, it is capable of replicating in HEK293E cells in which the gene encoding for *ebna1* has been stably introduced. Moreover, the transgene expression is driven by an optimised human cytomegalovirus promoter (CMV), which is particularly active in HEK293 cells, and it was described to provide expression levels reaching up to 20% of total cellular proteins (Massie *et al.*, 1998; Durocher *et al.*, 2002).

In the first vector (heavy chain encoding vector) the sequence encoding for a modified hIgG1 (ENST00000390549 carrying the mutations E233P / L234V / L235A / G236Δ / A327G / A330S / P331S / K214T / D356E / L358M) was synthesised by gene synthesis and inserted upstream of the SV40 polyA coding sequence, leaving convenient restriction enzyme cutting sites (*NotI-NheI*) for the in-frame sub-cloning of any amplified antibody variable heavy chain of interest (Fig. 3.1B). The mutations introduced into the constant heavy chain are to alter antibody effector functions and are therefore useful for therapeutic applications, as described in more detail in Chapter 4. Similarly, in the second plasmid (light chain encoding plasmid), the sequence encoding for hIgCκ (ENST00000390237) was ligated into the pTT3 backbone (Fig. 3.1B). The equivalent antibody variable region of interest can be sub-cloned between the *NotI* and *BsPEI* restriction sites.

3.3.2 Functional expression of two exemplar recombinant antibodies

The functionality of the plasmid system described above was tested by the recombinant expression of two exemplar recombinant antibodies. In this case, I am using two humanised monoclonal anti-Basigin antibodies (huMEM-M6/4 and huMEM-M6/8) that will be described in detail in Chapter 4. Briefly, plasmids encoding huMEM-M6/4 and huMEM-M6/8 were made by amplifying rearranged antibody variable regions from the anti-Basigin hybridomas MEM-M6/4 and MEM-M6/8 by RT-PCR, sequencing them, and synthesising the humanised V-regions by gene synthesis. These regions were cloned into the heavy and light chain encoding

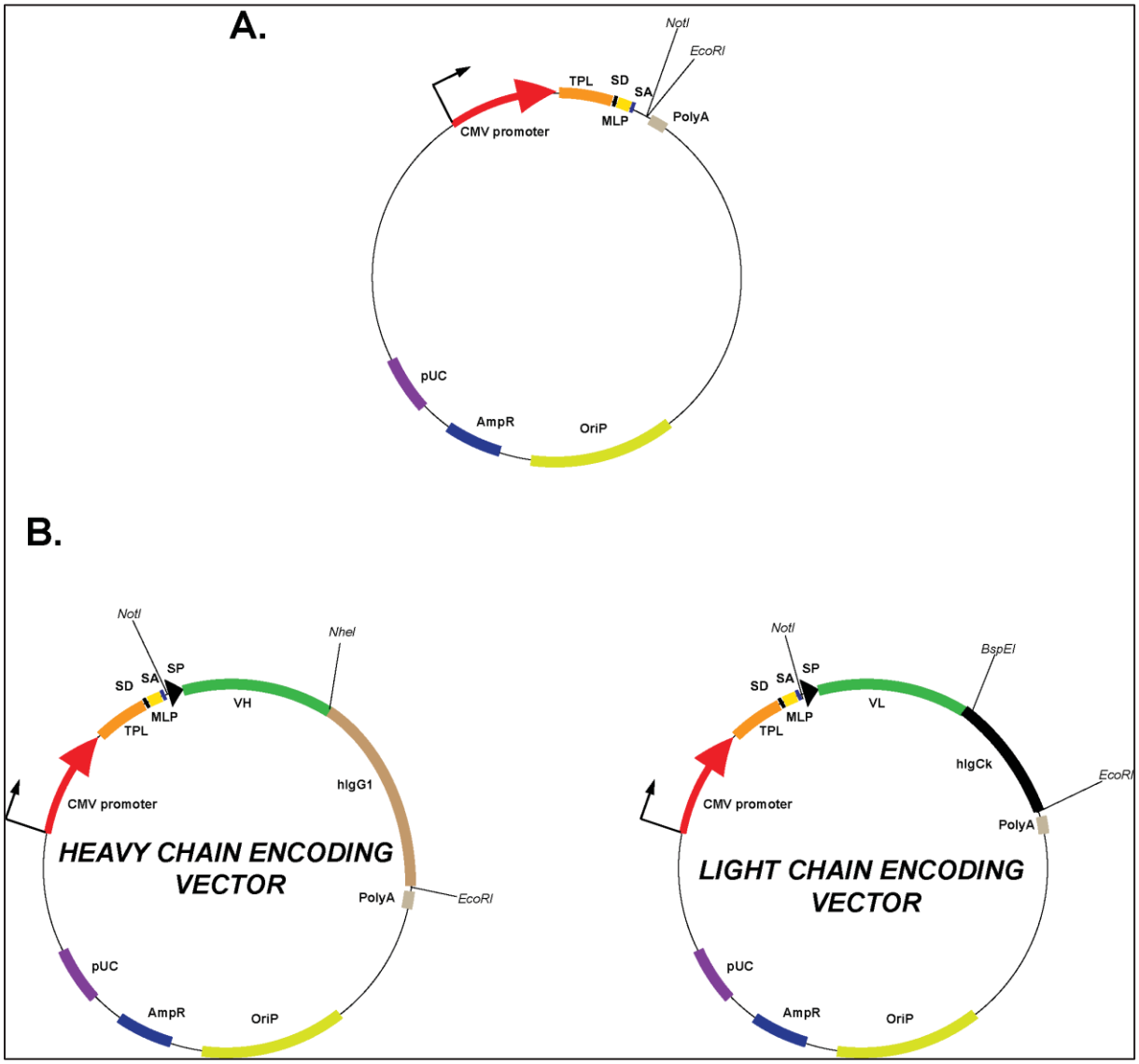

Figure 3.1 The episomal vectors which were developed for recombinant antibody expression.

Genetic map of the modified empty pTT3 vector backbone (***A.)*** and antibody heavy and light chain encoding vectors ***(B.)***. Abbreviations: VH, Variable heavy; VL, Variable Light; hIgG1, human Ig γ1 coding sequence; hIgCκ, human Ig costant κ coding sequence; CMV promoter, cytomegalovirus promoter; TPL, Tripartite Leader Sequence; SD, Splicing Donor; SA, Splicing Acceptor; MLP, Adenovirus Major Late Promoter enhancer; SP, Signal Peptide; NotI, NotI resctriction enzyme recognition site; NheI, NheI restriction enzyme recognition site; BspEI, BspEI resctriction enzyme recognition site; polyA, SV40 polyadenylation sequence; OriP, Epstein Barr virus origin of replication; AmpR, β-lactamase gene; pUC origin, bacterial origin of replication.

vectors respectively (Fig. 3.1). To test the functionality of the recombinant antibody expression system, both heavy and light chain encoding plasmids (Fig 3.3B) were co-transfected into HEK293F cells. Six days later, spent tissue culture supernatant was harvested and analysed for the presence of secreted antibody by western blot (Fig. 3.2A). Two bands at 55 and 28kDa, corresponding to antibody heavy and light chain were detected, confirming antibody expression and secretion at approximately stoichiometric amounts. Tissue culture supernatant containing the exemplar anti-basigin antibodies was then tested for their ability to bind BSG by ELISA. The entire ectodomain of the human BSG target was expressed as a soluble recombinant biotinylated protein by transient transfection of HEK293E cells (Fig. 3.2B) and quantitated by ELISA by using the anti-Cd4 antibody OX68 (Fig. 3.2C). Saturating amounts of the biotinylated BSG protein were immobilised on a streptavidin-coated microtitre plate and both exemplar antibodies, huMEM-M6/4 and huMEM-M6/8, demonstrated clear binding to Basigin relative to an anti-BSG positive control and isotype-matched negative control as determined by ELISA (Fig. 3.2D).

These data indicate that the plasmid system I established can be used for the rapid and cost effective recombinant expression of monoclonal antibodies. Antibody heavy and light chains can be readily detected in the tissue culture supernatant of transfected cells, and recombinant antibodies retain their capability of binding to the antigen they normally recognise.

3.4 Discussion

To achieve the aim of developing an anti-malarial therapeutic antibody targeting host proteins, we have selected the RH5 erythrocyte receptor Basigin as our target. Because these antibodies, if they are to be useful therapeutically, must be humanised (see Chapter 4) we need to develop a system of expressing functional recombinant antibodies. While there are several examples of similar expression systems that have been developed to produce functional recombinant antibodies (Karu *et al.*, 1995; Chadd and Chamow, 2001), we required one that could be used within the infrastructures of our available laboratory environment and, most importantly, be both rapid and cost-efficient to facilitate the testing of several different antibody sequences within a short timeframe.

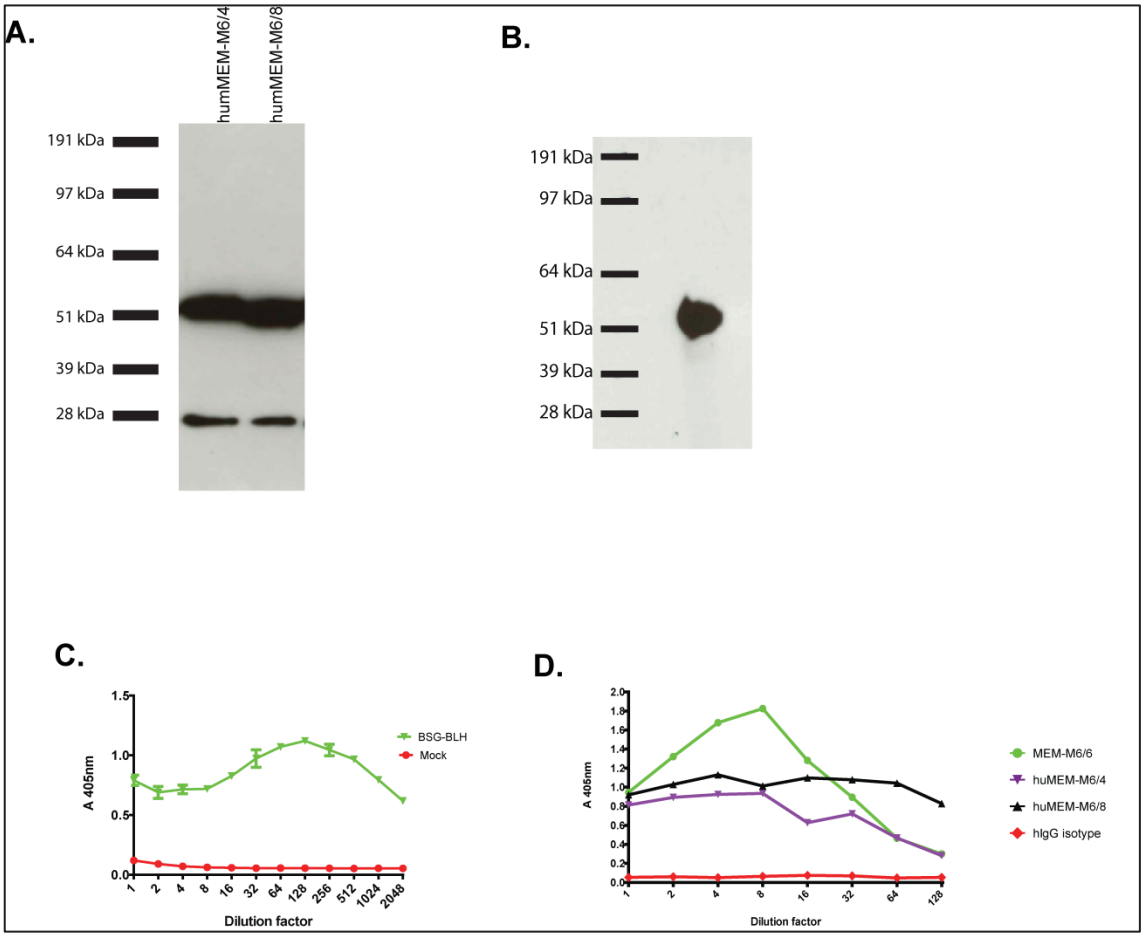

Figure 3.2 Recombinant expression and functional assessment of two exemplar recombinant monoclonal antibodies.

A. Recombinant antibody heavy and light chains were readily detected in approximately stoichiometric amounts in tissue culture supernatants, by western blot. Tissue culture supernatant containing either of the exemplar recombinant antibodies (huMEM-M6/4 or huMEM-M6/8) was analysed by denaturing SDS-PAGE, blotted and probed by using an anti-hIgG conjugated with Horseradish Peroxidase (HRP). Two bands at 55 and 28 kDa were obtained corresponding to antibody heavy and light chain, respectively.

B. BSG-BLH (Bio-Linker-His) was expressed at the correct size as determined by Western blot (a representative blot is shown). Tissue culture supernatant containing BSG-BLH was resolved under reducing conditions by SDS-PAGE, blotted, and probed using streptavidin-HRP. The expected molecular weight for BSG-BLH (including tags) was 56.3kDa.

C. Quantitation of recombinant monomeric BSG-BLH by ELISA (a representative graph is shown). Biotinylated BSG-BLH from tissue culture supernatant was serially diluted and immobilised on a streptavidin-coated plate. The wells of the plate were estimated to be saturated at a dilution of ~ 1:128. The anti-Cd4 mouse monoclonal OX68, was used as the primary antibody and an alkaline phosphatase-conjugated anti-mIgG as the secondary.

D. Basigin was bound by unpurified exemplar anti-BSG antibodies. BSG-BLH was immobilised on a streptavidin coated plate at concentrations sufficient for complete saturation of the available binding surface and incubated with a 2-fold dilution series of tissue culture supernatant containing each antibody. An alkaline phosphatase conjugated anti-hIgG was used as secondary. The anti-Basigin monoclonal MEM-M6/6 and a hIgG isotype (both at starting concentration 1µg/ml) were used as positive and negative control, respectively.

Using an expression system based on HEK293 cells grown at high density in suspension and the pTT3 plasmid (Durocher *et al.*, 2002), I designed expression vectors for both antibody heavy and light chains. A key design feature is the ability to use simple restriction enzymes to clone antibody variable regions that have been either amplified directly from hybridoma cDNA or (and which adds a great deal of flexibility) using gene synthesised fragments. This latter point enables antibody regions to be humanised by CDR grafting onto closely-matched human variable regions very rapidly. I estimate that an experienced researcher familiar with this plasmid and expression system could obtain large amounts (>30mg/L) of purified antibody within 15 days for direct cloning, and 30 days for a humanised antibody from successfully amplifying the heavy and light chain V-regions. Similarly, the cost of this system is not prohibitive, enabling a researcher to humanise an antibody for less than 400GBP, depending on the cost of gene synthesis. The expression plasmid system described herein may have various applications, like the rapid screening of antibody functions for infectious diseases (Liao *et al.*, 2011b) as well as the production of recombinant antibodies for structural studies (Liao *et al.*, 2013).

In conclusion, in this Chapter, I have described the successful development of a plasmid system which allows the recombinant expression of antibodies in a rapid and efficient manner. The functionality of this system was confirmed by the recombinant expression of two exemplar antibodies that will be described in more detail in Chapter 4, but that were both able to bind their target antigen. I envisage that this plasmid system will be a useful tool for the rapid expression of functional antibodies and will have many uses both within the laboratory itself and other laboratories that have already adopted the HEK293 expression system.