

Generation of human monoclonal antibodies: The development of methods to identify and isolate neutralising antibodies targeted to Ebolavirus from peripheral blood

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Abstract

Neutralising antibodies are an essential component in the immune response to virus infection and are often effective at preventing or attenuating viral diseases. The administration of cocktails of potently neutralising monoclonal antibodies (nAb) to abrogate viral infections has been demonstrated as an effective treatment to control disease progression caused by a diverse range of viruses. The mechanism of neutralisation is mediated predominantly by nAb binding to glycosylated envelope proteins displayed on the surface of virus particles, thereby preventing binding to host cell membrane bound receptor proteins and neutralising the entry process. Although a robust nAb response is elicited following infection with a number of viruses, many human viruses have evolved mechanisms to effectively evade the humoral immune response. The emergence or re-emergence of pathogenic human viruses poses a significant threat to the global population. The Ebolavirus epidemic of 2014-2015 arose from the emergence of a known virus in a new area, which rapidly spread through a naïve population with devastating consequences, resulting in 11,323 fatalities from a total of 28,646 confirmed cases of infection. A rapid induction of potent, broadly nAbs was a possible pathway for infection control in the 17,323 survivors of the epidemic. The isolation of potent nAbs from seroconverted individuals following antigenic exposure provides the potential to develop novel therapeutics for administration within a region affected by an epidemic. To this end, the aim of this project was to develop a platform of methods to isolate human monoclonal antibodies directly from peripheral blood through the isolation of memory B-lymphocytes and subsequent stimulation to secrete antibodies using an *in vitro* culture system. Identification

of DNA sequences encoding antibody heavy and light chain variable domains (V_H and V_L respectively), allowed for the production of recombinant antibody following the sub-cloning of V_H and V_L regions into plasmid expression vectors to provide a proof of principle of platform function. Secondly, the introduction of functional screens to the platform would provide a method to identify antibodies derived from memory B-cells with the ability to neutralise Ebolavirus pseudo-particles using *in vitro* entry inhibition assays.

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List of Abbreviations

5'RACE	5' Rapid amplification of cDNA ends
ADCC	Antibody dependant cellular cytotoxicity
ADCP	Antibody dependant cellular phagocytosis
AICDA	Activation-induced cytidine deaminase
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
BCR	B-cell receptor
BCL-6	B-cell lymphoma 6 protein
BSL	Biosafety Level
BLIMP-1	B-lymphocyte induced maturation protein 1
BDBV	Bundibugya ebolavirus
C1q	Complement component 1q
CD	Cluster of differentiation
CDC	Complement dependant cytotoxicity
cDNA	Complementary DNA
CDR	Complementarity determining region
CFR	Case fatality rate
C_H	Constant domain, heavy chain
CHO	Chinese hamster ovary cells
C_L	Constant domain, light chain
CMV	Cytomegalovirus
CpG	CpG oligodeoxynucleotides
CSR	Class switch recombination
D	Diversity gene segment, (heavy chain)

DNA PKcs	DNA-dependant protein kinase, catalytic subunit
EBOV	Zaire ebolavirus
EBV	Epstein Barr virus
EVD	Ebolavirus disease
Fab	Fragment antigen binding
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallisable
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FR	Framework region
Fv	Fragment variable
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
GP	Glycoprotein
HAT	Hypoxanthine-aminopterin-thymidine
HEK-293T	Human embryoniv kidney cells
HPS	Hantavirus pulmonary syndrome
HR	Hyper-variable region
Ig	Immunoglobulin
IRF-4	Interferon regulatory factor
ISAAC	Immunospot array assay on a chip
J_H	Joining gene segment, heavy chain
J_K	Joining gene segment, kappa chain
J_λ	Joining gene segment, lambda chain
mAb	Monoclonal antibody

MHC II	Major histocompatibility class II
mRNA	Messenger RNA
MyD88	Myeloid Differentiation Primary Response 88
NHEJ	Non-homologous end joining proteins
nAb	Neutralising monoclonal antibody
NP	Nucleoprotein
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PPMM	Phosphomanomutase
PNPP	*para*-Nitrophenyl Phosphate
RAG	Recombination activating gene
RESTV	Reston ebolavirus
RSS	Recombination signal sequences
RT-PCR	Reverse transcription polymerase chain reaction
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe acute respiratory Coronavirus
sGP	Secreted glycoprotein
SHM	Somatic hypermutation
SNV	Sin Nobre virus
ssGP	Small secreted glycoprotein
SUDV	Sudan ebolavirus

TAFV	Taï Forest ebolavirus
TCR	T-cell antigen receptors
TLR	Toll-like receptor
V_H	Variable gene segment, heavy chain
V_H	Variable domain, heavy chain
V_K	Variable gene segment, kappa chain
V_λ	Variable gene segment, lambda chain
V_L	Variable domain, light chain
XBP1	X-box binding protein

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1 General Introduction

1.1 Antibody structure and function

When a pathogen infects a vertebrate host, it is met with a number of immune responses, one of which is the antibody response. Antibodies, also referred to as immunoglobulins (Ig), are glycoproteins that are produced by B-lymphocytes when stimulated by a specific antigen. Humans express five antibody isotypes; IgA; IgD; IgE; IgG and IgM, each having different functional properties.

1.1.1 Immunoglobulin isotypes and their function

Predominantly associated with front line mucosal immunity, IgA accounts for up to 50% of the Ig isotypes produced within secretions and associated with mucosal membranes. IgD predominantly functions as a signalling receptor on the surface of B-lymphocytes although it also exists as soluble molecules within the serum, albeit at low levels (<0.5% of serum Igs). IgE accounts for an even smaller fraction of serum Igs (<0.01%) and is mainly associated with the allergic immune response. The main antibody produced during a primary immune response is the IgM isotype. IgM monomers are expressed on the surface of B-lymphocytes whilst a soluble secreted form exists in a multimeric form; usually with penta-valency. The multimeric nature of secreted IgM results in a molecule with high avidity binding to antigen, which overcomes the relative low affinity of the monomeric form (Schroeder and Cavacini, 2010).

IgG is by far the most abundant isotype, contributing around 75% of circulating antibodies (Pier et al., 2004). There are four subclasses of IgG; IgG_1, IgG_2, IgG_3 and IgG_4 with IgG_1 being the most abundant form.

1.1.2 Immunoglobulin G structure

The structure of an IgG molecule consists of four polypeptide chains; two heavy and two light chains covalently linked by disulfide bonds (Figure 1-1, panel A). Heavy and light chain anatomies are distinct, with heavy chains comprising three constant domains (C_{H1}, C_{H2}, C_{H3}) and one variable domain (V_H), whilst light chains consist of a single constant (C_L) and variable domain (V_L) (Figure 1-1, panel A). Two light chain variants exist in the human repertoire, termed kappa and lambda. The ratio of kappa to lambda variants in a healthy subject is approximately 3:1 (Leffel et al., 1997).

Each variable domain is divided into four framework regions (FR) which provide the scaffold for supporting three complementarity determining regions (CDR). The CDRs are hypervariable regions which provide the specificity of a particular IgG molecule for an antigen with high affinity. Short sequences of protein and carbohydrate molecules on the surface of the infecting organism are recognised by B-cell receptors (BCRs) (membrane bound Igs expressed on the surface of B-lymphocytes) which often produces a potent antibody response specific to the invading organism.

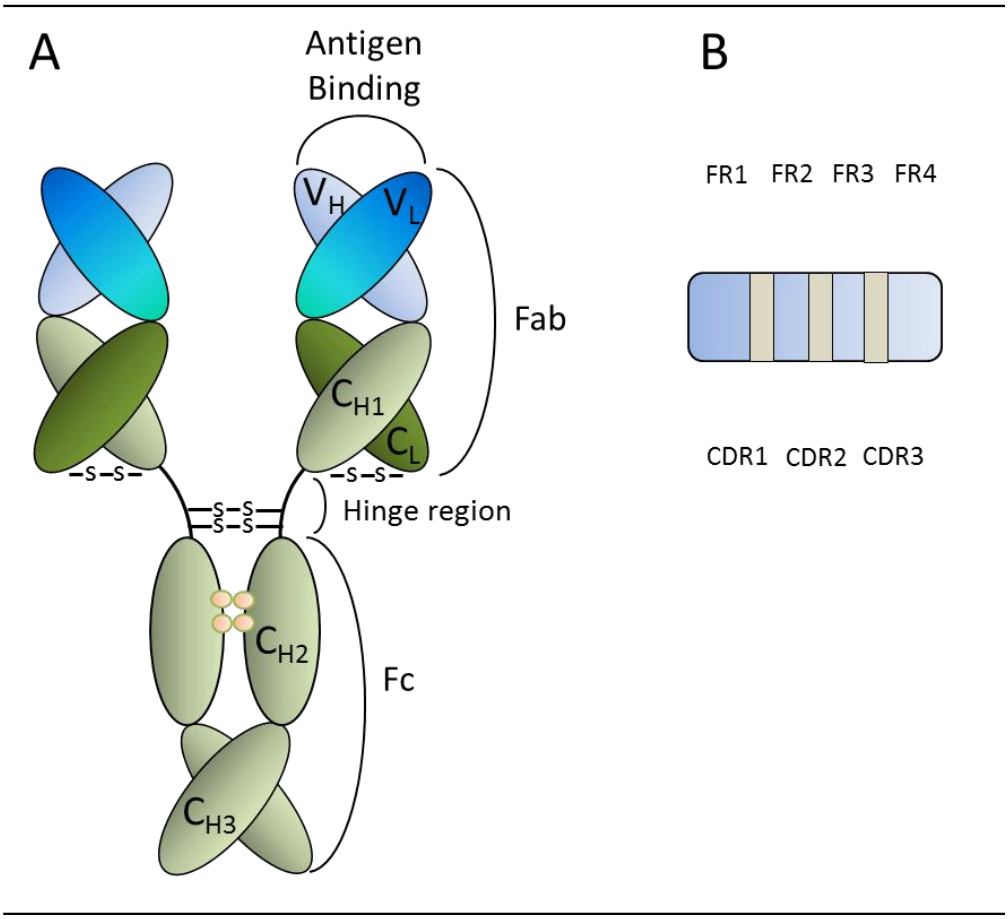


Figure 1-1| Schematic cartoon depicting IgG structure

A) The IgG molecule is composed of two identical heavy chains and two identical light chains connected by disulfide bonds. Carbohydrate moities (indicated by orange circles) link C_{H2} domains. A light chain contains one variable and one constant domain; a heavy chain contains one variable and three constant domains. Variable domains are shown in blue and constant domains shown in green. Antigen binding is mediated by the variable domains (V_H and V_L) which in conjunction with C_{H1} and C_L form the antigen binding fragment (Fab). Heavy chain constant domains (C_{H2} , C_{H3}) provide effector functions and are termed the fragment crystallisable (Fc) region. The Fc and Fab are joined by a short flexible linker which forms a hinge region. **B)** The three complementarity determining regions (CDR) are located within variable domains. Framework regions (FR) are situated adjacent to the CDRs providing the scaffold for the CDR loops.

1.1.3 Functional properties of immunoglobulins

Antibodies impart their protective effects through various mechanisms involving either their constant or variable domains. The variable domains of heavy and light chains are responsible for antigen binding and are referred to as the Fragment variable (Fv). Each variable domain comprises three CDRs (CDR1 to CDR3) and four FRs (FR1 to FR4) (Figure 1-1, panel B). The Fv in conjunction with C_{H1} and C_L domains is termed the Fragment antigen binding (Fab). The C_{H2} and C_{H3} domains provide antibody effector functions and are termed the fragment crystallisable (Fc) (Figure 1-1, panel A). The F_C region mediates cellular responses to pathogens through antibody dependant cellular cytotoxicity (ADCC), antibody dependant cellular phagocytosis (ADCP) or complement dependant cytotoxicity (CDC). The Fab and Fc regions are linked by a short, flexible region of amino acids which facilitates antigen binding by allowing slight movement of the Fab relative to the Fc region (Figure 1-1, panel A) (Eales, 2005, Schroeder and Cavacini, 2010, Klasse and Sattentau, 2002).

1.1.4 Generation of antibody diversity (primary repertoire)

Diversity in antibodies is produced during two stages of B cell development. Rearrangement of V_H diversity (D) and joining (J_H) gene segments and light chain V_L and joining (J_L) segments in pre-B cells provides the primary repertoire of immunoglobulins in a healthy individual. The heavy chain locus is located on chromosome 14q32.33 and encodes 39 V_H genes divided into seven families (Matsuda et al., 1998). Downstream of the variable genes are 27 D and six J_H genes (Matsuda et al., 1998, Schroeder and Cavacini, 2010)

.

(Figure 1-2, panel A). Constant (C_H) genes, which encode each of the Ig isotypes are located downstream of J_H genes. During developmental heavy chain recombination, *D* and J_H genes are the first to recombine, followed by the incorporation of a single V_H gene, thus forming the *VDJ* segment that comprises a complete V_H region (Tonegawa, 1983). Encoded within the V_H region are FR1, CDR1, FR2, CDR2, FR3 and the amino terminus of CDR3, whilst the *D* and J_H regions encode the remainder of CDR3 and the FR4 region. This arrangement of V_H, *D* and J_H genes provides a combinatorial diversity with an estimated 3.8 x 10^4 possible combinations of genes (Schroeder and Cavacini, 2010).

The kappa variable domain comprises of V_K and J_K genes only. The kappa chain locus is located on chromosome 2p11.2, encoding 35 functional V_K genes, divided into six families, and five J_K genes (Barbie and LeFranc, 1998) (Figure 1-2, panel B). The functional variable region (V_K) is formed through recombination of V_K and J_K segments before recombination with a single C_K gene (Tonegawa, 1983).

Similarly, the lambda variable domain is composed of V_λ and J_λ genes. Located on chromosome 22q11.2 (Kawasaki et al., 1997) the lambda chain locus encodes 30 functional V_λ genes in 11 families (Pallares et al., 1998) and four J_λ genes, each of which is associated with a single gene encoding a C_λ domain (Schroeder and Cavacini, 2010) (Figure 1-2, panel C). In contrast to kappa chain recombination, $J_\lambda - C_\lambda$ genes recombine with a V_λ gene (Tonegawa, 1983). In both light chain variants, FR1, CDR1, FR2, CDR2 and FR3 are encoded by the V_λ gene, whilst CDR3 and FR4 are encoded by the J_λ gene (Schroeder and Cavacini, 2010). Recombination of kappa and lambda

germline sequences produces a theoretical 175 and 120 gene recombinations respectively.

The recombination events described above are tightly regulated to ensure that the genes recombine in the correct order and preclude the possibility of gene segments recombining with themselves. Recombination of *D* and J_H genes followed by V_H genes is controlled by recombination signal sequences (RSS) located between the genes. These non-coding RSS sequences comprise a conserved heptamer sequence, followed by a spacer of either 12 or 23 nucleotides, and a conserved nonamer sequence. The 3' region of all V_H genes and the 5' region of all J_H genes contain an RSS, whilst *D* genes are flanked by an RSS at the 3' and 5' ends. The spacer regions within an RSS vary depending on their location; the RSS adjacent to V_H and J_H genes contain a 23 nucleotide spacer, whilst those flanking a *D* gene contain a 12 nucleotide spacer.

The RSS in kappa chain genes contain a 12 nucleotide spacer adjacent to V_K genes and a 23 nucleotide spacer adjacent to J_K genes. This arrangement is reversed in lambda chain genes. Productive rearrangements occur only between sequences flanked by a 12 nucleotide spacer with another flanked by a 23 nucleotide spacer. This mechanism therefore eliminates unproductive rearrangements between *V-V* genes or *J-J* genes (Tonegawa, 1983, Dudley et al., 2005). Two proteins, recombination activating gene (RAG)-1 and RAG-2, initiate recombination through binding to a 23-spacer RSS (RAG-1) and to a 12-spacer RSS (RAG-2) to bring DNA encoding two different gene segments into close proximity. Single stranded breaks are introduced into the DNA by the action of RAG-1 and RAG-2 to form a hairpin loop at the end of each gene

segment. Further single strand nicks are incorporated into the DNA through the action of non-homologous end joining proteins (NHEJ), Artemis and DNA-dependent protein kinase, catalytic subunit (DNA PKcs). The resulting 3' overhangs are modified by exonucleases, DNA polymersases and terminal deoxynucleotide transferases to incorporate or excise nucleotides to introduce mutations into the CDR3, producing significant variability in the length of this region. Finally, a number of NHEJ proteins repair the breaks that were introduced into the DNA to form the complete variable domain encoding sequences (Dudley et al., 2005).

1.1.5 Generation of antibody diversity (secondary repertoire)

The secondary repertoire is generated by modification of rearranged *V*(*D*)*J* sequences and the switching of heavy chain constant genes. Alteration of *V* gene sequences occurs through the process of somatic hypermutation (SHM) following exposure to antigen. The process of SHM, which introduces mutations at a rate of 10^3 changes per cell division, is mediated through the activity of activation-induced cytidine deaminase (AID) (Schroeder and Cavacini, 2010). The action of AID converts cytidine to uracil in single stranded DNA through deamination of cytidine residues. Uracil-DNA glycosylase is then able to remove the uracil to generate an abasic site which is randomly replaced by any nucleotide through the excision repair mechanism. Additional mutations, proximal to the original C-U mutation, may also be incorporated by the mismatch repair machinery. The process of SHM occurs in B-cell germinal centres within lymph nodes following exposure to antigen by follicular dendritic cells or T-helper cells and involves several rounds of

clonal expansion to select high affinity variants whilst mutant progeny with decreased affinity are removed from the cellular pool through apoptosis (McHeyzer-Williams and McHeyzer-Williams, 2005).

The germinal centres are also the location in which class switch recombination occurs (CSR). This process causes affinity matured, antigen specific variable regions to recombine with constant region genes to generate different antibody isotypes (Figure 1-2, panel A). Class switch recombination also requires the action of AID which localises to specific switch signals

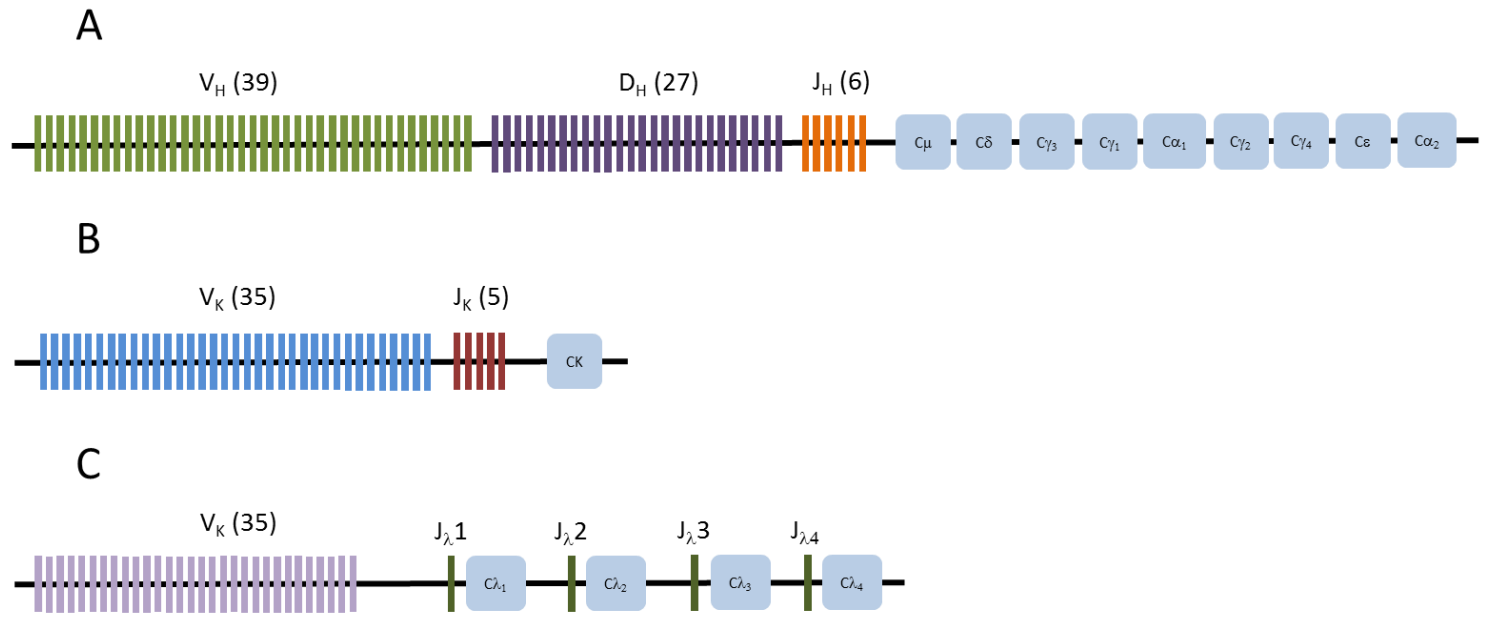


Figure 1-2| Schematic representation of immunoglobulin gene loci.

A) The heavy chain locus, located on chromosome 14q32.33, encodes V,D and J genes which combine in a random fashion to form a functional variable domain. The initial recombination event incorporates the Cm gene to produce IgM. Subsequent recombination events during B-cell maturation involves class switching of the constant domains to produce any of the different Ig isotypes. **B)** The kappa chain locus, located on chromosome 2p11.2, contains V and J genes that combine to form the functional variable domain encoding gene. A single gene encoding the constant domain is incorporated to form the functional kappa light chain. **C)** The lambda chain locus, located on chromosome 22q11.2m, contains variable genes that combine with any one J gene and associated constant gene to form the functional lambda light chain.

between constant gene regions (Dudley et al., 2005). Germinal centre maturation of the B cell response results in the generation of plasma cells which secrete high affinity antibodies in response to antigenic challenge. The process also produces long-lived, high affinity memory B-cells. IgG^+ memory B cells migrate from the germinal centres within lymph nodes throughout the body and become activated through re-exposure to antigen. Activation drives clonal expansion and differentiation into plasma cells through co-stimulatory signals derived from T-helper cells and the upregulation of cytokine signalling (McHeyzer-Williams and McHeyzer-Williams, 2005, Aiba, 2010). The result of these diversity inducing mechanisms is estimated to generate an antibody repertoire in excess of 10^{16} different immunoglobulins (Schroeder and Cavacini, 2010).

1.2 Antibodies as directly acting antiviral therapeutics

Antibody variable domains provide highly selective interactions with a particular epitope within a target viral antigen. This property has the potential to neutralize viral infections through a number of mechanisms, including the immobilization and agglutination of virus particles through antibody mediated crosslinking (Klasse and Sattentau, 2002). Neutralization can occur when a sufficient number of virus surface epitopes are bound to antibodies resulting in a broad and non-specific inhibition of receptor binding or membrane fusion interactions (Klasse and Sattentau, 2002).

Alternatively, an antibody which binds to a specific, targeted virus epitope could also lead to neutralization. This mechanism relies on a more potent antibody and requires a lower antibody titre. These binding interactions

can occur with either virus or host cell surface proteins, reviewed by Edwards et al., (Edwards et al., 2012). Antibodies that bind to a virus/host cell complex prior to membrane fusion could also lead to a potent mechanism of neutralization, however these complexes are transient in their nature.

1.2.1 Polyclonal sera containing neutralizing antibodies

Passive infusions of antibodies to protect against infection have been carried out for millions of years through their transfer in the breast milk of mammals. The concept is therefore not a recent one, however the first medical transfer occurred approximately 125 years ago by von Behring and Kitasato (Von Behring and Kitasato, 1890). Since this event, polyclonal serum taken from an immunized or recovering individual has been used as a therapeutic to either control current or prevent future infection (Casadevall et al., 2004). This method of passive immunization does not rely on the recipient's immune system to elicit a response but instead provides an immediate effect due to the presence of protective antibodies derived from the donor. Whilst polyclonal sera can be effective against viral infection, this method of therapy does have a number of disadvantages including:

1) as the sera are un-refined mixtures, there can be a large degree of batch-batch variation between preparations, and an individual administration requires a large dose of serum (Both et al., 2012);

2) due to the inherent risk associated with the use of blood products, polyclonal sera have to be carefully screened and treated before use; which is both expensive and time consuming. Even after careful preparation, it is not uncommon for a recipient to suffer from serum sickness; an immune response

to the serum resulting in a number of symptoms including, fever and localized inflammation (Karelitz, 1949);

3) most of the anti-virus antibodies present will be non-neutralizing and could potentially interfere with the action of neutralizing antibodies (Marasco, 2007).

To eliminate the disadvantages associated with polyclonal serum therapy, purified monoclonal antibodies (mAbs) offer a much more attractive therapeutic alternative. Monoclonal antibodies are derived from clones of a single B-lymphocyte and therefore recognize a unique antigenic epitope.

1.3 Monoclonal antibodies as therapeutics

As of November 2014, 47 monoclonal antibody products have approval for use in the US or Europe for therapeutic applications in humans (Ecker et al., 2015). The current rate of approval is approximately four new products every year. If this rate of approval was to continue, by 2020 approximately 70 monoclonal antibody therapeutics would be available on the global market, with a net worth of approximately $125 billion (Ecker et al., 2015). There is little doubt that mAbs are emerging as one of the most significant classes of antiviral therapeutic, however their discovery and development as a pharmaceutical is far from straightforward. The following section describes some of the current methods for identifying new therapeutic monoclonal antibodies.

A number of display platforms for identifying mAbs reactive to specific antigens have been developed. These include phage, yeast and mammalian display approaches, with phage display being the most widely used (Griffiths and Duncan, 1998, Hoogenboom, 2005). Single chain variable fragments (scFv) or Fabs are cloned into suitable vectors and displayed as fusion proteins

with a partner derived from the system in use, for example a bacteriophage coat protein. Fusion proteins are then screened against a binding partner to isolate clones of interest. Antibody genes can be derived from mRNA extracted from the B-cells of individuals harbouring a viral infection (Barbos and Burton, 1996) and cloned into a phagemid vector, in frame with the fusion protein, to generate a library of clones (O'Connell et al., 2002). Large libraries have the potential to generate a great number of antibodies to a given target with a range of binding affinities. Due to the fact that V_H and V_L sequences are cloned individually, non-naturally occurring rearrangements can be generated and isolated during screening. Subsequent rounds of selection result in the isolation of mAbs with increasing affinity for the target. The process of affinity maturation can also be mimicked using this technique by employing a variety of methods during cloning, including error-prone PCR, variable-gene mutagenesis and CDR mutagenesis (Hawkins et al., 1992, Low et al., 1996). Combinatorial display technologies therefore provide the ability to identify antibody coding sequences for cloning into an antibody expression system and provide the potential to identify and isolate broadly neutralizing antibodies against a particular virus (Johansson et al., 2007). A significant disadvantage of display technologies in the development of mAbs for treatment is that the requirement of a binding partner inhibits the discovery of novel, neutralizing antibodies with previously uncharacterized epitopes.

1.3.1 Hybridoma cell lines to generate mAb

The seminal methodology of mAb production was developed by Kohler and Millstein (Kohler and Millstein, 1975) in 1975 for which they were awarded the Nobel Prize for Medicine and Physiology in 1984. Their research utilised

B-lymphocytes derived from the spleen of an immunised mouse. The lymphocytes actively produced antibodies targeted to sheep erythrocytes but could not be sustained in cell culture. However, fusion of the lymphocytes, using Sendai virus, to a mouse myeloma cell line resulted in the production of an antibody producing immortalized cell known as a hybridoma. The significant technological advance was that hybridoma cells could be maintained easily in cell culture. Hybridoma cells were selected using Hypoxanthine-aminopterin-thymidine (HAT) medium which is permissive to the reproduction of hybridoma cells, but not to parental myeloma cells. This seminal research created the possibility to produce mAbs targeted to almost any antigen. Hybridoma technology however, has not resulted in a high success rate of drug development (Reichert et al., 2005). One of the most significant drawbacks with the technique is that when administered in humans, murine antibodies often elicit a harmful immune response (Hwang and Foote, 2005). The reason for this is because mAbs produced in mice are highly immunogenic to the recipient's immune system, leading to the production of human anti-mouse antibodies and rapid clearance of the therapeutic mAb. Further to their immunogenicity, murine mAbs are unable to generate sufficient effector functions due to their weak interactions with human complement component 1q (C1q) or with cellular IgG receptors (FcγR) expressed on effector cells (Reichert et al., 2005). Due to their inability to bind to the human salvage receptor FcRn, murine antibodies also have a very short half-life of around 20 hours within human circulation (Presta, 2003).

1.3.2 Chimerization and humanization technologies

To circumvent the problems associated with mouse hybridoma derived mAbs, humanization technologies were developed during the second half of the 1980s. The goal of these technologies was to decrease mAb immunogenicity when applied to humans, whilst retaining the binding specificity and affinity of the original, non-human antibody. This relied on rational design methods and genetic engineering to combine murine antigen binding regions with human constant domains to yield human/mouse antibody chimeras (Presta, 2003, Almagro and Fransson, 2008). The antigen binding region is composed of specific amino acid residues belonging to the V_H and V_L domains. Therefore, by engineering murine variable domains onto the relevant human constant domains, recombinant antibodies with almost 70% human sequence could be generated. Although this was an improvement over purely murine antibodies, administration of chimeras still resulted in a human anti-chimeric antibody response (Hwang and Foote, 2005). Further refinement of chimeric antibodies was achieved by reducing the mouse encoded sequence. The three CDR loops of each variable domain (V_H and V_L) provide the epitope binding region. Therefore, by replacing only the CDRs of human antibodies with murine sequences specific to a particular antigen, molecules with lower immunogenicity could be created (Jones et al., 1986, Verhoeyem et al., 1987). Technologies for CDR grafting were rapidly applied to existing mAbs to improve their efficacy (Reichmann et al., 1988, Queen et al., 1989) and are continuing to be developed and refined.

1.3.3 Transgenic mice

An alternative approach to genetically engineering human/mouse chimeric antibodies is to recover human mAbs from hybridoma B-cell lines established from transgenic mice possessing complete human antibody repertoires. The mice are generated by modification at the embryonic stem cell stage. Gene targeting technology is used to inactivate immunoglobulin heavy and light chain loci resulting in a mouse strain incapable of expressing murine immunoglobulin genes. A second strain is generated by incorporating human germline immunoglobulin genes into embryonic stem cells using yeast artificial chromosomes resulting in a strain that produces both human and mouse antibodies (Green et al., 1994). Selective breeding of the two parental strains allows for the isolation of an experimental strain which produces only human antibodies (Jakobovits, 1995). An *in vivo* humoral immune response can then be stimulated by immunising the mouse with target antigens resulting in the production of a repertoire of mature B-cells. Screening of the B-cells subsequently identifies the productive source of a mAb of interest which can then be cloned using established methods such as hybridoma technology (Lonberg, 2005).

Although a number of Food and Drug administration (FDA) approved human antibodies, for example Panitumumab and Zalutumumab (Jakobovits et al., 2007) have been identified using this technology, it is possible that they may not possess exactly the same characteristics as naturally occurring human antibodies. This is because the immune systems of transgenic mice cannot completely replicate the human immune response due to unavoidable background effects derived from a host immune response to the immunising

antigens. To completely eliminate the limitations associated with murine systems, a number of technologies have been developed to engineer mAbs derived from a purely human immune system.

1.3.4 Immortalization of human B cells using Epstein-Barr virus

Epstein Barr virus (EBV) is a lymphotrophic virus belonging to the family *Herpesviridae.* It was shown that during *in vitro* infection, B-cells were transformed by the virus resulting in the production of an immortalised cell line, capable of indefinite growth (Steinitz et al., 1977). Following screening for specific antigen reactivity, B-lymphocytes of interest are immortalised and cultured to generate a human antibody producing cell line. Monoclonal antibodies obtained using this technique are therefore fully human, affinity matured molecules comprising naturally occurring V_H and V_L pairings. Whilst this approach offers numerous advantageous, transformation efficiency by EBV is not always adequate, resulting in the immortalization of only a percentage of antibody producing cells (Tremblay and Khandjian, 2008). Techniques such as electroporation (Yu et al., 2008) and the activation of B-cells using CpG oligodeoxynucleotides (CpG) (Traggai et al., 2004) however, have significantly improved fusion efficiency.

1.3.5 Single B-cell technologies

The isolation of an individual antibody secreting lymphocyte from a human donor and subsequent identification of the antibody variable domain coding sequences, overcomes all limitations of the technologies previously discussed (Jiang, 2006, Meijer et al., 2006, Tiller et al., 2008, Tiller et al., 2009). Single B-cell technologies provide the ability to isolate functional human, affinity

matured mAbs possessing heavy and light chain combinations derived *in vivo* that may be difficult to reproduce *in vitro*. The main limitation is the requirement of a readily available access to suitable donors. The source of B-cells can be from either peripheral blood mononuclear cells (PBMC) or from lymphoid tissue, for example bone marrow biopsies. The majority of B-lymphocytes have a half-life of around 10-40 days in circulation, making them intractable for identifying B-cells with desirable specificities (Lanzavecchia, 2006). Memory B-cells however, provide a long lasting record of an individual's exposure to an antigen due to the expression of B-cell receptors containing paratopes for a particular antigen, and can therefore be utilized to identify individual antibody secreting cells with appropriate specificity. Isolation of memory B-cells can be achieved using a number of different strategies. Non-targeted techniques for isolating single B-cells in a random manner include; isolation using magnetic beads linked to B-cell selective markers (Jiang, 2006); extraction of single cells from histological sections (Kuppers et al., 1993); laser capture and micro-dissection (Obiakor et al., 2002); and fluorescence activated cell sorting (FACS) (Wardemann et al., 2003, Smith et al., 2009).

Where a particular antigen of interest is known it can be used as bait to isolate only B-cells that are reactive to that particular antigen. Techniques for antigen-targeted selection of B-cells include the use of magnetic beads coated with antigen (Lagerkvist et al., 1995); the haemolytic plaque assay (Babcook et al., 1996) and fluorochrome labelled FACS (Herzenberg et al., 2002, Scheid et al., 2009b). Targeted selection of B-cells allows the development of high-throughput technologies for isolating individual cells on a large scale. These

include, micro-engraved arrays (Love et al., 2006) and the immunospot array assay on a chip (ISAAC) technique (Jin et al., 2009). A significant limitation of targeted B-cell selection however, is that it ultimately limits any antibody produced by this method to a specific target molecule and is therefore not suited to identifying broadly reactive, neutralising antibodies.

Due to single B-cell technologies presenting significant advantages over the other antibody discovery techniques described, the procedures required will be described in more detail. Following single B-cell isolation, gene transcripts encoding IgG V_H and V_L domains are extracted and reverse transcribed into complementary DNA (cDNA). Individual cells are washed and lysed before the addition of random hexamer primers and other components required for reverse transcription (Tiller et al., 2008). The cDNA is used as a template for subsequent rounds of nested polymerase chain reactions (PCR). During the first round of PCR, a mixture of forward primers and a single reverse primer is used for the independent amplification of heavy and light chains (Wang and Stollar, 2000, Wardemann et al., 2003, Tiller et al., 2008). Forward primers are designed using consensus sequences derived from known IgG heavy and light chain variable domains, usually within the leader sequence or FR1 region. Reverse primer sequences are complimentary to the respective constant domains.

One of the largest technical challenges posed by this system is forward primer design. It is likely that primer mixtures will not amplify all functional IgG V_H and V_L genes. An alternative method of amplifying unknown 5' regions of messenger RNA (mRNA) is the 5'Rapid amplification of cDNA ends (5'RACE). This technique has the advantage of using non-specific

primers during first round PCR amplification thereby removing potential deficiencies in forward primer annealing. Template mRNA concentration from a single B-cell is too low for standard 5'RACE, however an optimised procedure has been demonstrated to amplify V_H and V_L genes from a single B-cell origin (Ozawa et al., 2006). Second round PCR is carried out to increase specificity at the 3' constant region and to introduce 5' initiation and leader sequences, if 5'RACE has not been used. The forward and reverse primers used during second round PCR can also be used to introduce restriction enzyme sites required for cloning into expression vectors.

1.3.6 Expression of recombinant antibodies

The production of linear expression cassettes using overlapping PCR of second round amplicons has been demonstrated to remove the requirement to sub-clone V_H and V_L regions into expression vectors for recombinant antibody production (Liao et al., 2009). This technique eliminates the need for restriction enzyme cloning, which is often time consuming, as the linear expression cassettes can be directly transfected. A significant limitation of linear expression cassettes however is their inability to establish stable integration into mammalian cells. Where stable integration is required therefore, the need for sub-cloning into suitable expression vectors remains.

1.3.6.1 Cell lines for monoclonal antibody expression

Post translational glycosylation is critical for the efficacy of recombinant mAbs in respect of stability, potency, potential immunogenicity and effector functions (Abès and Teillaud, 2010). Prokaryote cell based and cell free expression systems are therefore limited to the production of single chain antibodies or antibody fragments (Jiang et al., 2006, Ali et al., 2005), although

it is reported by Simmons et al., (Simmons et al., 2002), that full length, aglycosylated mAbs lacking ADCC function can be produced on a large scale in *Escherichia Coli* (*E.Coli*). Expression of mAbs in eukaryotic cells provides the ability to produce post-translationally modified proteins. Expression systems utilizing yeast and plant cells exist to produce mAbs with modified glycosylation patterns (Li et al., 2006a, Potgiieter et al., 2009, Hamorsky et al., 2013, Both et al., 2013). These systems notwithstanding, the majority of mAbs are produced using mammalian cell lines. Various mammalian cell lines are available for mAb expression to produce high yields of recombinant protein including NSo murine myeloma cells, human embryonic kidney cells (HEK-293), and Chinese hamster ovary cells (CHO) (Spens and Haggstrom, 2007, Grunberg et al., 2003). The majority of therapeutic proteins are produced using CHO cells, with around 70% of current recombinant protein therapeutics being currently expressed in these cells (Jayapal et al., 2007). A number of CHO cell lineages have been developed including DUX-B_{11}, DG_{44} and CHOK1. All of these cell lineages are permissive to transient transfection with mAb expression vectors, however not all are suitable for developing stable cells lines as they result in decreased yields of recombinant mAb (Hu et al., 2013).

1.3.6.2 Expression vector construction

Suitable mammalian expression vectors contain an expression cassette for introducing an antibody variable domain upstream of appropriate immunoglobulin constant domains. In order to drive high levels of gene transcription, strong promoter sequences are used, such as the cytomegalovirus (CMV) immediate early promoter sequence (Boshart et al., 1985). Poly-

adenylation signals are required to maximise transcript levels. Common signal sequences are the SV_{40} early or late sequence or the bovine growth hormone poly-adenylation sequence (McLean et al., 2000). Translation levels are enhanced by the inclusion of a consensus Kozak sequence immediately upstream of the initiation codon (Kozak, 1986) and secretion of the translated mAb is directed by a signal peptide sequence located immediately upstream of the mature antibody (Birch and Racher, 2006).

1.4 Application of monoclonal antibody discovery to emerging viruses

A significant global risk to the human population is the emergence or re-emergence of enveloped RNA viruses. There have been several cases over recent years of emerging viruses spreading rapidly through naïve populations causing varying degrees of casualties. Examples of RNA viruses which have caused recent episodes of disease with the potential for future outbreaks are presented in the following sections.

1.4.1 Sin Nombre virus

Sin Nombre virus (SNV) is a negative-sense single-stranded RNA virus belonging to the genus *Hantavirus* within the family of *Bunyaviredae*. Of the five genera within the family, all include arthropod borne viruses with the exception of *Hantavirus*, (being transmitted by rodents). Sin Nombre virus was first isolated in 1993 from one of its main reservoir hosts – the deer mouse *(Peromyscus maniculatus)* following an outbreak of hantavirus pulmonary syndrome (HPS) in the Four Corners region of the Western United States (Dale et al., 1998). This previously unknown hantavirus spread through a population

for a period of two months, infecting 24 individuals and killing 14 of the reported cases before the routes of transmission were broken. Although relatively few confirmed infections were reported, the most alarming feature associated with the outbreak was a case fatality rate (CFR) of 58%.

1.4.2 Severe acute respiratory syndrome Coronavirus

Severe acute respiratory virus (SARS-CoV) is a positive sense, single stranded RNA virus belonging to the genus *Betacoronavirus.* Prior to 2002, severe acute respiratory syndrome (SARS) was not a documented illness. In November of that year however, the first cases of the respiratory illness were identified in China (Drosten et al., 2003, Fouchier et al., 2003, Ksiazek et al., 2003, Lee et al., 2003). Within a few months, the coronavirus causing the disease had spread globally and by July 2003, a total of 8437 individuals had become infected in over 30 countries spanning five different continents. The virus killed almost 10% of those it infected with a recorded 813 deaths during the pandemic (Cherry, 2004). Fortunately, spread of the disease was rapidly contained and since 2004 there have been no new recorded outbreaks of SARS. This fact notwithstanding however, there is significant potential for a re-emergence of the disease. The original transmission route is likely to have been through zoonotic transfer between market place animals, specifically palm civets (*Paguma larvata),* to humans in the Guangdong region of China where cases of the disease were first reported (Li et al., 2006b). Although strong evidence of SARS-CoV infection of palm civets has been documented it is thought that the natural reservoirs of the virus are several species of horseshoe bats of the *Rhinolophus* genus (Li et al., 2006b). Mutations within the SARS-CoV envelope proteins allowed for the transmission chain to be

established from the bats, through intermediate hosts (Palm civets) into humans.

1.4.3 Lujo virus

In 2008, an isolated case of unexplained hemorrhagic fever was reported in Zambia. The patient was transferred to a health facility in South Africa resulting in a fatal nosocomial outbreak. Four individuals who had close contact with the index case also became infected. In total, four out of the five patients died from the infection. The etiologic agent of this small but devastating (CFR of 80%) outbreak was identified as a novel *Arenavirus*, subsequently named Lujo virus (Briese et al., 2009). It is unknown how the infection was established in the index case, however a likely cause was through inhalation of dust or aerosolized particles of rodent excreta that contained the virus. Although the number of cases studied to date is small, Lujo virus is highly lethal in humans. The reason for the emergence of this virus is unclear, but possible reasons include the increased contact between humans and wild animal species coupled with more advanced detection methods.

1.4.4 Zika virus

Zika virus disease is caused by a positive sense, single stranded RNA virus in the family *Flaviviridae* called Zika virus. The virus was first isolated in 1947 in the Zika forest in Uganda from captive rhesus monkeys (*Macaca mulatta*) during routine surveillance for yellow fever virus (Dick et al., 1957). In 1952 the first cases of disease in humans were reported in two regions of central Africa. During the following 60 years the virus spread through Asia and into the Pacific rim and most recently, in March 2015, in South America. This geographical distribution of the virus is due to the global range of its host

species of mosquito (*Aedes aegypti).* Symptoms of Zika virus disease are typically relatively mild, with infected individuals suffering from mild fever, rash, joint pain and conjunctivitis. However, infection during pregnancy can cause a severe birth defect called microcephaly as well as other severe foetal malformations and neurological disorders (Johannessen et al., 2016, Rasmussen et al., 2016). A link has also been made between Zika virus infection and Guillain-Barré syndrome, a rare autoimmune disorder which affects the peripheral nervous system (Cao-Lormeau et al., 2016).

1.4.5 Ebola virus

The first confirmed cases of Ebola virus disease (EVD) in humans (previously termed Ebolavirus heamorraghic fever) resulting from Ebolavirus infection, occurred in two simultaneous outbreaks in 1976. The locations of these outbreaks were Nzara, South Sudan and Yambuku in the Democratic Republic of the Congo (formally Zaire).

Ebolaviruses are enveloped, negative sense, single stranded RNA viruses belonging to the family *Filoviridae.* Along with *Ebolaviruses*, there are two other genera in the family: *Marbugvirus* and *Cuevavirus.* Since the initial discovery in 1976, a total of five species of Ebolavirus have been identified: *Zaire ebolavirus* (*EBOV*); *Sudan ebolavirus* (*SUDV*) (both species first identified in the initial 1976 outbreaks); *Reston ebolavirus* (*RESTV*) (first identified in 1989); *Taï Forest ebolavirus* (*TAFV*) (first identified in 1994) and *Bundibugya ebolavirus* (*BDBV*) (first identified in 2007).

Following the initial discovery of Ebolavirus in 1976, there have been periodic outbreaks of EVD attributable to all five Ebolavirus species. With the exception of incidents occurring within research laboratories, all outbreaks

have been centred within sub-Saharan Africa. Most of the naturally occurring outbreaks (between 1976 and 2012) affected relatively few individuals (ranging from 6 to 425). The CFRs for these outbreaks however ranged from 25% (Uganda, (*BDBV*)) to 89% (Democratic Republic of the Congo, (*EBOV*)), with a mean CFR of 60.8%. There was a paradigm shift in 2013 when an outbreak of *EBOV* in West Africa spread rapidly from the index case in Guéckédou, in the Nzékoré region of Guinea, through neighbouring Liberia and into Sierra Leone. Cases originating from the outbreak were also reported in Senegal, Mali, Nigeria, the US, Spain, Germany, France, the UK, Switzerland, the Netherlands, Norway and Italy after individuals became infected in West Africa and travelled from the region. The outbreak was declared a public health emergency of international concern by the World Health Organisation on 8 August 2014 (although the first recorded case of the outbreak occurred on 2 December 2013) and continued until 29 March 2016 when the public health emergency was declared to have ended. A small number of isolated cases persist however at the time of writing. During the outbreak, a total of 28,646 confirmed cases were reported with 11,323 confirmed deaths (CFR = 39.5%). This was an outbreak of Ebolavirus of unprecedented magnitude. Phylogenetic analysis of viral DNA sequence data suggests that the epidemic arose from a single introduction of the virus into the human population (Baize et al., 2014), likely to have arisen from zoonotic transfer from a bat reservoir species (*Hypsignathus monstrosus, Epomops franqueti,* and *Myonycteris torquata)* (Leroy et al., 2005). It is suspected that human to human transmission of the virus occurred for a period of several months before the outbreak became apparent, allowing multiple transmission

chains to become established, thereby increasing the number of cases of disease.

After an infection event, an asymptomatic incubation period of between 2 – 21 days follows before symptoms manifest. The virus is not transmittable before symptoms begin. In the majority of cases, symptoms comprised fever, vomiting and severe diarrhea. Cases of severe internal or external haemorrhage were not documented in many confirmed cases (Baize et al., 2014)

1.5 Ebolavirus structure

Viruses in the family *Filoviridae* are filamentous, with *Ebolaviruses* being approximately 970nm long. The negative sense, single stranded RNA genome is approximately 19,000 nucleotides in length and encodes seven structural proteins: nucleoprotein (NP); polymerase cofactor (VP35); matrix protein (VP40); envelope glycoprotein (GP); replicon-transcription protein (VP30); interferon inhibitor (VP24) and RNA dependant RNA polymerase (L) (Figure 1-3). Although Ebolaviruses demonstrate a range of cellular tropism, dendritic cells, monocytes, macrophages, hepatocytes and endothelial cells are thought to be the primary target cells. Infection of these cell types is important in the manifestation of haemorrhagic and immune disorders (Geisbert and Hensley, 2004). Entry into cells is a multistep process requiring the binding of host surface receptors, internalisation into endosomes and membrane fusion. The surface glycoprotein, GP_{1-2} is responsible for all of these processes. The fourth gene of the genome, GP, encodes three functionally different proteins; the 676 amino

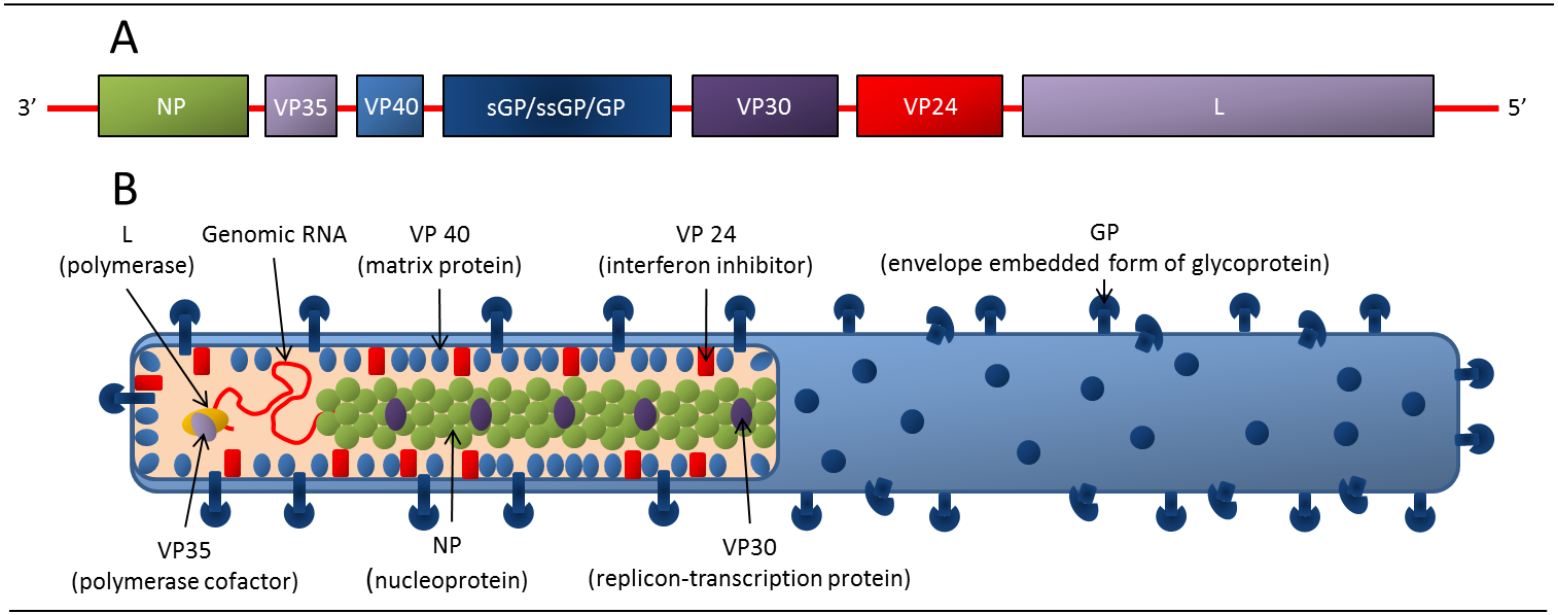


Figure 1-3| Schematic representation of an Ebolavirus genome and virion.

A) The Ebolavirus genome consists of negative sense, single stranded RNA, approximately 19 kb in length. The genome contains seven genes separated by discrete intergenic regions containing initiation and termination signals leading to the production of individual mRNA transcripts encoding seven structural proteins; nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replicon-transcription protein (VP30), replication inhibitor (VP24), RNA dependant RNA polymerase (L). **B**) The replication competent virion requires the interaction of the seven structural proteins with viral RNA and host derived membrane envelope. Membrane embedded GP mediates entry into a host cell. The most abundant protein within a virion is VP40 which provides structural integrity to the particle. VP24 plays a role in subverting the host innate immune response. The ribonucleoprotein complex consists of NP, VP35, VP30 and L and mediates transcription and replication of the viral genome.

acid, full length surface glycoprotein (GP_{1-2}); a 364 amino acid pre-secreted glycoprotein (sGP) (Sanchez et al., 1996), and a 298 amino acid small secreted protein (ssGP) (Volchkov et al., 1995, Volchkov et al., 1998). A region of seven consecutive uridine residues within the GP gene causes the RNA dependant RNA polymerase to stutter at this site during transcription resulting in the insertion of an additional adenosine nucleotide to the mRNA. The resulting frameshift within the coding region results in the production of mRNA encoding GP_{1-2} (Sanchez et al., 1996, Mehedi et al., 2011). Unedited (no slippage) transcripts lead to the expression of sGP, whilst the addition of two adenosine residues at the slippage site results in the production of ssGP. The ratio of the production of transcripts encoding the three proteins (sGP:GP_{1-2}:ssGP) has been shown to be 71:24:5 respectively in Vero E6 cells (Mehedi et al., 2011). This ratio has not been demonstrated at the protein level in either *in vitro* or *in vivo* studies however. The role of sGP in an infection is likely to be varied and include antigenic subversion, (by competing for anti-GP_{1-2} antibodies (Mohan et al., 2012) and by directing the host immune response to epitopes located on sGP but not GP_{1-2}) and by restoring endothelial cell barrier function (Wahl-Jensen et al., 2005). The role of ssGP still remains unclear (Mehedi et al., 2013).

GP_{1-2} is expressed as a 626 amino acid polyprotein before proteolytic cleavage by host cell encoded furin produces the glycoprotein subunits GP1 and GP2 (Volchkov et al., 1998). The two subunits remain covalently attached via a disulphide bond between Cys53 of GP1 and Cys609 of GP2 (Jeffers et al., 2002). The resulting GP1-GP2 heterodimer associates non-covalently with two other heterodimers into a 450kDa higher-order trimeric complex to form the

functioning virus envelope protein at the surface of nascent virions (Schornberg et al., 2006).

The roles of the surface exposed GP1 and the membrane spanning GP2 subunits in cellular entry are distinct. GP1 mediates attachment to host cell receptors through the putative receptor binding domain located within this subunit. At the C-terminus of GP1 is a highly glycosylated domain, called the mucin domain (Lee and Saphire, 2009a).

GP2 contains the machinery to drive fusion of viral and endosomal membranes in order to deliver the ribonucleocomplex into the cytoplasm of an infected cell (Lee and Saphire, 2009a).

The glycoprotein is the only viral protein found on the surface of the Ebola virion (Figure 1-3, panel B) and is therefore the main antigenic target for the host immune response. High resolution crystallographic models for the *EBOV* and *SUDV* glycoproteins have been elucidated (Lee et al., 2008, Dias et al., 2011). In these models, the three GP1 subunits form a core globular, chalice like structure (Figure 1-4, panel A). The fusion loop and hyper-variable region 1 (HR1) of GP2 fold around the outside of this core to stabilise the complex. The GP1 encoded receptor binding domain orientates at the top of the complex with critical binding residues facing upwards. Occluding this domain is a small glycosylated region called the glycan cap.

1.5.1 Ebolavirus monoclonal antibody therapeutics

A number of antibodies have been identified which bind to *EBOV* GP. KZ52 was isolated from a human survivor of a 1995 outbreak in Kikwit, Democratic Republic of the Congo (formally Zaire) (Maruyama et al., 1999). KZ52 has been shown to neutralise *EBOV using in vitro* experiments, and demonstrates

protection from lethal *EBOV* challenge in rodent models (Parren et al., 2002), but has minimal effects in non-human primates (Oswald et al., 2007). A cocktail of nAbs (2G4, 4G7 and 13C6), termed Zmapp, was formulated in 2014 in response to the West African Ebolavirus epidemic (Qiu et al., 2014). The combination was derived from two previous cocktails; MB-003 (comprising the human or human-mouse chimeric mAbs 13C6, 13F6, 6D8) (Olinger et al., 2012) and Zmab (comprising murine mAbs 1H3, 2G4 and 4G7) (Qui 2012). The murine mAbs 2G4 and 4G7 were chimerised with human constant regions to facilitate clinical acceptance and to extend the half-life following administration. In non-human primate experiments, the Zmapp cocktail provided protection against lethal challenge as late as 5 days post exposure (Qiu et al., 2014).

Epitope mapping studies have identified three major sites on the surface of EBOV GP to which the antibodies bind; the glycan cap, the mucin-like domain and the region of GP1 and GP2 subunit interaction at the base of the functional trimeric protein (Murin et al., 2014) (Figure 1-4, panel B). Only antibodies that bind to the GP1-GP2 interface have displayed neutralising phenotypes however, with non-neutralising (in the absence of complement mediated neutralisation) antibodies directed to the glycan cap and mucin-like regions (Wilson et al., 2000, Lee and Saphire, 2009b, Keck et al., 2015, Reynard and Volchkov, 2015), Figure 1-4, panels B and C). These regions of the Ebolavirus glycoprotein represent at least three sites of vulnerability on the virus surface. It is likely that other, hitherto undiscovered neutralising epitopes exist within the glycoprotein that confer higher degrees of neutralisation to monoclonal antibodies that have yet to be isolated.

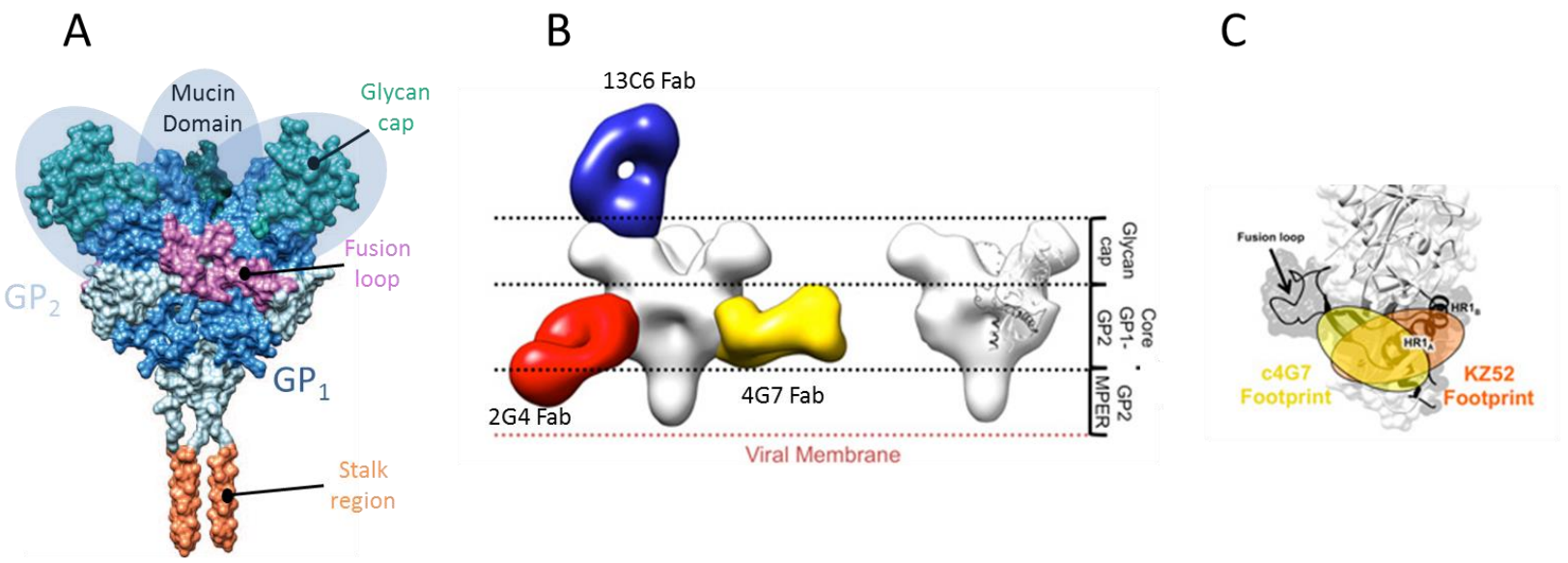


Figure 1-4| Electron microscopy surface reconstructions of the EBOV trimer in isolation and in complex with the neutralising antibodies comprising Zmapp

A) *Negative stain electron microscopy derived surface representation of the EBOV GP trimer at 18Å resolution. The mucin domain, glycan cap of GP1, GP1 core, GP2 core, the fusion loop region of GP2 and the stalk region are mapped onto the density map. The poorly defined, unstructured mucin domains are represented as spheres only.* **B)** Hybrid reconstructions of EBOV GP crystal structure in complex with single-particle negative stain EM reconstructions of Fab 2G4, 4G7 and 13C6 **C)** Comparison of EM reconstructions and crystal structures of 2G4 and KZ52 binding to the base of the EBOV trimer with overlapping footprints.
Figure A modified from Bornhodt et al. (2016) Science. Figures B and C modified from Murin et al. (2014) PNAS

1.6 Project Aims

In all of the examples given above, animal reservoirs allow the viruses causing diseases in humans to retreat, mutate and potentially re-emerge into the human population. For all of the above examples there are no effective vaccines and treatments are at early stages of development. There is little doubt that these and other viruses will re-emerge in the future with the potential to cause significant disease in humans. Due to the reservoirs in which all of these viruses reside between outbreaks, they are able to mutate into previously un-encountered strains and spread rapidly through naïve hosts.

A rapid response to a moving target is therefore required. B-cell technologies can be used to identify effective nAbs which arrest virus replication for emerging or re-emerging viruses. The identification of these antibodies can also aid vaccine design through elucidation of epitopes susceptible to neutralisation.

The aims of this project are to design a platform of techniques designed to identify and isolate nAbs directly from the peripheral blood of human donors. The platform is required to provide rapid progression from the receipt of a blood sample to the characterisation of isolated antibodies. Once established, the platform would be utilised to identify human monoclonal antibodies that bind to Ebolavirus envelope protein and neutralise infection during *in vitro* entry inhibition assays.

2 Memory B-lymphocyte Platform Development

2.1 Introduction

As described in the previous chapter, the aim of this project was to develop a platform utilising several different molecular and cellular biology methods to identify and isolate human recombinant monoclonal antibodies directly from the peripheral blood of a human donor. This chapter presents the evolution of the methods through stages of optimisation to achieve the basis of the final platform. The methods are presented in the sequence as follows: feeder cell culture; memory B-lymphocyte culture; PCR amplification of IgG variable domain sequences; sub cloning IgG variable domains into an expression vector; recombinant IgG expression and characterisation.

2.1.1 Feeder Cell Culture

Memory B-cells require the presence of secondary cells in primary culture conditions in order to be stimulated to proliferate and to secrete antibodies. The feeder cell culture is required to coat the surface of culture wells onto which memory B-lymphocytes are seeded. This is in order to provide a cellular surface on which the memory B-lymphocytes can settle and also to provide stimulatory factors to induce clonal expansion and differentiation into antibody secreting plasma cells. Feeder cells must be in a quiescent state to prevent undesired proliferation and outgrowth of the relatively slowly proliferating memory B-lymphocytes. To achieve this, feeder cells were gently irradiated using a fixed source of gamma radiation at a critical dose, high enough to prevent cell division whilst maintaining basal metabolism at a rate

sufficient to provide stimulatory factors to the memory B-lymphocytes. The type of feeder cells was paramount to the success of the cultures and a number of sources were investigated.

Initial cultures were established using macrophages derived from primary monocytes isolated from PBMC preparations taken from healthy donors. Differentiation of monocytes into macrophages was achieved using granulocyte macrophage–colony stimulating factor (GM-CSF). Differentiated macrophages were gently irradiated using a fixed source of gamma radiation prior to plating. Seeding densities required to provide a confluent monolayer of healthy, adherent macrophages on the base of culture wells were determined empirically using varying concentrations of seed stock. Primary macrophage cultures were observed over time using a light microscope to determine their stability in cell culture conditions.

Macrophage-like feeder cells were also generated from THP-1 cells. THP-1 is a human cell line derived from the peripheral blood of a patient with acute monocytic leukaemia (Tsuchiya et al., 1980). The cells are highly plastic and can be stimulated to differentiate into multiple lineages (Bruck et al., 1995, Berges et al., 2005). Characterisation studies carried out on THP-1 cells have indicated that they resemble primary monocytes and macrophages in both morphology and differentiation potential (Tsuchiya et al., 1980). The use of an immortalised cell line was adopted in order to remove the requirement to isolate primary monocytes from PBMC and to provide a more stable source of feeder cells in culture conditions. Undifferentiated cells grew steadily as suspended cultures and provided sufficient numbers for differentiation into macrophage-like cells within 3-4 days. Differentiation was achieved using

phorbol 12-myristate 13-acetate (PMA). Following differentiation, cultures were irradiated and plated and their stability observed over time using a light microscope.

Potent stimulation of B-lymphocytes to proliferate and differentiate into antibody secreting plasma cells can be partially achieved by activation of CD-40 molecules expressed on the surface of B-lymphocytes by CD-154 (also referred to as CD-40L). During the initial stages of an infection, antigen presenting cells (APCs) display polypeptides derived from the infectious agent on their surface in complex with major histocompatibility class II (MHC II) molecules. Complexes of MHC II proteins with peptide fragments are subsequently engaged by T-cell antigen receptors (TCRs) located within surface membranes of $CD4^+$ T-lymphocytes. Activation of $CD4^+$ T-lymphocytes in this way results in a cascade of secondary signalling events. One such event is the upregulation of CD-154 expression by the activated T-lymphocyte and subsequent engagement of CD-40 receptors expressed by activated B-lymphocytes. Ligation of CD-40 to CD-154 results in the recruitment of adapter proteins known as TNFR-associated factors (TRAFS) to the cytoplasmic domain of CD-40 receptors, resulting in a cascade of signalling pathways inducing cellular proliferation, differentiation, and isotype switching and somatic hypermutation of IgG encoding genes. A soluble form of CD-154, secreted from both activated T-lymphocytes and B-lymphocytes, has also been described to elicit similar responses as the transmembrane form (Graf. et al., 1995, Mazzie et al., 1995, Wykes, 2003). The effects of CD40 stimulation of B-lymphocytes during an immune response can be mimicked *in vitro* to provide the stimuli required for B-cell proliferation (Wiesner et al., 2008). An

efficient method of providing *in vitro* CD-154 stimulation to B-lymphocytes is through direct cell–cell interactions with the feeder cells. A suitable CD-154 expressing cell line, termed LL8 cells (Banchereau et al., 1994, Rancan et al., 2015) was kindly donated by Dr Andreas Moosmann. The cell line was prepared by the stable transfection of L929 murine fibroblasts with an expression plasmid encoding CD-154 using (CMV) promotor control of expression and selection with Geneticin (G418). The murine fibroblasts grew rapidly in culture and required a relatively high dose of gamma radiation to yield a non-proliferating phenotype prior to plating. Suitable seeding densities were investigated by plating out a range of cell concentrations and observing the cultures over time using a light microscope. The presence of surface expressed CD-154 was also analysed using flow cytometry.

2.1.2 Memory B-lymphocyte culture

During a primary infection, a T-lymphocyte dependent antibody response is established when naïve B-lymphocytes receive stimulatory signals through antigen binding to the B-cell receptor and via CD-40 activation from surface expressed CD-154 on $CD4^+$ T-lymphocytes. Activation of B-lymphocytes initially leads to rapid cellular expansion and the production of transient antibody secreting cells (Parker, 1993, Mills and Cambier, 2003). A more sustained response to the infection is achieved following germinal centre reactions which produce high affinity memory B-lymphocytes and a resulting population of long-lived plasma cells (Banchereau et al., 1994, Manz et al., 2005). Memory B-lymphocytes continue to circulate for up to the lifetime of an individual following antigen exposure, even in the absence of subsequent re-exposure. For example, memory B-lymphocytes specific to vaccinia virus

have been detected more than 50 years following vaccination (Crotty et al., 2003). The expression of class-switched IgG B-cell receptors defines the majority of circulating memory B-lymphocytes (Xu et al., 2014b). This pool of circulating memory B-lymphocytes therefore provides a source of cells within the peripheral blood that can be cloned *in vitro* and stimulated to produce antibodies in culture.

It is possible to purify memory B-lymphocytes from peripheral blood within a pool of PBMC. During the period of method optimisation, PBMC were isolated from healthy donors because it was not necessary to identify memory B-lymphocytes carrying a paratope specific to a particular antigen.

Flow cytometry can be used to isolate memory B-lymphocytes from a PBMC pool by utilising known antigen specificity. For example, HIV-specific memory B-cells can be isolated by specifically labelling B-Cell receptors of cognate cells with fluorescently labelled GP-140 (Scheid et al., 2009a). Whilst this method is effective at selecting cells with known antigen specificity, it has the significant disadvantage of not recognising memory B-lymphocytes activated by a previously uncharacterised antigen. One example of this would be a transient epitope only exposed during virus envelope protein/receptor binding, membrane fusion or other short-lived event during the entry of a virus into a host cell. A method with broader scope to identify memory B-cells with previously undescribed or transient epitopes is to isolate cells expressing BCRs without incorporating specific pre-selection events. To achieve this during this study, memory B-lymphocytes were discriminated from other B-cells by the expression of a generic IgG BCR only.

Memory B-lymphocytes were purified from PBMC using a magnetic beads-column based separation in a two-step method; depletion followed by enrichment. Initially, bulk PBMC were labelled with magnetic beads conjugated to antibodies raised against cluster of differentiation markers specific to all cell types present in the PBMC pool other than B-lymphocytes. Passing the differentially labelled PBMC over a column within a magnetic field retained all non-B-cells, while unlabelled B-lymphocytes were free to flow through the column to be collected. The resulting pool of un-labelled B-lymphocytes was then mixed with magnetic beads, conjugated to anti-IgG antibodies. Passing this pool over a second column within a magnetic field resulted in IgG expressing memory B-lymphocytes to be retained on the column. Removal of the magnetic field allowed the memory B-cells to be washed from the column. This provided an efficient method of purifying memory B-lymphocytes. Effective separations at each step were assessed using flow cytometry to ensure that a homogenous pool of memory B-lymphocytes resulted from the purification.

Purified memory B-lymphocytes were seeded into multi well plates coated with quiescent feeder cells. An important requirement of the platform was to identify monoclonal antibodies which bind to a target virus and neutralise virus replication. In order to achieve this, each well of the plate must be seeded with B-cells at clonal density. Seeding with multiple cells would result in the production of polyclonal antibodies, with the number of specific antibodies within the pool being dependant on the number of cells within a well that were stimulated to secrete antibodies. The requirement to culture individual cells introduced two significantly challenging problems. Firstly,

because memory B-lymphocytes were not pre-selected, the ability to introduce single cells into each well required accurate cell density measurements and dilution steps before distributing cell suspensions into the culture plates. Secondly, it is inherently more difficult to produce antibodies from cultures derived from single cells than from bulk B-cell populations. To achieve single cell distribution across culture plates, a serial dilution was carried out on a cell suspension with known concentration. The target was to provide an average of 1.3 cells per unit volume to be added to each well of the plate. Assuming a Poisson distribution, all wells in a plate would therefore be seeded with at least one cell with 30% of wells receiving two cells (Jones et al., 2013). Due to the fact that not all memory B-lymphocytes that were seeded would be successfully stimulated to produce detectable levels of antibody molecules, all memory B-lymphocyte culture plates included a number of wells that were seeded with at least a 10-fold increase in cell number to provide a positive control for antibody production.

During T-lymphocyte dependent activation of B-lymphocytes, soluble cytokines are secreted by activating T-cells which in turn stimulate B-cells to enter rounds of cell division and differentiate into antibody secreting plasma cells. In a natural infection, naïve B-lymphocytes require three stimulatory events to become activated. Two of these events are antigen specific. It is possible however for memory B-lymphocytes to become activated to proliferate and differentiate without encountering antigen specific signals.

An important early signal required for B cell proliferation is the induction of phosphoinositide 3-kinase (PI3K) activity (Donahue and Fruman, 2003). PI3K activation leads to a signalling cascade resulting in progression from a

G0 (resting phase) of the cell cycle into the DNA synthesis phase (S phase) and is therefore a crucial survival signal initiating cell proliferation. The transition into plasmablasts, and then mature plasma cells has been well described and is governed mainly by cytokines that decrease expression levels of B-cell lymphoma 6 protein (BCL-6) and increase B-lymphocyte induced maturation protein 1 (BLIMP-1; also referred to as PDRM1) (Shaffer et al., 2002, Nutt et al., 2011). BCL-6 and BLIMP-1 are mutually exclusive transcription factor regulators. BLIMP-1 downregulates BCL-6 expression which in turn increases the expression of differentiation inducing factors such as Interferon regulatory factor 4 (IRF4) and X-box binding protein 1 (XBP1) (Hodgkin et al., 1996, Tangye et al., 2002). To simulate these effects *in vitro*, a mixture of soluble cytokines and Toll-like receptor agonists were added to culture media to enhance stimulatory signals provided by the feeder cells. The signalling molecules investigated were: Interleukin (IL) 2, IL-4, IL-21 and Resiquimod (R-848).

IL-2 and IL-4 have been shown to activate PI3K and therefore drive resting memory B-lymphocytes into a proliferative phase (Tadmori et al., 1989, Brennan et al., 1997, Donahue and Fruman, 2003).

IL-21 is a pleiotropic cytokine that exerts activating signals for many different lymphocyte populations (Chan and Brink, 2012). Following T-lymphocyte priming and activation, IL-21 has been shown to induce the expression of activation-induced cytidine deaminase (AICDA), BLIMP 1 and XBP1.

R-848 exhibits potent antiviral activity. Belonging to the imidazoquinoline group of compounds, R-848 activates immune cells through

the Toll-like receptor (TLR) 7 and 8, Myeloid Differentiation Primary Response 88 (MyD88)-dependant pathway (Bishop et al., 2000, Ettinger et al., 2005).

All of these signalling molecules have been demonstrated to direct memory B-lymphocytes down a differentiation pathway resulting in the formation of antibody secreting plasma cells (Bryant et al., 2007, Ettinger et al., 2005, Avery et al., 2008). Different combinations of the signalling molecules were tested to identify the most effective method of inducing proliferation and antibody secretion. In addition to the different combinations of signals, two strategies of administration of the stimulating factors were employed. The first strategy was to introduce all stimulants during the initial establishment of the cultures to provide proliferation and differentiation signals simultaneously. The second strategy was to introduce temporal separation of the proliferation and differentiation signals by introducing proliferation factors at culture establishment and differentiation factors several days later. This was done in order to test whether uncoupling of the two phases increased antibody yields from the cultures.

In order to determine the efficiency of antibody production from memory B-lymphocyte cultures, thereby providing a method of assessing the different stimulation strategies in terms of antibody production, an assay to detect IgG within culture supernatants was required. A simple sandwich ELISA described in the literature (Metheringham et al., 2009), was adapted for use in the B-cell cultures. Incorporation of an internal IgG standard curve also provided a mechanism for semi-quantification of IgG produced from the culture wells. The assay provided a robust and reproducible method of identifying wells

within B-lymphocyte cultures that were producing sufficient amount of IgG to target for the next stage of the platform. It was not necessary to target wells producing IgG with a particular specificity during the development process, therefore the only assay required at this stage was to assess antibody production.

Although it has been reported that B-lymphocytes can be conditionally immortalised through maintenance of CD-40 receptor activation via CD-154 (Wiesner et al., 2008), the same results have not been demonstrated for populations of B-cells derived from clonal expansion of single cells within a culture. The optimal time period for maintaining culture conditions to provide maximum IgG yields was therefore determined. Cultures established using all permutations of stimulant combinations, as well as introducing temporal variations were tested for a number of days. IgG production was assessed at different time points using an IgG ELISA to estimate levels of antibody present within culture supernatants.

2.1.3 Polymerase Chain Reaction amplification of IgG variable domain sequences

The experiments described in the previous section elucidated the most productive methods for producing antibody from B-cell cultures seeded with single B-lymphocytes. Identification of antibody variable domains for heavy and light chains was then required in order to provide a reproducible source of recombinant antibodies. In order to achieve this, DNA sequences encoding antibody variable domains were identified. It was not necessary to identify constant domain sequences as only variable domains confer binding specificity to a given antigen.

At the end of the culture period, productive wells identified from the B-lymphocyte cultures using an IgG ELISA were targeted for mRNA extraction and cDNA synthesis. Total mRNA containing transcripts from many actively transcribed genes was liberated from the clonally expanded B-cell pool by lysing all of the cells within a targeted well. The mRNA was then used as a template for reverse transcription polymerase chain reaction (RT-PCR) to generate a pool of cDNA. A critical aspect of this step was to ensure that no mRNA was lost due to the inherent instability of RNA molecules. The pool of cDNA provided a template for PCR to amplify V_H and V_L region encoding sequences from the complex mixture of products.

In order to elucidate specific DNA sequences encoding the variable domains, gene specific primers were required to anneal into regions flanking the variable region. In principle this was straightforward, however the choice of primer sequence was critically important. Using this technique, one primer is required to anneal to the 5' end of the gene. This primer must have a sequence complementary to either the leader sequence or to conserved regions within FR1. Due to the leader and FR1 regions differing in sequence between immunoglobulin families, mixtures of primers, some of which containing degenerate positions, were required. The sequence of the second primer which annealed to the 3' end of the gene presented less difficulty in design as this primer can anneal to a highly conserved C_H or C_L region. Separate reaction mixtures, and therefore primer mixtures, were assembled for each of the three possible variable domains; heavy, kappa or lambda and PCR carried out separately for each chain.

Following amplification, PCR products were visualised using agarose gel electrophoresis, thereby providing a method of determining whether mRNA isolation, cDNA synthesis and IgG variable domain specific PCR steps were successful for heavy and light chains from each well that was interrogated. PCR mixtures yielding positive bands could be analysed using DNA sequencing and stored at -20°C before advancing to the subsequent step of the platform.

The time point at which to harvest mRNA from the B-cell cultures was critical to determine. Harvesting too early reduced the number of clonally expanded cells and therefore IgG variable domain encoding transcripts present. Harvesting too late limited the number of viable cells present as they began to enter apoptosis and necrosis, again limiting transcript numbers. Determination of the time point at which IgG concentration was at its highest was not a useful indicator for mRNA harvesting. This was because the half-life of IgG in culture conditions is typically around 30 days whilst the plasma cells secreting antibody were likely to have expired well before this time. In order to identify the optimum time point, mRNA was harvested on alternate days throughout a culture period and PCR products (for lambda chains) were identified using agarose gel electrophoresis.

2.1.4 Sub-cloning IgG variable domains into an expression vector

Purified and sequence verified PCR products derived from V_H and V_L specific PCR must be cloned into an expression vector in order to be reproduced as recombinant antibody molecules. The choice of expression vector was very important. A suitable expression vector must contain C_H and C_L domains due

to the isolation of only the variable domains from the B-lymphocyte cultures. Cloning the variable domains in frame with corresponding constant domains reproduced a full length coding sequence. The vector must also contain a suitable promotor sequence and antibiotic resistance gene for both mammalian cell expression and bacterial cell plasmid propagation.

Suitable expression vectors for IgG1 heavy (pORIgHIB huIgG1) and IgG1 light (pORIgLIB huIgG1) chains (Figure 2-1) were kindly provided by Professor Lindy Durant (Metheringham et al., 2009) and were used to clone all variable domains into.

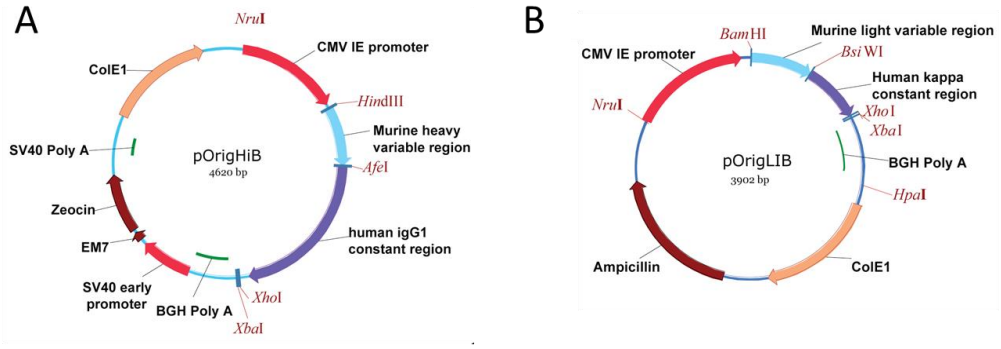


Figure 2-1| Plasmid map of the IgG1 expression vectors used for recombinant antibody expression (Scancell Ltd., Nottingham, UK)

A) Plasmid map of the pORIgHIB huIgG1 expression vector. The human IgG1 constant region is shown in dark blue and is encoded immediately downstream and in frame with a murine heavy variable region, shown in light blue. The murine variable domain is excised during linearization of the vector with *HindIII* and *AfeI* restriction enzymes prior to homologous recombination cloning of a human variable domain. Antibiotic resistance is provided by the *Sh ble* gene regulated by an SV40 early promoter. **B)** Plasmid map of the pORIgLIB huIgG1 expression vector. The human kappa constant region (dark blue) is located downstream and in frame with a murine variable domain (light blue). The variable domain is removed using *BamHI* and *AfeI* restriction enzymes in preparation for homologous recombination with a human variable domain.
Both expression vectors utilise a cytomegalovirus immediate early promotor to control expression of IgG variable and constant region genes. A BGH polyadenylation (poly A) signal ensures that transcripts are processed correctly. The ColE1 coding region enables replication of both plasmids in *E.Coli*.
Figure adapted from Metheringham et al., (2009).

In order for antibody molecules to be secreted from a producing cell, a signal peptide/leader sequence was required at the 5' end of the gene to direct nascent protein to the secretory system for export out of the cell. Due to the 5' primers annealing downstream of the translation initiation site, a suitable sequence was required to contain a translation initiation signal (Kozak, 1986) and signal peptide, incorporating a signal peptidase cleavage signal upstream of the FR1 region of the variable domain. DNA sequences encoding a translation initiation and signal peptide sequence for V_H and V_L domains were designed *in silico* from similar sequences taken from the literature (Klatt and Konthur, 2012). A standard sequence was designed for each of the two vectors to be used for any variable domains that were cloned. Translation of the sequences using the ExPASy translate tool, (Swiss Institute of Bioinformatics, Switzerland) provided primary structures that were interrogated for signal peptide cleavage using SignalIP 4.0 software (Petersen et al., 2011). The outputs from the SignalIP 4.0 server, showing the amino acid sequences and cleavage sites for each chain are given in Figure 2-2. The DNA sequences were then incorporated into the second round PCR forward primers in order to place the translation initiation/signal peptide sequence in frame with FR1 of isolated V_H and V_L domains.

Although the two pORIg vectors contained restriction enzyme sites located at suitable positions to allow the cloning of variable domain sequences into the expression vector, an alternative cloning strategy based on homologous recombination of complementary DNA sequence was employed using In-fusion HD cloning (Takara Bio, California, USA). The strategy was based on a 15bp sequence, homologous to the appropriate region of the vector, being

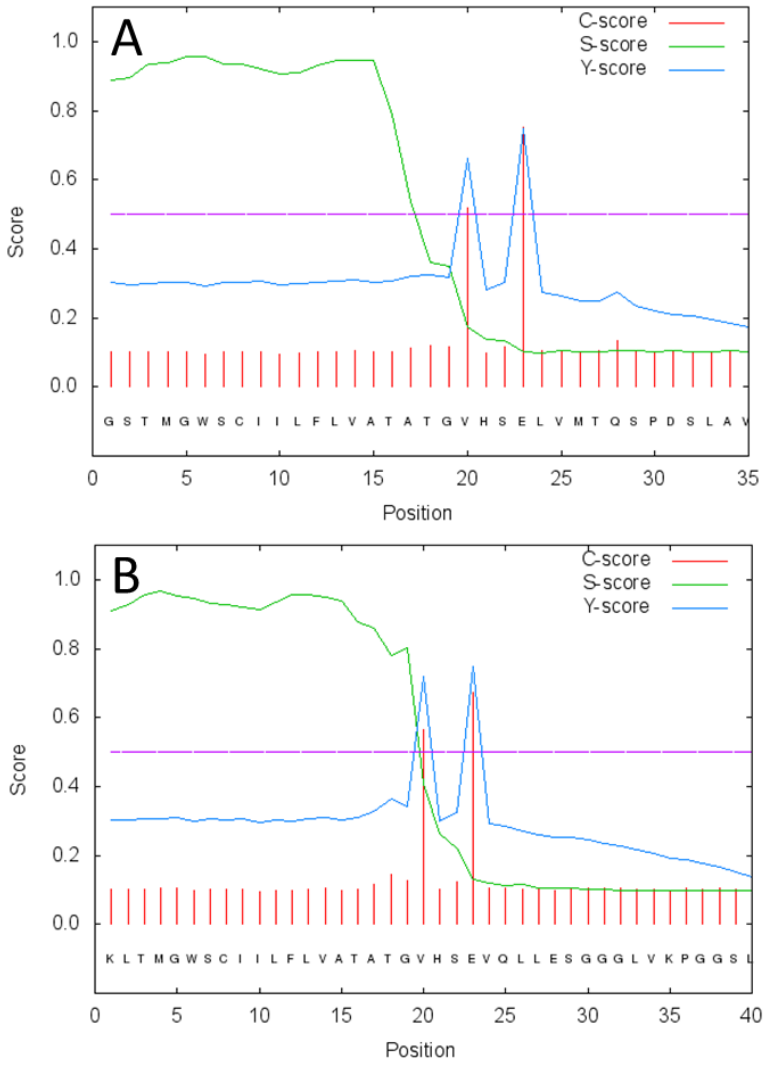


Figure 2-2| Identification of signal peptide cleavage sequences incorporated into all VH and VL regions.

Output from SignalIP 4.0 analysis identifying signal peptide cleavage sites within the translation initiation/signal peptide cleavage sequences that were inserted into variable domains isolated from B-cell cultures. The C-score distinguishes signal peptide cleavage sites. The S-score discriminates between sequences within signal peptides and those within mature proteins. The Y-score provides a geometric average of the C-score and the slope of the S-score.

The primary cleavage site was identified between residues at positions 20 and 21 (Val-His). A secondary cleavage site was incorporated between residues 23 and 24 (Glu-Leu for the V_H; Glu-Val for the V_L). The position of translation initiation is shown at position 4.

A) Heavy chain sequence. **B)** Light chain sequence.

inserted into variable domain sequences at the 5' and 3' ends. This sequence was introduced during PCR on specifically designed second round primers. Purified PCR products were then incubated with linearised vector and a proprietary mixture of enzymes used to introduce the cloned variable domains into the vector in frame with the relevant constant domain.

To validate the methods described above for cloning IgG variable domains into the expression vectors, the sequences of monoclonal antibodies taken from the literature were sub cloned. The antibodies chosen were KZ52, 2G4, 4G7 and 13C6 (Maruyama et al., 1999, Qiu et al., 2011, Qiu et al., 2014, Olinger et al., 2012). As described in Chapter 1, these monoclonal antibodies have been shown to neutralise Ebola infection *in vitro* and will therefore also provide positive control antibodies for Ebola specific experiments described in the next chapter.

2.1.5 Recombinant antibody expression and characterisation for binding interactions with immobilised EBOV GP

Post translational glycosylation is critical for the efficacy of recombinant mAbs, in respect of stability, potency, potential immunogenicity and effector functions (Abès and Teillaud, 2010). Prokaryote cell based and cell free expression systems are therefore limited to the production of single chain antibodies or antibody fragments (Jiang et al., 2006, Ali et al., 2005). Expression of monoclonal antibodies in eukaryotic cells provides the ability to produce post-translationally modified proteins. Expression systems utilizing yeast and plant cells exist to produce mAbs with modified glycosylation patterns (Li et al., 2006a, Potgiieter et al., 2009, Hamorsky et al., 2013, Both et

al., 2013, Grunberg et al., 2003, Jayapal et al., 2007). These systems notwithstanding, the majority of monoclonal antibodies are produced using mammalian cell lines. Various suitable cell lines are available to produce high yields of recombinant antibody, including NSo murine myeloma cells, HEK-293, and CHO cells (Grunberg et al., 2003, Spens and Haggstrom, 2007).

The cells chosen for antibody expression was the cell line HEK-293T. These cells express the SV40 large T-antigen (DuBridge et al., 1987) allowing producer cells to support episomal replication of pORIg vectors which contain an SV40 origin of replication. This episomal plasmid replication therefore extends the expression of IgG following transient transfections. To accelerate the process of producing sufficient IgG for characterisation, transient transfections of the HEK-293T cells were adopted. This method removed the requirement for generating clonal cell lines thereby dramatically decreasing culture periods for protein expression to 48 hours. Yields from transient transfections were variable, however the majority of expression cultures produced in the region of 500 – 1000ng of IgG per mL. The efficiency of recombinant IgG expression in HEK-293T cells following transient transfections with IgG expression vectors was assessed using the IgG ELISA described in Section 2.1.2.

A sandwich ELISA based binding assay was used to identify potential interactions between recombinant IgG molecules and immobilised Ebolavirus glycoprotein. The assay incorporated a wheat germ agglutinin, which was coated onto the wells of the assay plate and has previously been shown to capture Ebolavirus glycoprotein (Maruyama et al., 1999). The source of envelope protein was lysed HEK-293T cells that were used to produce

Ebolavirus pseudo-particles as described in Section 3.2.4. During particle production, excess glycoprotein not incorporated into pseudo-particles become embedded within the cell surface membrane and can be utilised following lysis of the producer HEK-293T cells.

2.2 Materials and Methods

2.2.1 Feeder cell cultures

2.2.1.1 PBMC Isolation from peripheral blood

Approximately 30mL of whole blood was diluted with pre-warmed (37°C) RPMI 1640 (Gibco, Paisley, UK) at a blood:media ratio of 2:1. Diluted blood was layered gently onto 10mL of pre-warmed Histopaque®-1077 (Sigma Aldrich, Gillingham, UK) in a 50mL centrifuge tube. The tube was centrifuged at 800 x g for 22 minutes at the lowest rates of acceleration and deceleration in order to separate the blood/Histopaque® mixture into four layers; (i) a lower layer containing red blood cells, (ii) a layer of Histopaque®-1077, (iii) a narrow white layer containing PBMC, (iv) an upper layer of plasma (Figure 2-3). PBMCs were aspirated carefully using a 2mL Pasteur pipette without disturbing the Histopaque®-1077 and plasma layers. PBMC were washed by transferring the cells into a 50mL centrifuge tube and topped up to 50mL with RMPI 1640 and centrifuged at 300 x g for 10 minutes. The supernatant was removed and resuspended in 5mL of pre-warmed RMPI 1640 supplemented with 10% foetal bovine serum (Gibco, Paisley, UK).

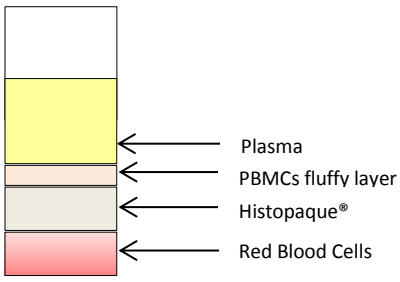


Figure 2-3| Schematic representation of the separation of a whole blood sample into plasma, PBMC and red blood cells following centrifugation over Histopaque®.

A cell count was carried out using a 10μL aliquot of the PBMC preparation using a haemocytometer. The total PBMC preparation was topped up to 30mL of RPMI 1640/FBS and washed by centrifugation at 300 x g for 10 minutes.

2.2.1.2 Differentiation of primary monocytes into macrophages

PBMC were transferred into a Corning T75 culture flask (Fisher Scientific, Loughborough, UK) containing 20mL RPMI 1640/FBS supplemented with 50ng/mL GM-CSF (R&D Systems, Abingdon, UK). The flask was incubated for 5-7 days at 37°C with 5% CO_2 in static cultures to allow macrophages to settle onto the base of the culture flask. Supernatant was removed and adherent cells were washed once using cold PBS (Gibco, Paisley, UK). Macrophages were removed from the plastic by the addition of 5mL pre-warmed cell dissociation buffer and incubated at 37°C for 5-10 minutes. Macrophages were removed from the flask with gentle tapping and a 10μL aliquot removed for counting using a haemocytometer. The remaining cells were washed in 30mL pre-warmed RPMI 1640/FBS by centrifugation at 300 x g for 5 minutes. The cell pellet was resuspended in 5mL pre-warmed RPMI 1640/FBS and transferred into a sterile polystyrene tube wrapped in Parafilm M (Alpha Laboratories, Eastleigh, UK). Macrophage cells were irradiated (12Gy) using a caesium-137 sealed source of gamma radiation and plated into 96 well culture plates (BD Biosciences, Oxford, UK).

2.2.1.3 Production of macrophage feeder culture from THP-1 cells

Cryopreserved THP-1 cells (Tsuchiya et al., 1980) were partially thawed in a 37°C water bath, before the addition of 1mL of pre warmed RPMI 1640/FBS medium. Thawed cells were transferred into a Corning T25 flask (Fisher

Scientific, Loughborough, UK) containing 5mL of RPMI 1640/FBS medium and incubated overnight at 37°C with 5% CO_2. Recovered cells were pelleted at 300 x g for 10 minutes before transferring the cell pellet into a Corning T75 flask containing 19mL of RPMI 1640/FBS medium supplemented with 2 mM L-glutamine (Sigma Aldrich, Gillingham, UK) and incubating for 5-7 days until the cell density reached between $5x10^5$ – $5\ x10^6$ cells/mL. At this cell density, the THP-1 cells were differentiated into macrophage like cells by the addition of 100ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, Gillingham, UK) to the culture medium and incubated at 37°C with 5% CO_2 for up to 7 days. Culture supernatant was removed and adherent macrophages washed with 10mL of cold PBS before the addition of 5mL of pre-warmed cell dissociation buffer. The cells were incubated at 37°C for 5-10 minutes and removed from the plastic with gentle tapping.

2.2.1.4 Establishment of feeder culture cells using the mouse fibroblast cell line, LL8

LL8 fibroblasts (received as a kind donation from Dr Andreas Moosmann) were resuscitated using the procedure described in Section 2.2.1.3. Thawed cells were incubated overnight at 37°C with 5% CO_2 in a Corning T25 flask containing 5mL of RPMI 1640/FBS medium. Recovered cells were pelleted at 300 x g for 10 minutes before transferring the cell pellet into a Corning T75 flask containing 19mL of RPMI 1640/FBS medium supplemented with 500μg/mL G418 antibiotic (Gibco, Paisley, UK) to select for CD-154 producing cells. LL8 cells were transferred into a Corning T225 culture flask (Fisher Scientific, Loughborough, UK) containing 50mL RPMI 1640/FBS medium supplemented with 250μg/mL G418 antibiotic and incubated at 37°C

with 5% CO_2 until reaching a cell density of approximately $3x10^7$ cells/mL. Culture supernatant was removed and adherent LL8 cells washed with 10mL of cold PBS before the addition of 5mL of pre-warmed trypsin-EDTA (0.25) (Gibco, Paisley, UK). The cells were incubated at 37°C for 5-10 minutes and removed from the plastic with gentle tapping and a 10μL aliquot removed for counting using a haemocytometer. The remaining cells were washed in 30mL pre-warmed RPMI 1640/FBS by centrifugation at 300 x g for 5 minutes. The cell pellet was resuspended in 5mL pre-warmed RPMI 1640/FBS and transferred into a sterile polystyrene tube wrapped in Parafilm M. LL8 cells were irradiated (180Gy) using a caesium-137 static source and plated into 96 well culture plates.

2.2.1.5 Flow cytometry analysis of LL8 cells for the surface expression of CD-154 molecules

LL8 cells were counted and $5x10^5$ cells resuspended in 1mL of PBA buffer (96.2% phosphate buffered saline, 3.3% bovine serum albumin (Sigma Aldrich, Gillingham, UK), 0.5% sodium azide (Sigma Aldrich, Gillingham, UK)) and transferred into 12mm x 75mm sterile culture tubes with lids (Elkay, Basingstoke, UK). The cells were washed using centrifugation at 300 x g for 5 minutes before staining with 5μL of either; α-CD154(IgG1) monoclonal antibody conjugated to phycoerythrin (PE) (Beckman-Coulter, High Wycombe, UK) or an IgG1 isotype control antibody conjugated to fluorescein isothiocyanate (FITC) (Beckman-Coulter, High Wycombe, UK). The stained cells were incubated at room temperature for 30 minutes. PBA buffer was added to the cells before washing at 300 x g for 5 minutes. The supernatant was carefully discarded before fixing the cells in 500μL of 0.5% formaldehyde

solution (ThermoFisher Scientific, Loughborough, UK). The stained cells were analysed using a Beckman Coulter FC500 Flow Cytometer (Beckman Coulter, High Wycombe, UK). Results were analysed using WEASEL v3.0 for Windows (Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) and presented as two-dimensional dot plot. Unstained LL8 cells were included in the experiment to provide baseline measurements.

2.2.2 Memory B-lymphocyte culture

2.2.2.1 Purification of IgG^+ memory B-lymphocytes from PBMC

IgG^+ Memory B-cells were isolated from PBMC using a MACS IgG^+ Memory B-cell isolation kit (Miltenyi Biotech, Bisley, UK) according to the manufacturer's instructions. PBMCs were resuspended in 400μL of MACS buffer (PBS, pH 7.2; 0.5% BSA; 2mM EDTA) plus 100μL of MACS Memory B-cell Biotin-Antibody Cocktail microbead suspension containing antibodies to CD2, CD14, CD16, CD36, CD43 and CD235a per 10^8 PBMCs. The sample was gently homogenised and incubated at 4°C for 10 minutes. Following incubation, 300μL of MACS buffer plus 200μL of MACS Anti-biotin microbead suspension were added per 10^8 PBMCs. The sample was gently homogenised and incubated at 4°C for 15 minutes. Cells were washed by the addition of 10mL cold MACS buffer and centrifuging at 300 x g for 10 minutes. Supernatant was aspirated completely and the cell pellet resuspended in 1mL MACS buffer.

An LD column was prepared by inserting it into a MACS separator and washing with 2mL cold MACS buffer. The PBMC/microbead suspension was added to the column and washed with 2 x 1mL cold MACS buffer. The total B-cell fraction was washed off the column with the flow through and captured

in a 50mL tube. The total B-cell fraction was mixed with 7mL of cold MACS buffer and centrifuged at 300 x g for 10 minutes to pellet the cells. Supernatant was aspirated completely and the cell pellet resuspended in 50µL cold MACS buffer to which 50µL MACS Anti-IgG microbeads were added per 10^8 starting PBMCs. The sample was gently homogenised before incubating at 4°C for 15 minutes. Cells were washed with 10mL cold MACS buffer with centrifugation at 300 x g for 10 minutes. Supernatant was aspirated completely and the pellet resuspended in 500µL cold MACS buffer.

An MS column was prepared by inserting it into a MACS separator and washing with 500µL cold MACS buffer. The cell suspension was added to the column and washed with 3 x 500µL cold MACS buffer to remove IgG^- B-cells. The column was removed from the separator and placed over a sterile collection tube. IgG^+ Memory B-cells were displaced from the column using 1mL of cold MACS buffer. A 10µL aliquot was removed for counting using a haemocytometer and the remaining cells centrifuged at 300 x g for 10 minutes before resuspending at an appropriate concentration for seeding multi-well culture plates.

2.2.2.2 Flow cytometry analysis of IgG^+ memory B-cell purification from a PBMC pool

Peripheral blood mononuclear cells were isolated from a whole blood sample using the method described in Section 2.2.1.1 and an IgG^+ memory B-cell purification was carried out as described in Section 2.2.2.1. Four types of cellular pools were tested at different stages during the purification process to analyse the cell types present. The four pools of cells tested were; i) PBMC, ii) B-cell depleted PBMCs, iii) Total B-cells, iv) IgG^+ B-cells. Cells were

prepared for flow cytometry analysis as described in Section 2.2.1.4. To identify populations of B-lymphocytes within the pools, cells were stained with 5μL of an α-CD20 (IgG2a) monoclonal antibody conjugated to phycoerythrin (PE) (Beckman-Coulter, High Wycombe, UK) and 5μL of an α-IgG (IgG1) monoclonal antibody conjugated to PE-Cy5. Anti-IgG1 and α-IgG2a isotype control antibodies conjugated to fluorescein isothiocyanate (FITC) (Beckman-Coulter, High Wycombe, UK) were included in the experiment. A Beckman Coulter FC500 Flow Cytometer (Beckman Coulter, High Wycombe, UK) was used to analyse the labelled cells after gating the PBMC population and the results analysed using WEASEL v3.0 for Windows (Walter and Eliza Hall Institute of Medical Research, Parkville, Australia). Data were presented as two-dimensional dot plot. A population of unstained cells were analysed for each pool in order to provide baseline measurements.

2.2.2.3 *In vitro* culturing of IgG^+ memory B-cells for antibody production

IgG^+ memory B-cells were seeded onto irradiated LL8 cells in 96 well culture plates at a density of 1.3, 13, 80 or 130 cells per well in 100μL of RPMI 1640/FBS. A further 100μL of RPMI 1640/FBS containing various combinations of; 665ng/mL IL-2 (R&D Systems, Abingdon, UK); 25ng/mL IL-4 (R&D Systems, Abingdon, UK); 100ng/mL IL-21 (R&D Systems, Abingdon, UK); 2500ng/mL R848 (Sigma Aldrich, Gillingham, UK) were added to each well. The cultures were incubated at 37°C with 5% CO_2 for up to 16 days.

2.2.2.4 Screening cell culture supernatants for antibody production

A Nunc, maxisorp-96 well plate (Fisher Scientific, Loughborough, UK) was coated with 50μL of coating antibody (goat α-human IgG-Fc) (Sigma Aldrich, Gillingham, UK) at 230X dilution in PBS and incubated overnight at 4°C in a humidity chamber. Following equilibration to room temperature, the plate was washed 3 x using PBS/5% Tween-20 (Sigma Aldrich, Gillingham, UK). Non-specific binding sites were blocked with 200μL of 1% fish skin gelatin (Sigma Aldrich, Gillingham, UK) dissolved in PBS/5% Tween-20 and incubated at room temperature for 60 minutes. The plate was washed 3 x using PBS/5% Tween-20 before adding 50μL of B-cell culture supernatant to corresponding wells of the assay plate. The plate was incubated at room temperature for 60 minutes. Wells were washed 3 x using PBS/5% Tween-20. Bound primary antibody from culture supernatants was detected by the addition of secondary antibody (goat α-human IgG–conjugated to alkaline phosphatase) (Sigma Aldrich, Gillingham, UK) at a 2000X dilution in PBS/5% Tween-20 to each well in 50μL aliquots. The plate was incubated at room temperature for 60 minutes before washing 3 x using PBS/5% Tween-20. Bound secondary antibody was identified by the addition of 50μL *para*-Nitrophenyl Phosphate (pNPP) (Sigma Aldrich, Gillingham, UK). Plates were incubated in the dark at room temperature for approximately 30 minutes until a colour change was observed for positive reactions. The absorbance at 405nm was recorded using a Fluorostar Optima plate reader (BMG Labtech, Aylesbury, UK). Samples were run in either duplicate or triplicate and the results analysed using Microsoft excel and Graphpad Prism 6.0 (Graphpad Software, San Diego,

USA). The standard deviation of replicate values was used to calculate error bars.

Standard curves for the estimation of total antibody production per culture well were produced using a dilution series of a monoclonal antibody (AP33) at concentrations of; 2000ng/mL, 1000ng/mL, 500ng/mL, 250ng/mL, 125ng/mL, 62.5ng/mL and 0ng/mL.

2.2.3 Identification of IgG variable domain sequences from B-cell cultures

2.2.3.1 Messenger RNA extraction from culture wells and cDNA synthesis

B-cell supernatants testing positive for IgG production were selected for isolation and analysis of the IgG genes expressed. The entire cell population was extracted from a culture well, with the remaining supernatant (approximately 100μL), and transferred into a 0.7mL tube. Any remaining cells were washed from the culture well in 100μL of cell wash buffer (1xPBS, 0.8units/μL RNAse OUT (Invitrogen, Paisley, UK). A further 300mL of cell wash buffer was added to the cells before washing with centrifugation at 300 x g for 5 minutes. Residual wash buffer was carefully aspirated by pipetting. The cells were lysed by the addition of 10μL of lysis buffer (20units RNAseOUT, 2.5μL 5x Thermoscript RT buffer (Invitrogen, Paisley, UK), 0.625μL 0.1M dithiothreitol (DTT) (Invitrogen, Paisley, UK), 0.03125μL Igepal CA-630 (Sigma Aldrich, Gillingham, UK), made up to 10μL with diethyl pyrocarbonate (DEPC)-treated H_2O (Invitrogen, Paisley, UK)). Samples were transferred into 0.2mL PCR tubes and snap frozen on dry ice for

approximately 30 seconds. At this point the samples could be stored at -80°C or used immediately for cDNA synthesis.

RNA samples were thawed on ice before adding cDNA synthesis buffer (10mM dNTPs (Invitrogen, Paisley, UK), 7.5units Thermoscript reverse transcriptase (RT) (Invitrogen, Paisley, UK) and either; 75ng Random hexamer primers (Invitrogen, Paisley, UK), or 50pmoles Oligo$(dT)_{20}$ primers (Invitrogen, Paisley, UK). The samples were incubated on a thermal cycler at 25°C for 25 minutes if random hexamer primers were used to allow the primers to anneal to complementary sequences. cDNA synthesis was carried out at 50°C for 45 minutes before terminating the reaction by heating to 85°C for 5 minutes. RNA template was removed from the reaction mixtures by the addition of 1μL RNAse H (Invitrogen, Paisley, UK), and incubating at 37°C for 20 minutes.

2.2.3.2 IgG variable region specific amplification using PCR

IgG variable regions were amplified from cDNA using primer sets specific for heavy, kappa and lambda light chains. Primer names and sequences are listed in Tables 2-1 to 2-3. Heavy, kappa and lambda chain variable domains were amplified in separate reactions containing 2.5μL cDNA in a total volume of 25μL containing 0.625units Taq DNA polymerase (Invitrogen, Paisley, UK), 10mM dNTPs (Invitrogen, Paisley, UK), 2.5μL 10 x reaction buffer (Invitrogen, Paisley, UK) and 5pM of each primer and making up to volume using DEPC-treated H_2O (Invitrogen, Paisley, UK). PCR amplification was carried out in a 3-step reaction consisting of an initial DNA polymerase activation step at 95°C for 15 minutes, followed by 45 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 55 seconds. A final extension soak of

72°C for 55 seconds completed the reaction. The presence of PCR products was detected using agarose gel electrophoresis. Negative control reactions were provided by the omission of cDNA into reaction mixtures. A GeneRuler DNA Ladder Mix molecular weight standard (ThermoFisher Scientific, Loughborough, UK) was included in all agarose gel electrophoresis experiments.

Table 2-1| Heavy chain specific primer set

Sense		Anti-sense	
VH1 LEADER-A	ATGGACTGGACCTGGAGGAT	Cγ CH1	GGAAGGTGTGCACGCCGCTGGTC
VH1 LEADER-B	ATGGACTGGACCTGGAGCAT		
VH1 LEADER-C	ATGGACTGGACCTGGAGAAT		
VH1 LEADER-D	GGTTCCTCTTTGTGGTGGC		
VH1 LEADER-E	ATGGACTGGACCTGGAGGGT		
VH1-LEADER-F	ATGGACTGGATTTGGAGGAT		
VH1-LEADER-G	AGGTTCCTCTTTGTGGTGGCAG		
VH3 LEADER-A	TAAAAGGTGTCCAGTGT		
VH3 LEADER-B	TAAGAGGTGTCCAGTGT		
VH3 LEADER-C	TAGAAGGTGTCCAGTGT		
VH3 LEADER-D	GCTATTTTTAAAGGTGTCCAGTGT		
VH3 LEADER-E	TACAAGGTGTCCAGTGT		
VH3 LEADER-F	TTAAAGCTGTCCAGTGT		
VH4 LEADER-A	ATGAAACACCTGTGGTTCTTCC		
VH4 LEADER-B	ATGAAACACCTGTGGTTCTT		
VH4 LEADER-C	ATGAAGCACCTGTGGTTCTT		
VH4 LEADER-D	ATGAAACATCTGTGGTTCTT		
VH5 LEADER-A	TTCTCCAAGGAGTCTGT		
VH5 LEADER-B	CCTCCACAGTGAGAGTCTG		
VH6 LEADER-A	ATGTCTGTCTCCTTCCTCATC		
VH7 LEADER-A	GGCAGCAGCAACAGGTGCCCA		

All primers were synthesised commercially by Sigma Aldrich, Gillingham, UK. Primer sequences taken from Scheid *et al.,* Science , (2011).

Table 2-2| Kappa chain specific primer set

Sense		Anti-sense	
5'L-Vκ1/2	ATGAGGSTCCCYGCTCAGCTGCTGG	3′ Cκ 543	GTTTCTCGTAGTCTGCTTTGCTCA
5′ L-Vκ3	CTCTTCCTCCTGCTACTCTGGCTCCCAG		
5' L-Vκ4	ATTTCTCTGTTGCTCTGGATCTCTG		

All primers were synthesised commercially by Sigma Aldrich, Gillingham, UK. Primer sequences taken from Wardemann *et al.* Science, (2003)

Table 2-3| Lambda chain specific primer set

Sense		Anti-sense	
5' L-Vλ1	GGTCCTGGGCCCAGTCTGTGCTG	3′ Cλ	CACCAGTGTGGCCTTGTTGGCTTG
5′ L-Vλ2	GGTCCTGGGCCCAGTCTGCCCTG		
5' L-Vλ3	GCTCTGTGACCTCCTATGAGCTG		
5′L-Vλ4/5	GGTCTCTCTCSCAGCYTGTGCTG		
5′ L-Vλ6	GTTCTTGGGCCAATTTTATGCTG		
5′ L-Vλ7	GGTCCAATTCYCAGGCTGTGGTG		

All primers were synthesised commercially by Sigma Aldrich, Gillingham, UK. Primer sequences taken from Wardemann *et al.* Science, (2003)

2.2.3.3 Analysis of PCR products using agarose gel electrophoresis

A 5μL aliquot of PCR products was removed and mixed with 6x gel loading buffer (50% glycerol in H_2O, 0.002% bromophenol blue) and loaded onto a 2% agarose (Bioline, London, UK) gel prepared with 1x Tris Acetate-ethylene diamine tetraacetic acid (EDTA) buffer (Sigma Aldrich, Gillingham, UK) and 5μL ethidium bromide solution (Fisher Scientific, Loughborough, UK). The molecular weight of PCR products was estimated using 5μL of a Generuler DNA ladder mixture (Fermentas, St. Leon-Rot, Germany) which was added to the gel. PCR products were resolved by exposure to an electric current of 95V for approximately 45 minutes and visualised using a UV trans-illuminator. Results were recorded photographically.

2.2.3.4 Purification of PCR products

PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Crawley, UK) using centrifugation according to the manufacturer's instructions. Five volumes of Buffer PB were mixed with 1 volume of PCR product and loaded onto a QIAquick spin column. DNA was bound to the silica membrane by centrifugation at 13,000 x g. The sample was washed with 750μL of Buffer PE by centrifugation to remove residual primers or impurities. DNA elution was achieved using 50μL of Buffer EB and centrifuging at 13,000 x g. A Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, Loughborough, UK) was used to estimate DNA concentration.

2.2.3.5 DNA sequencing using the dideoxy chain terminator method

The dideoxy chain terminator method (Sanger, 1977) was used to sequence DNA samples. For PCR products, 1ng/μL per 100bp was required. For plasmid DNA, 100ng/μL was required. Samples were prepared accordingly and submitted, along with appropriate sequencing primers to Source BioScience, Nottingham, UK, who carried out the sequencing reactions. Sequencing reads were downloaded as read-only applied BioSystems (ABI) trace formatted files and analysed using Finch TV 1.4 (PerkinElmer, London, UK).

2.2.3.6 Analysis of V_H and V_L domains using the Immunogenetics database

DNA sequence data were analysed using the Immunogenetics (IMGT®) database (Lefranc et al., 2009) in order to characterise the isolated V_H and V_L domains. The isolated sequences were aligned to known Ig genes using the IMGT/V-QUEST (Brochet et al., 2008) sequence alignment software. Percentage identity to germline sequences for V, J and D regions were assigned for heavy chain sequences and V and J regions for light chain sequences. Gene functionality was indicated through the assignment of a productive rearrangement (no stop-codons, an in-frame junction with no defects in gene regulatory elements).

2.2.4 PCR modification of isolated variable domains and sub-cloning into expression vectors

2.2.4.1 Second round PCR amplification of PCR products

Second round PCR amplification was carried out using bespoke primers designed according to DNA sequence data corresponding to the 5' region of the variable domains. Sense primers for heavy and light chain variable domains incorporated universal sequences of 16bp of homology to the pORIgHIB huIgG1 and pORIgLIB huIgG1 expression vectors respectively, to allow PCR modified sequences to be inserted into the vectors. Adjacent to the homology regions, each sense primer also incorporated a translation initiation signal (Kozak, 1986) and a signal peptide cleavage signal containing a region of complementarity with variable domain sequences isolated from B-cell samples. Anti-sense primers also incorporated a 16bp region of homology with the expression vectors and a constant domain complementary region.

Second round PCR mixtures were assembled in a total volume of 25μL containing 1μL of first round PCR product diluted approximately 1000x, 0.625units Taq DNA polymerase, 10mM dNTPs, 2.5μL 10 x reaction buffer and 5pM of each primer before making up to volume using DEPC-treated H_2O. Amplification was carried out in a 3-step reaction consisting of an initial DNA polymerase activation step at 95°C for 15 minutes, followed by 45 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 55 seconds. A final extension soak of 72°C for 55 seconds completed the reaction. The presence of PCR products were detected by agarose gel electrophoresis.

2.2.4.2 Homologous recombination of PCR products into expression vectors`

In preparation for homologous recombination cloning, expression vectors were linearised by digestion with restriction enzymes as follows; approximately 800ng of pORIgHIB huIgG1 plasmid DNA were digested with 2 units of *HindIII* (New England Biolabs, Hitchin, UK) and 2 units of *AfeI* (New England Biolabs, Hitchin, UK) in NEBuffer 2.1 made up to a final volume of 50µL with DEPC-treated H_2O and incubated for 30 minutes at 37°C, and approximately 800ng of pORIgLIB huIgG1 plasmid DNA were digested with 2 units of *BamHI* (New England Biolabs, Hitchin, UK) in NEBuffer 3.1, made up to a final volume of 50µL with DEPC-treated H_2O and incubated for 30 minutes at 37°C before adding 2 units of *BsiWI* and incubating for 30 minutes at 55°C. All restriction enzyme digestion reactions were terminated by heating to 80°C for 20 minutes. Plasmid digestion was confirmed using agarose gel electrophoresis as described in Section 2.2.3.3 and column purified using the method described in Section 2.2.3.4.

Homologous recombination was achieved using an In-fusion HD cloning kit (Takara Bio, California, USA) according to the manufacturer's protocol. Briefly, 100ng of linearized vector were mixed with 200ng of purified second round PCR products, 2µL of 5 x In-Fusion HD enzyme premix and made up to a total volume of 10µL using dH_2O. The reaction mixture was incubated for 15 minutes at 50°C before placing on ice.

2.2.4.3 Transformation of expression vector plasmids into Escherichia Coli TOP10 cells

Chemically competent *E.Coli* cells (Invitrogen, Paisley, UK), were thawed slowly on ice in 50μL aliquots before adding 2μL of plasmid DNA to the cells and incubating on ice for 20 minutes. The plasmid DNA was transformed into the cells by heat-shocking at 42°C for 30 seconds followed by a 2 minute incubation on ice. The cells were recovered by the addition of 200μL of SOC medium (Invitrogen, Paisley, UK), before incubating at 37°C with shaking at 200rpm for 45 minutes. Cell suspensions were plated onto LB (Sigma Aldrich, Gillingham, UK) agar plates containing either 50μg/mL zeocin or 25μg/mL ampicillin for heavy and light chain expression vectors respectively. The plates were incubated at 37°C overnight.

2.2.4.4 Plasmid purification from Escherichia Coli TOP10 cell cultures

Colonies obtained from expression vector transformations were inoculated into 5mL of LB (Sigma Aldrich, Gillingham, UK) media containing either 50μg/mL zeocin or 25μg/mL ampicillin for heavy and light chain expression vectors respectively and incubated for 8 hours at 37°C with shaking at 200rpm. A 1mL aliquot of the culture was transferred into 49mL of LB medium containing either 50μg/mL zeocin or 25μg/mL ampicillin and incubated overnight at 37°C with shaking at 200rpm. Plasmid DNA was purified from the cultures using a GenElute Sigma Midiprep kit according to the manufacturer's instructions. Bacterial cells were pelleted by centrifugation at 4000 x g for 10 minutes. Cell pellets were resuspended in 4mL of cold Resuspension/RNAse A Solution before the addition of 4mL of Lysis Solution.

The mixture was gently homogenised by inverting 6 – 8 times and incubating for a maximum of 5 minutes. The lysis reaction was neutralised by the addition of 4mL of cold Neutralisation Solution and inverted 4 – 6 times to mix before adding 3mL of Binding Solution. Mixtures were gently inverted 1 – 2 times before transferring immediately into the barrel of a filter syringe and incubated at room temperature for 5 minutes. A GenElute Midiprep Binding Column was prepared by the addition of 4mL of Column Preparation Solution and centrifuging at 3,000 x g for 2 minutes. The neutralised lysis solution was transferred onto a binding column and plasmid DNA allowed to adsorb to the membrane during centrifugation at 3,000 x g for 2 minutes. Residual primers and impurities were removed from the column by the addition of 4mL of Wash Solution 1 and centrifuging at 3,000 x g for 2 minutes. A second wash step was carried out by adding 4mL of Wash Solution 2 to the column and centrifuging at 3,000 x g for 2 minutes. Plasmid DNA was eluted from the column in 1mL of Elution Solution and centrifuging at 3,000 x g for 5 minutes. The concentration of eluted plasmid DNA was estimated using a Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, Loughborough, UK).

DNA sequence data were obtained as described in Section 2.2.3.5 using plasmid specific primers to confirm the presence of the correct insert into each expression vector.

2.2.5 Expression of whole IgG in mammalian cells

2.2.5.1 Maintenance of Human Embryonic Kidney cells (HEK-293T) for recombinant antibody production

Cryopreserved Human Embryonic Kidney Cells (HEK-293T) were partially thawed in a 37°C water bath before the addition of 1mL of pre-warmed Dulbecco's Modified Eagle medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% FBS. The cells were immediately transferred into a Corning T25 flask containing 5mL of DMEM/FBS and incubated overnight at 37°C with 5% CO_2. The cells were pelleted at 300 x g for 10 minutes before resuspending in 19mL of DMEM/FBS in a Corning T75 flask and incubating for 2 – 3 days until a 85-95% confluent cell monolayer was achieved. Culture supernatant was removed and adherent HEK-293T cells washed with 10mL of cold PBS before the addition of 5mL of pre-warmed trypsin-EDTA (0.25) (Gibco, Paisley, UK). The cells were incubated at 37°C for 5-10 minutes and removed from the plastic with gentle tapping and a 10μL aliquot removed for counting using a haemocytometer. The remaining cells were washed in 30mL pre-warmed DMEM/FBS by centrifugation at 300 x g for 5 minutes. Following resuspension in DMEM/FBS, $1.5x10^6$ cells were transferred into 50mL of DMEM/FBS in a Corning T225 flask and incubated at 37°C with 5% CO_2 until a 85-95% confluent monolayer was achieved. The cells were thus maintained until a maximum of 60 passages was reached.

2.2.5.2 Transient transfection of HEK-293T cells to produce recombinant IgG molecules

In preparation for transient transfections, cells were seeded into 10cm diameter tissue culture dish (BD Biosciences, Bedford, USA) at a density of 1.2 x 10^6 cells per dish and allowed to reach a confluency of 65-75%. Transfections were achieved using polyethylenimine (PEI) *in vitro* transfection reagent (Fermentas, St. Leon-Rot, Germany). For each transfection, 24μL of PEI was mixed with 300μL of OptiMem medium (Gibco, Paisley, UK) and added to 300μL aliquot of OptiMEM containing 2μg of the plasmid DNA to be transfected before incubating at room temperature for 45 minutes to allow for the formation of PEI-DNA complexes. Following the incubation, the DMEM/FBS medium was carefully aspirated from HEK-293T cells and replaced with 7mL of OptiMEM to which the 600μL of PEI-DNA mixture were added. The cells were incubated at 37°C with 5% CO_2 for 6 hours to allow for the uptake of DNA into the cells. Finally, the OptiMEM transfection mixture was removed from the cells and replaced with 7mL of DMEM/FBS. Recombinant antibody expression was carried out for 48 hours by incubating the cells at 37°C with 5% CO_2 before harvesting the IgG molecules with the culture supernatant. To generate a fully functioning antibody molecule, 2μL of plasmid DNA for a heavy and a corresponding light chain were co-transfected into HEK-293T cells. Recombinant antibody expression was assessed using the methods in Sections 2.2.2.4.

2.2.6 Production of plasmid expression vectors containing the control mAbs KZ52, 2G4, 4G7 and 13C6

The primary structures of V_H and V_L domains of control antibody KZ52 were obtained from the Worldwide Protein Data Bank (PDB) (Berman et al., 2003), specifically: KZ52, PDB: 3CSY (Lee et al., 2008). Amino acid sequences for V_H and V_L regions of antibodies, 2G4 and 4G7 were taken directly from those published in (Audet et al., 2014) whilst the sequences for 13C6 were kindly provided by Professor Gary Kobinger. In each case, the amino acid sequence was back translated using EMBOSS Backtranseq software (EMBL-EBI, Cambridge, UK) into the corresponding DNA sequence. The constant domain region for each antibody was identified by aligning each sequence in FASTA format to the corresponding expression vector backbone using MEGA 6 software (Tamura et al., 2013). Translation initiation and signal peptide sequences were added to the 5' region of the DNA sequence as described in Section 2.2.4.1. Finally, a 16bp region of homology to the corresponding expression vector was incorporated at the 5' and 3' regions to allow for homologous recombination into the expression vectors. Each sequence, corresponding to the V_H and V_L domain of each control antibody, was synthesised using the gBlocks® Gene Fragment synthesis service (Integrated DNA Technologies, Leuvan, Belgium). The synthetic genes were inserted into expression vectors using the methods described in Sections 2.2.4.2 to 2.2.4.4 and the DNA sequences analysed according to the methods in Sections 2.2.3.5 and 2.2.3.6. Recombinant control antibodies were expressed as described in Sections 2.2.5.1 and 2.2.5.2.

2.2.7 Binding assay to identify interactions between recombinant mAbs, KZ52, 2G4, 4G7 and 13C6 with EBOV GP.

Wheat germ agglutinin (Sigma Aldrich, Gillingham, UK) was solvated in bicarbonate buffer (pH 9.5) (Sigma Aldrich, Gillingham, UK) at a concentration of 50μg/mL and added to a Nunc maxisorp-96 well plate (Fisher Scientific, Loughborough, UK) in 50μL aliquots before incubating overnight at 4°C in a humidity chamber. Following equilibration to room temperature, the plate was washed 3 x using PBS/5% Tween-20. Non-specific binding sites were blocked with 200μL of 5% skimmed milk suspension and incubated in a humidity chamber at either room temperature for 3 hours or overnight at 4°C. The plate was washed 3 x using PBS/5% Tween-20 before adding 50μL of a 1:5 dilution of clarified HEK-293T cell lysates (transfected with EBOV GP corresponding to Zaire ebolavirus H.sapiens-tc/COD/1976/Yambuku-Mayinga 344 (Mayinga EBOV; GenBank accession NC_002549)) to each culture well. HEK 293T cells were lysed by the addition of 1mL of cold cell lysis buffer (50mM Tris.HCl pH 8.0, 140mM NaCl, 1mM EDTA, 1% (v/v) IgePal, 100mM iodoacetamide (all Sigma Aldrich, Gillingham, UK) to medium free HEK-293T cells and incubating for 45 minutes at room temperature with gentle rocking.

The plate was incubated for 1 hour at room temperature in a humidity chamber. Following washing 3 x using PBS/5% Tween-20, a 50μL aliquot of HEK-293T culture supernatant containing each of the recombinant mAbs was transferred to each well of the plate, which was subsequently incubated for 1

hour at room temperature. Wells were washed 3 x using PBS/5% Tween-20. Bound primary antibody from the culture supernatants was detected following the addition of secondary antibody (goat α-human IgG–conjugated to alkaline phosphatase) at a 2000X dilution in PBS/5% Tween-20 to each well in 50μL aliquots. Plates were incubated at room temperature for 60 minutes before washing 3 x using PBS/5% Tween-20. The addition of 50μL of pNPP was used to identify bound secondary antibody. Plates were incubated in the dark at room temperature for approximately 30 minutes until a colour change was observed for positive reactions. The absorbance at 405nm was recorded using a Fluorostar Optima plate reader (BMG Labtech, Aylesbury, UK). Samples were run in either duplicate or triplicate and the results analysed using Microsoft excel and Graphpad Prism 6.0 (Graphpad Software, San Diego, USA). The standard deviation of replicate values was used to calculate error bars.

2.3 Results

2.3.1 Feeder Cell Culture

Optimal seeding densities for irradiated primary macrophage cells were identified by plating cells at three different densities into 96 well plates. The cell densities investigated were; 1) 312,502 cells per well ($3x10^6$ per plate), 2) 15,625 cells per well ($1.5x10^6$ per plate), 3) 7,913 cells per well ($7.5x10^5$ per plate) (FIG 2-3 panels A-C respectively). After allowing the cells to settle on the base of the well for 72 hours, images were captured using a light microscope. Examination of the images showed that seeding at 312,502 cells per well provided a monolayer with 95-100% confluence. Interestingly, the lower two seeding densities produced monolayers with similar degrees of confluence of around 50-60%.

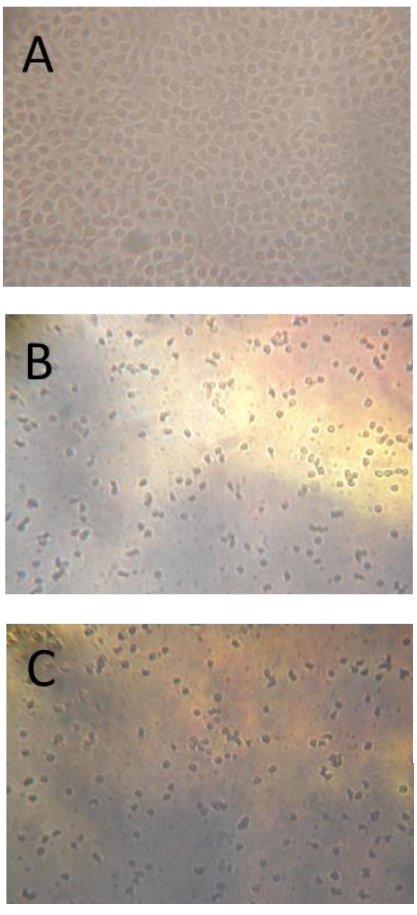


Figure 2-3| Light microscope analysis of irradiated primary macrophage feeder cells

Cells were seeded at different densities into 96 well culture plates in order to identify the optimum seeding density for B-cell co-culture experiments. All cells were irradiated at 18Gy using a caesium-137 sealed source of gamma radiation prior to seeding and subsequently cultured for 72 hours. All images were captured at the end of the culture period. Magnification 100X.

A) Gamma irradiated primary macrophages seeded at a density of 312,502 cells per well. **B)** Gamma irradiated primary macrophages seeded at a density of 15,625 cells per well. **C)** Gamma irradiated primary macrophages seeded at a density of 7,913 cells per well.

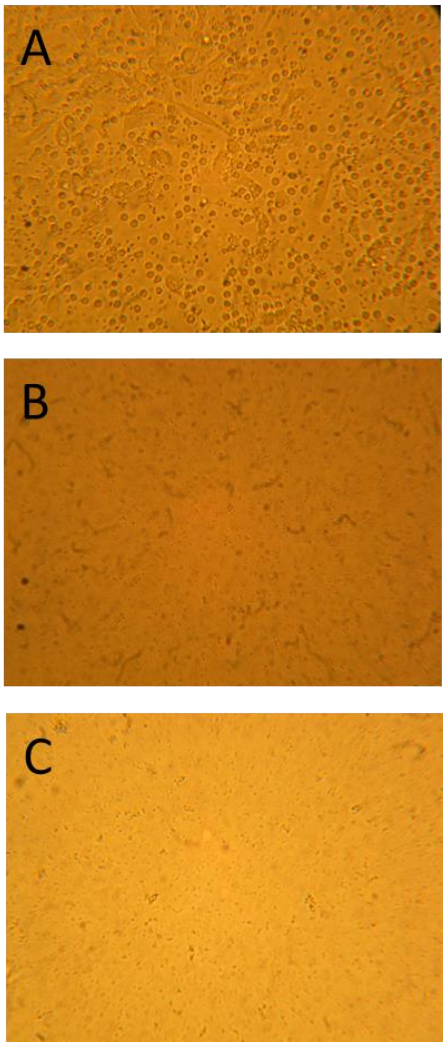


Figure 2-4| Light microscope images showing variability between primary macrophage feeder cell populations

All culture wells were seeded with irradiated macrophages at a density of 312,502 cell per well into 96 well culture plates in preparation for B-cell co-culture experiments. The cells were irradiated at 18Gy using a caesium-137 sealed source of gamma radiation prior to seeding and subsequently cultured for approximately 72 hours using identical culture conditions. Images were captured at the end of the culture period. Magnification 100X

A) Gamma irradiated primary macrophage culture 1. **B)** Gamma irradiated primary macrophage culture 2. **C)** Gamma irradiated primary macrophage culture 1.

Irradiated primary macrophage feeder cultures were established in preparation for co-culture with memory B-lymphocytes. Macrophage cultures were seeded into 96 well plates at a cell density of 312,502 cells per well. The cultures proved to be difficult to maintain for periods longer than 72 hours however. Large degrees of variation in cell viability were observed in cultures established using identical methods (Figure 2-4). After approximately 72 hours of incubation, some primary macrophage cultures exhibited a healthy morphology, (Figure 2-4, panel A) however, equivalent cultures observed at

the same time point displayed levels of necrosis between approximately 50% and 90% (Figure 2-4, panels B and C).

Due to primary macrophage cultures providing variable results, the next cell type assessed was the monocyte-like THP-1 cell line. The cells were grown in suspension culture for between 3 to 7 passages until the cell density reached between $5x10^5$ – $5\ x10^6$ cells/mL before being stimulated to differentiate into macrophage-like cells using PMA. The degree of differentiation was assessed under a light microscope on days 2, 5 and 7 following the addition of 100nM PMA (Figure 2-5). The morphology of monocytes (rounded and non-adherent) and macrophages (elongated and adherent) are sufficiently different for each cell type to be identified using a light microscope. After 2 days in culture, no differentiated macrophages were observed (Figure 2-5, panel A). On day 5, no differentiated macrophages were observed, although approximately 10% of total monocytes appeared to be entering a transitional stage between cell types (Figure 2-5, panel B). On day 7, progression towards differentiation was observed, with approximately 10% of total monocytes displaying macrophage-like cell morphology (Figure 2-5, panel C), however there had been no observable increase in the percentage of monocyte-like cells entering the differentiation pathway.

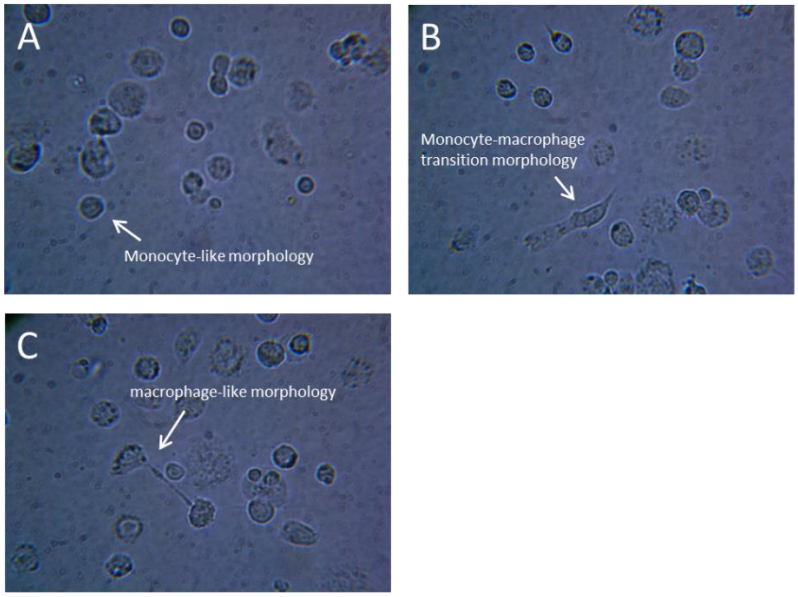


Figure 2-5| Light microscope analysis of THP-1 monocyte-like cell differentiation

Undifferentiated cells were cultured until reaching a cell density of between $5x10^5$ to $5 x10^6$ cells/mL. Differentiation was initiated by the administration of 100ng/mL of PMA. The cultures were incubated for 7 days and monocyte to macrophage-like cell differentiation was assessed from morphological changes to the cells. Light microscope images were recorded from a single culture at approximately the same positioning within the culture flask after 2, 5 and 7 days of incubation. Magnification 100X

A) THP-1 cell line derived macrophage-like cells following 2 days of culture. The entire cell population demonstrated a monocyte-like morphology. A THP-1 cell with typical monocyte-like morphology is indicated. **B)** THP-1 cell line derived macrophage-like cells following 5 days of culture. Although THP-1 cells with a macrophage-like morphology were completely absent from the culture, approximately 10% of the cell population demonstrated a monocyte to macrophage transition morphology. A typical cell with this morphology is indicated in the figure. **C)** THP-1 cell line derived macrophage-like cells following 7 days of culture. Approximately 10% of the total cell population displayed a macrophage-like cell morphology. A representative cell is indicated in the figure.

The routine failure of THP-1 monocyte-like cell differentiation into macrophage-like cells rendered the cell line unsuitable as a source of feeder cells. A second cell line, LL8, was investigated for feeder cell suitability. Ectopic CD-154 expression on LL8 fibroblasts was visualised using flow cytometry. The cells were cultured for either 6 or 12 passages, in the absence of geneticin selection, before fixing and staining with anti-CD-154 antibodies

conjugated to PE. Stained and unstained cells were examined for CD-154 surface expression using a flow cytometer (Figure 2-6). Comparison of CD-154 stained and unstained cell populations showed approximately 80% of fibroblasts expressed the molecule in their surface membrane for at least 12 passages in the absence of antibiotic selection.

Following confirmation that CD-154 molecules were expressed on the surface of LL8 fibroblasts, the seeding density required to achieve a 95 – 100% confluent monolayer in 96 well culture plates was established (Figure 2-7). The level of gamma irradiation required by the LL8 cells (180Gy) was taken from the literature and did not require optimisation (Rancan et al., 2015). Irradiated cells were seeded into 96 well culture plates at seeding densities of 3,906; 7,812; 15,625; 31,250 and 62,500 cells per well and allowed to settle onto the substrate for 24 hours. Following this period, the wells were investigated using a light microscope to assess how confluent the cells were within a monolayer. The lower three seeding densities (3,906; 7,812; 15,625 cells per well) resulted in monolayers of less than 90% confluent cells (Figure 2-7, panels A to C). Seeding with 31,250 cells per well achieved a monolayer with 90-100% confluency (Figure 2-7, panel D), whilst the highest density of

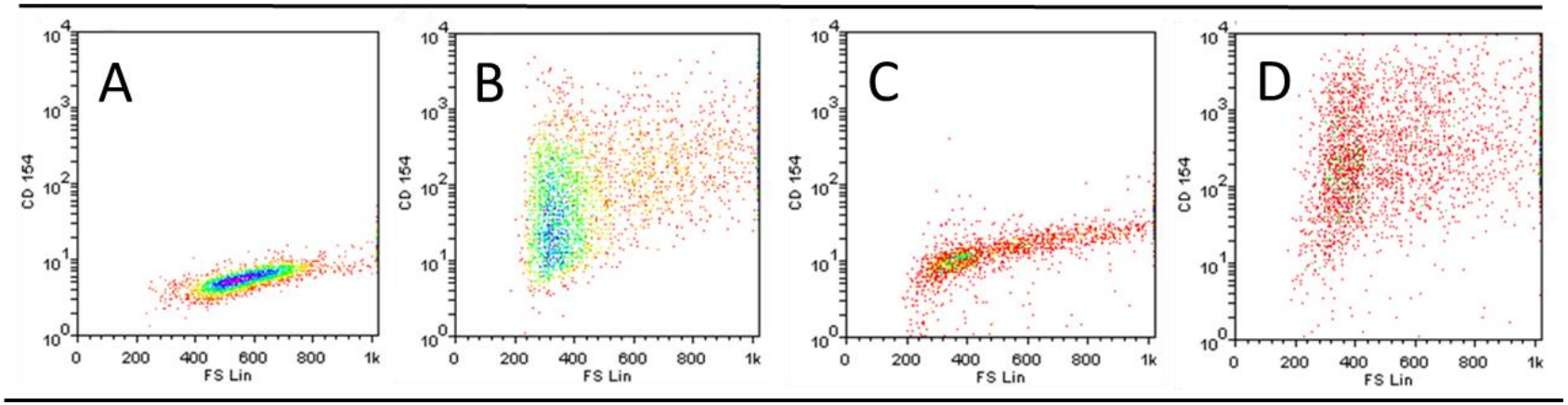


Figure 2-6| Analysis of ectopic CD-154 expression on the surface of LL8 murine fibroblast feeder cells

The cells were cultured for 12 passages in the absence of antibiotic selection for cells containing the CD-154 expression plasmid. Cell populations of approximately $5x10^5$ were stained using an anti-CD-154 monoclonal antibody conjugated to PE at passage number 6 and 12. A Beckman Coulter FC500 flow cytometer was used to measure the the fluorescence of un-gated stained cells. Unstained cells were used to identify the population of non-fluorescent LL8 cells. Approximately 80% of LL8 cells fluoresced and each experiment captured approximately 50,000 events.

A) Fluorescence of unstained LL8 cells measured after 6 passages. **B)** Fluorescence of anti-CD-154-PE stained LL8 cells measured after 6 passages. **C)** Fluorescence of unstained LL8 cells measured after 12 passages. **D)** Fluorescence of anti-CD-154-PE stained LL8 cells measured after 12 passages.

62,500 cells per well resulted in 100% confluency, however cells located adjacent to the edge of the well had formed multiple layers (Figure 2-7, panel E).

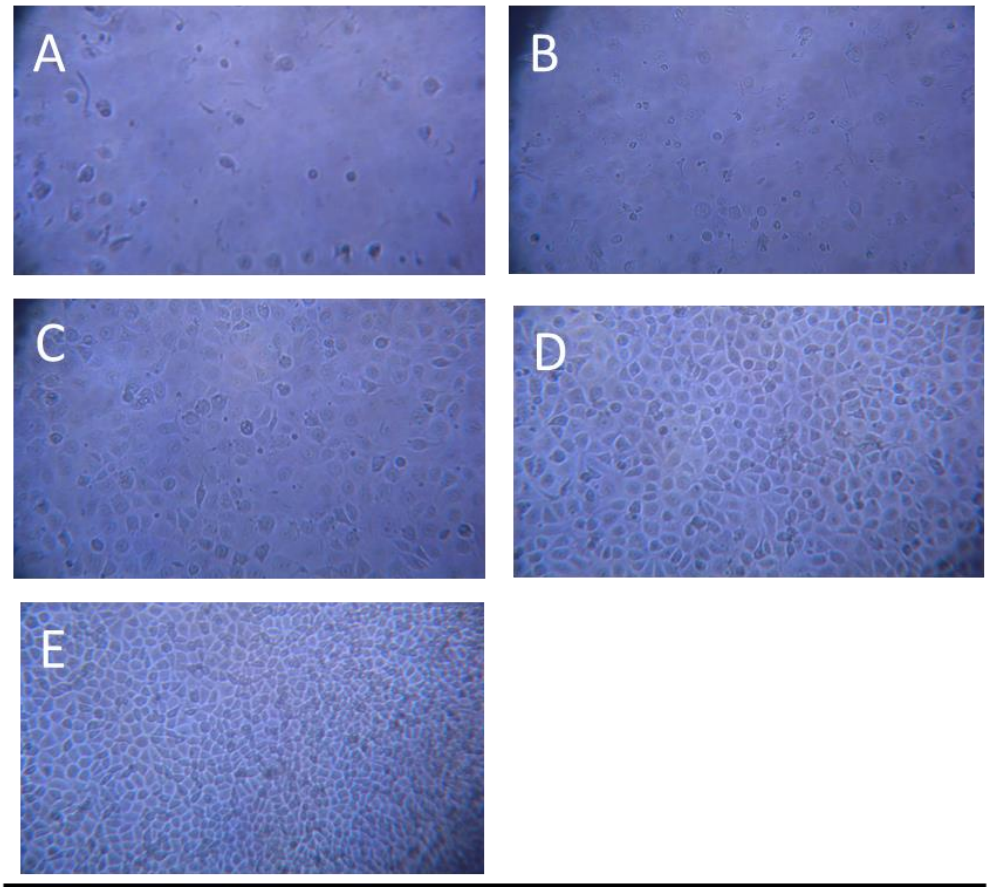


Figure 2-7 |Light microscope images of LL8 feeder cell monolayers in culture wells established using different seeding densities

Different seeding densities of LL8 cells were used to determine the optimum number of feeder cells to seed into a culture well prior to co-culturing with memory B-lymphocytes. The LL8 cells were grown to a density of $3x10^7$ cells/mL before irradiating at 180 Gy using a caesium-137 static source of gamma radiation. Irradiated cells were transferred into a 96 well plate at one of 6 different cell densities and allowed to settle onto the substrate by incubating at 37°C with 5% CO_2 for 24 hours. The degree of confluence was then estimated using a light microscope. Magnification 100X.

A) Culture well seeded with 3,906 cells per well. Estimated degree of confluency: 15%. **B)** Culture well seeded with 7,812 cells per well. Estimated degree of confluency: 25%. **C)** Culture well seeded with 15,625 cells per well. Estimated degree of confluency: 80%. **D)** Culture well seeded with 31,250 cells per well. Estimated degree of confluency: 100%. **E)** Culture well seeded with 62,500 cells per well. Estimated degree of confluency: >100%.

To optimise the seeding density of gamma irradiated LL8 cells further, the two cell densities which achieved a confluency of 100% or greater (31,250 and 62,500 cells per well) were used to seed a 96 well plate. The plate was incubated for ten days and light microscope images captured on culture day 3 and 10 to compare levels of necrosis and apoptosis within culture wells (Figure 2-8). On day 10 of the culture, the degree of confluency had decreased to approximately 80 – 90% for both seeding densities (Figure 2-8, panels B and D). Due to the differences in the starting number of cells, the higher seeding density would result in a greater number of dead cells in each well by culture day 10.

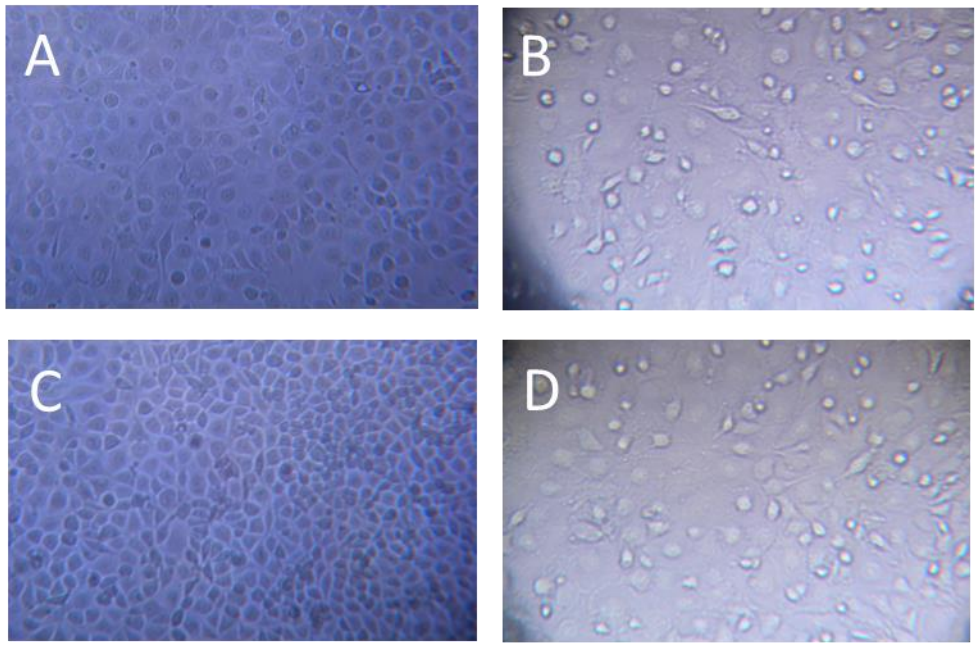


Figure 2-8 |Light microscope images of cultures of LL8 feeder to estimate levels of cell death over time

Irradiated cells were aliquoted into a 96 well plate and allowed to settle onto the substrate. The cultures were incubated at 37°C with 5% CO_2 for a period of 10 days and images captured on day 3 and day 10. Magnification 100X.
A) Culture well seeded with 31,250 cells. The image was captured after 3 days of culture. **B)** Culture well seeded with 31,250 cells. The image was captured after 10 days of culture. **C)** Culture well seeded with 62,500 cells. The image was captured after 3 days of culture. **D)** Culture well seeded with 62,500 cells. The image was captured after 10 days of culture.

2.3.2 Memory B-lymphocyte culture

Memory B-lymphocytes expressing IgG molecules in their surface membrane were purified from PMBC using magnetic beads coupled to antibodies targeting lymphocyte specific surface markers (defined by cluster of differentiation molecules), therefore depleting the PBMC of non-B-cells. IgG^+ memory B-lymphocytes were further purified from total B-cells by labelling with an anti-IgG micro-bead, thus providing a two-step method of purifying IgG^+ memory B-lymphocytes. The degree of purity at each step of the procedure was analysed using flow cytometry. Each of four discrete cell populations generated during the procedure (total PBMC; non-B-cells; total B-cells and IgG^+ memory B-cells) were incubated with anti CD-19–PEcy5 (to label all B-cells) and anti IgG–PEcy7 (to label IgG+ memory B-cells) before analysing using a Beckman Coulter FC500 flow cytometer (Figure 2-9). The pool of IgG+ memory B-cells was shown to increase in purity at each successive step with the final compartment exhibiting a purity of 85% IgG^+ memory B-cells. These cells were used in subsequent short term co-culture experiments.

Purified memory B-lymphocytes required *in vitro* stimulation in order to produce antibodies in detectable quantities. The individual stimulants tested were; IL-2 (665ng/mL); IL-4 (25ng/mL), IL-21 (100ng/mL) and R-848 (2500ng/mL). Four different combinations were assessed for antibody production;

i) IL2 + R848 + IL-21; ii) IL-4 + R848 + IL-21; iii) IL-2 + IL-21; iv) IL-4 + IL-21:

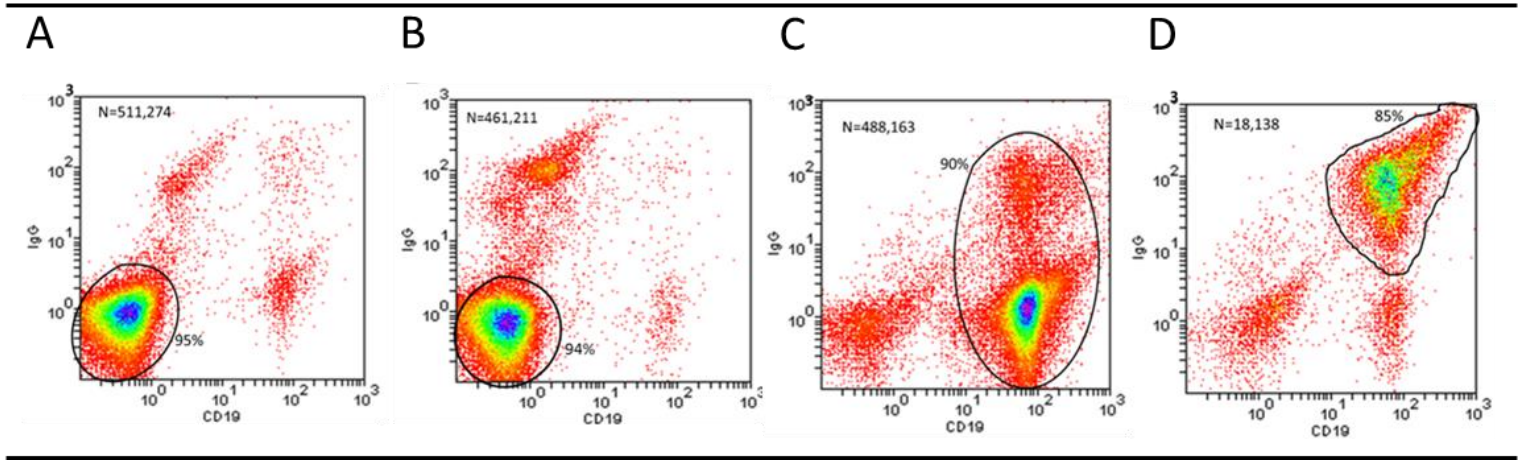


Figure 2-9|Flow cytometry analysis of IgG+ memory B-lymphocytes from PBMCs

Four cell populations were analysed at different stages during the purification procedure corresponding to PBMCs, B-cell depleted PBMCs, total B-cells and IgG+ B-cells. Cells were stained with anti-IgG monoclonal antibodies conjugated to PE-Cy5 and anti-CD20 antibodies conjugated to PE. The cells analysed using a Beckman Coulter FC500 flow cytometer.

A) The initial population of PBMC from which IgG^+ memory B-cells were purified. The majority (95%) of cells in this pool did not demonstrate fluorescence (circled) indicating that 95% of initial PBMCs were non-IgG B-cells. A population of free IgG were present in the top left quadrant. A population of non-IgG B-cells was present in the bottom right quadrant and the IgG^+ positive memory B-cell population in the top right hand quadrant. **B)** The fraction of cells isolated during the purification representing the initial cell population depleted of B-cells. Ninety four percent of the cells within this population did not demonstrate fluorescence (circled) and corresponded to non B-cells. A small population (~1%) of non-IgG B-cells was present in the bottom right hand quadrant. **C)** The total B-cell population. A small population of non-B-cells was present in the bottom right hand quadrant, however 90% of the population were B-cells (circled). **D)** The final purified cell fraction. A total of 85% of cells present were IgG^+ memory B-cells (circled). Small populations corresponding to non-B-cells (bottom left hand quadrant) and non-IgG B-cells (bottom right hand quadrant) were also observed.

The cocktails were added to the culture medium at the beginning of memory B-cell cultures seeded with 130, 13 and 1.3 cells per well. The different seeding densities were achieved using a limiting dilution of memory B-cells dilution. Following nine days of incubation, cell culture supernatants were probed for IgG production using an IgG ELISA (Figure 2-10). A minimum value of 5 standard deviations above background absorbance (shown as a red line) was set for each experiment as the threshold above which a well could be classed as producing IgG.

For all combinations of stimulants, 100% (64/64) of wells seeded with either 13 or 130 memory B-cells were classed as producers. Wells seeded with 1.3 memory B-cells however displayed varying numbers of IgG producing wells. The combination of IL-2 + R-848 + IL-21, produced antibody in 31.3% (15/48) of wells (Figure 2-10, panel A), whilst the combination of IL-4 + R848 + IL-21 had a production rate of 56.3% (27/48) of wells (Figure 2-10, panel B). When R-848 was omitted, IL-2 + IL-21 stimulated B-cells produced antibody in 64.6% (31/48) of wells (Figure 2-10, panel C) and IL-4 + IL-21 resulted in 70.8% (34/48) of wells producing IgG (Figure 2-10, panel D).

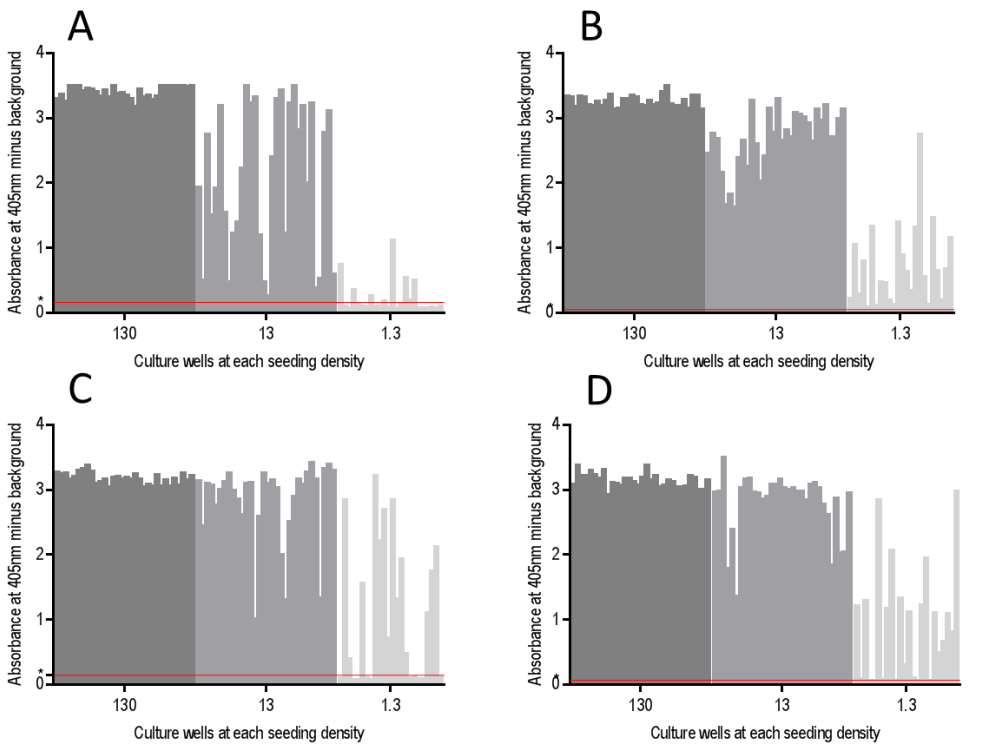


Figure 2-10| IgG ELISA data to indicate levels of antibody production from B-cell cultures using different combinations of stimulants

Purified IgG^+ memory B-cells were grown in co-cultures with irradiated LL8 feeder cells (31,250 cells per well) for 10 days. The production of IgG was tested for four different combinations of IL-2; IL-4; IL-21 and R848 from IgG^+ memory B-cell cultures seeded with either 130, 13 or an average of 1.3 cells per well. IgG secretion into the culture supernatant was measured on day 9 using the ELISA. The presence of IgG was identified by measuring the absorbance at 405nm (NB the maximum limit of absorbance at 405nm for the spectrophotometer was 3.5). Wells were classified as producing IgG in the A_{405} measured was greater than 5 standard deviation units above background absorbance for each assay. Graphs were generated from the data using GraphPad Prism 6.

A) IgG ELISA data recorded for B-cell cultures stimulated using 665ng/mL IL-2; 2500ng/mL R848 and 100ng/mL IL-21. **B)** IgG ELISA data recorded for B-cell cultures stimulated using 25ng/mL IL-4; 2500ng/mL R848 and 100ng/mL IL-21. **C)** IgG ELISA data recorded for B-cell cultures stimulated using 665ng/mL IL-2 and 100ng/mL IL-21. **D)** IgG ELISA data recorded for B-cell cultures stimulated using 25ng/mL IL-4 and 100ng/mL IL-21

The B-cell cultures were repeated with temporal separation of proliferation and differentiation signals to identify whether uncoupling of the two phases required for antibody production would increase total IgG yields. Individual combinations of stimulants were the same as previously described

with the exception of IL-21 being administered on day 5 of the cultures. An IgG ELISA was used to identify wells producing antibody. Wells with an absorbance greater than 5 standard deviation units above background absorbance for each assay (indicated by a red line) were classed as producing IgG (Figure 2-11). The maximum absorbance at 405nm recorded by the spectrophotometer was 3.5 which accounted for the plateaux on the graphs at this value. Every culture seeded with 130 memory B-lymphocytes produced antibody in 100% (48/48) of wells. A slight reduction in the number of producing wells seeded with 13 B-cells was observed for the combination of IL-2 + R-848 (day 0) + IL-21 (day 5), with 90.6% (29/32) producing above the threshold (Figure 2-11, panel A) and IL-4 + R-848 (day 0) + IL-21 (day 5), with 93.8% (30/32) of wells producing (Figure 2-11, panel B). The combination of IL-2 (day 0) + IL-21 (day 5) also had a reduction in the number of producing wells seeded with 13 B-cells, with 95.8% (23/24) of wells producing (Figure 2-11, panel C).

For wells seeded with 1.3 memory B-lymphocytes, the combination of IL-2 + R-848 (day 0) + IL-21 (day 5) resulted in 54.2% (13/24) wells producing (Figure 2-11, panel A). The mixture of IL-4 + R-848 (day 0) + IL-21 (day 5) generated 41.7% (10/24) productive wells (Figure 2-11, panel B). When R-848 was omitted from the mixture, a total of 16.7% (4/24) productive wells were observed for IL-2 (day 0) + IL-21 (day 5) stimulated cells (Figure 2-11, panel C) and 52.1% (25/48) productive wells for IL-4 (day 0) + IL-21 (day 5) stimulated cells (Figure 2-11, panel D).

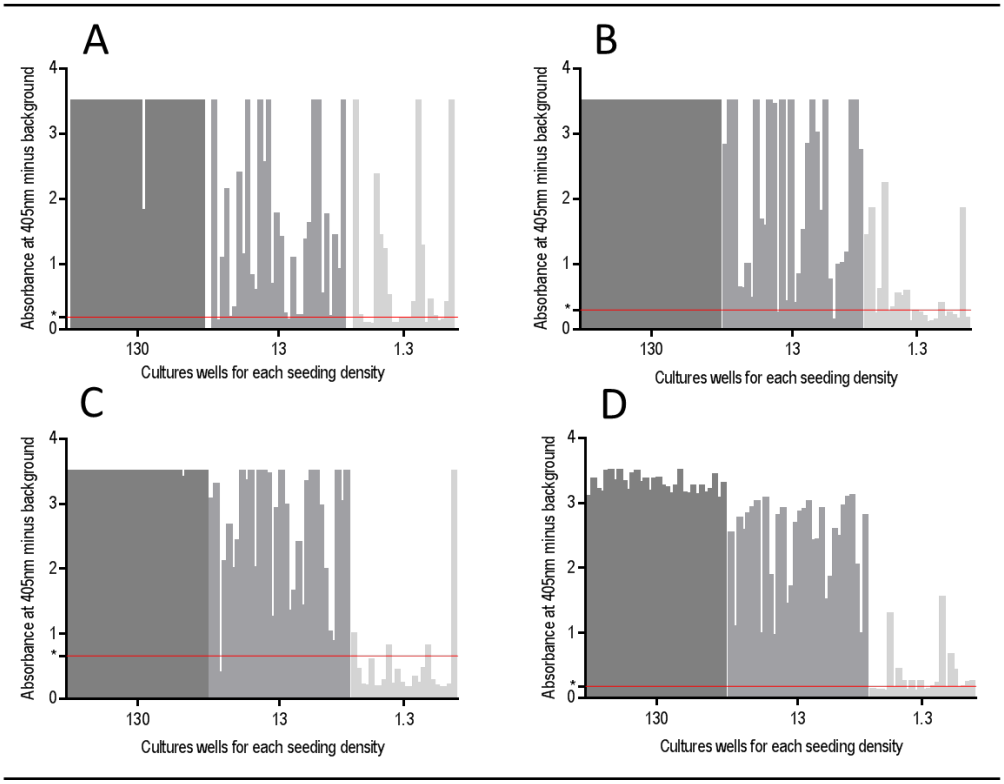


Figure 2-11|IgG ELISA data to indicate levels of antibody production from B-cell cultures using temporal variation of the different combinations of stimulants used in the previous experiment

Purified IgG^+ memory B-cells were seeded into wells containing irradiated LL8 feeder cells (31,250 cells per well) at either 130, 13 or an average of 1.3 B-cells per well. The co-cultures were incubated for either 10 or 16 days. The same combinations of IL-2; IL-4; IL-21 and R848 used in the previous experiment were tested, however R848 and IL-21 were added to the cultures on day 5. An IgG ELISA was used to measure secretion of antibody into the culture supernatant on day 15. Absorbance measurements at 405nm were recorded to indicate the presence of IgG (NB the maximum limit of absorbance at 405nm for the spectrophotometer was 3.5). Wells were classified as producing IgG in the A_{405} measured was greater than 5 standard deviation units above background absorbance for each assay. Graphs were generated from the data using GraphPad Prism 6.

A) IgG ELISA data recorded for B-cell cultures stimulated using 665ng/mL IL-2 (day 0); and 2500ng/mL R848, 100ng/mL IL-21 (day 5). **B)** IgG ELISA data recorded for B-cell cultures stimulated using 25ng/mL IL-4 (day 0); 2500ng/mL R848 and 100ng/mL IL-21 (day 5). **C)** IgG ELISA data recorded for B-cell cultures stimulated using 665ng/mL IL-2 (day 0) and 100ng/mL IL-21 (day 5). **D)** IgG ELISA data recorded for B-cell cultures stimulated using 25ng/mL IL-4 (day 0) and 100ng/mL IL-21 (day 5).

From these results, summarised in Figure 2-12, the combination of IL-4 and IL-21 added to the cultures on day 0, was the optimal stimulation strategy

for antibody production, measured as the percentage of antibody producing wells.

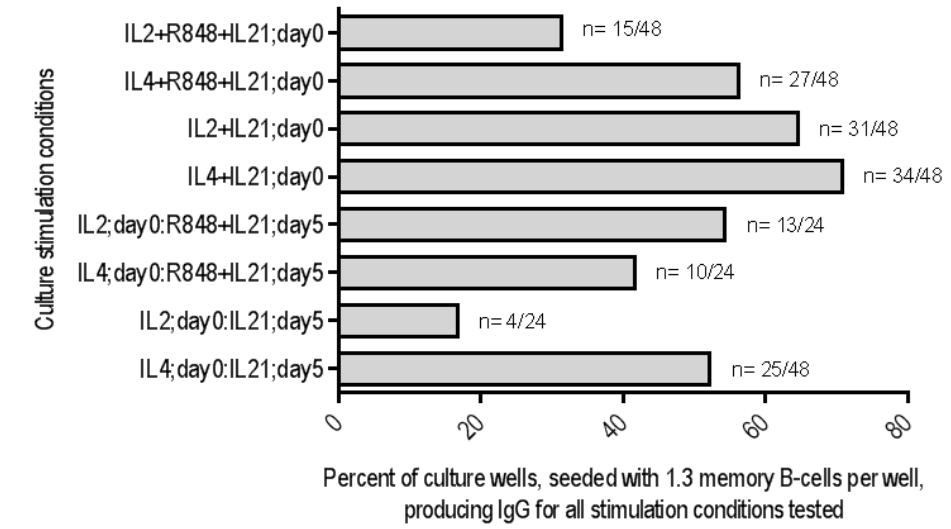


Figure 2-12|Summary of IgG ELISA data showing IgG production from memory B-cell cultures using different combinations of stimulants introduced to the cultures at different time points

The data were recorded for cultures seeded with an average of 1.3 memory B-cells per well and expressed as the number of wells for each condition producing IgG greater than 5 standard deviation units above background absorbance as a percentage of the total number of wells tested. The graph was generated using GraphPad Prism 6.

To test the requirement of IL-4 and IL-21 individually within the stimulation cocktail, memory B-lymphocyte cultures were established to test the effect of each cytokine. IgG^+ memory B-cells were grown in co-culture with irradiated LL8 cells. The cultures were established with memory B-cells seeded at either 80 or an average of 1.3 cells per well and incubated for 10 days. The cultures were stimulated on day 0 with either: 25ng/mL IL-4 and 100ng/mL IL-21; 100ng/mL IL-21 only; 25ng/mL IL-4 only or neither cytokine was added. A total of 44 wells for each condition tested were assayed

for antibody production using an IgG ELISA. Wells were identified as producing IgG if the A_{405} measurement was greater than 5 standard deviation units above background absorbance and expressed as a percentage of the total number of wells tested. The results are displayed in Table 2-4. For wells stimulated using both cytokines, 100% of the wells seeded with 80 B-cells produced detectable levels of antibody, whilst 62.5% of the wells seeded with an average of 1.3 B-cells produced antibody. Stimulation with IL-21 alone produced antibody in 25% of wells seeded with 1.3 B-cells. IL-4 stimulation alone produced antibody in 6.25% of wells seeded with 1.3 B-cells. The omission of both cytokines resulted in a failure to produce IgG from any wells.

Table 2-4| Summary of IgG ELISA data showing antibody production from B-cell cultures using different combinations of IL-4 and IL-21

Number of B-cells seeded	80	1.3	1.3	1.3	1.3
IL-4 (25ng/mL)	+	+	-	+	-
IL-21 (100ng/mL)	+	+	+	-	-
Percentage of wells producing antibody	100	62.5	25	6.25	0

IgG^+ memory B-cell cultures were stimulated using different combinations of IL-4 and IL-21 and antibody production measured using an IgG ELISA. The cultures were seeded with either 80 or an average of 1.3 B-cells per well and a total of 44 culture wells were assayed for each culture condition. Antibody producing wells were defined as wells recording an absorbance at 405nm greater than 5 standard deviation units above background absorbance and expressed as the percentage of IgG producing wells as a percentage of the total number of wells tested.

Following the addition of 25ng/mL IL-4 and 100ng/mL IL-21 at the beginning of a memory B-lymphocyte culture using an average of 1.3 cells per well, a time course experiment was established to sample IgG production in culture wells across the course of the culture period. Supernatants were tested for IgG production using an IgG ELISA after 5, 7, 9, 12 and 15 days of incubation. Wells which recorded an absorbance value greater than 5 standard

deviation units above the background absorbance (indicated by a red line) were classified as producing antibody (Figure 2-13 panels A to E). On day 5 post stimulation, 35.9% (23/64) of wells were producing antibody. This increased on day 7 to 68.8% (55/80) and again on day 9, to reach a maximum of 77.5% (62/80) of wells producing IgG above the background threshold. The number of antibody producing wells decreased after this point with day 12 recording 75% (60/80) of wells and on day 15 the number had dropped to 63.8% (51/80) of wells. The results for each day are summarised in Figure 2-13, panel F.

2.3.3 Polymerase Chain Reaction amplification of IgG variable domain sequences

Memory B-lymphocyte cultures were established with a seeding density of an average of 1.3 cells per well and stimulated to produce antibody using 25ng/mL IL-4 and 100ng/mL IL-21. Complementary DNA was reverse transcribed from mRNA extracted from cells within the culture after 10 days of incubation and used as a template for IgG variable domain gene specific PCR. Heavy, kappa and lambda chain reactions were carried out independently for five culture wells, B5; D10; F3; H3; I3. PCR products were analysed using agarose gel electrophoresis and visualised under UV radiation (Figure 2-14). All heavy chain specific reactions resulted in the presence of an amplified product (Figure 2-14, panel A). Kappa chain reactions generated products for two of the wells (Figure 2-14, panel B). Lambda chain reactions produced products

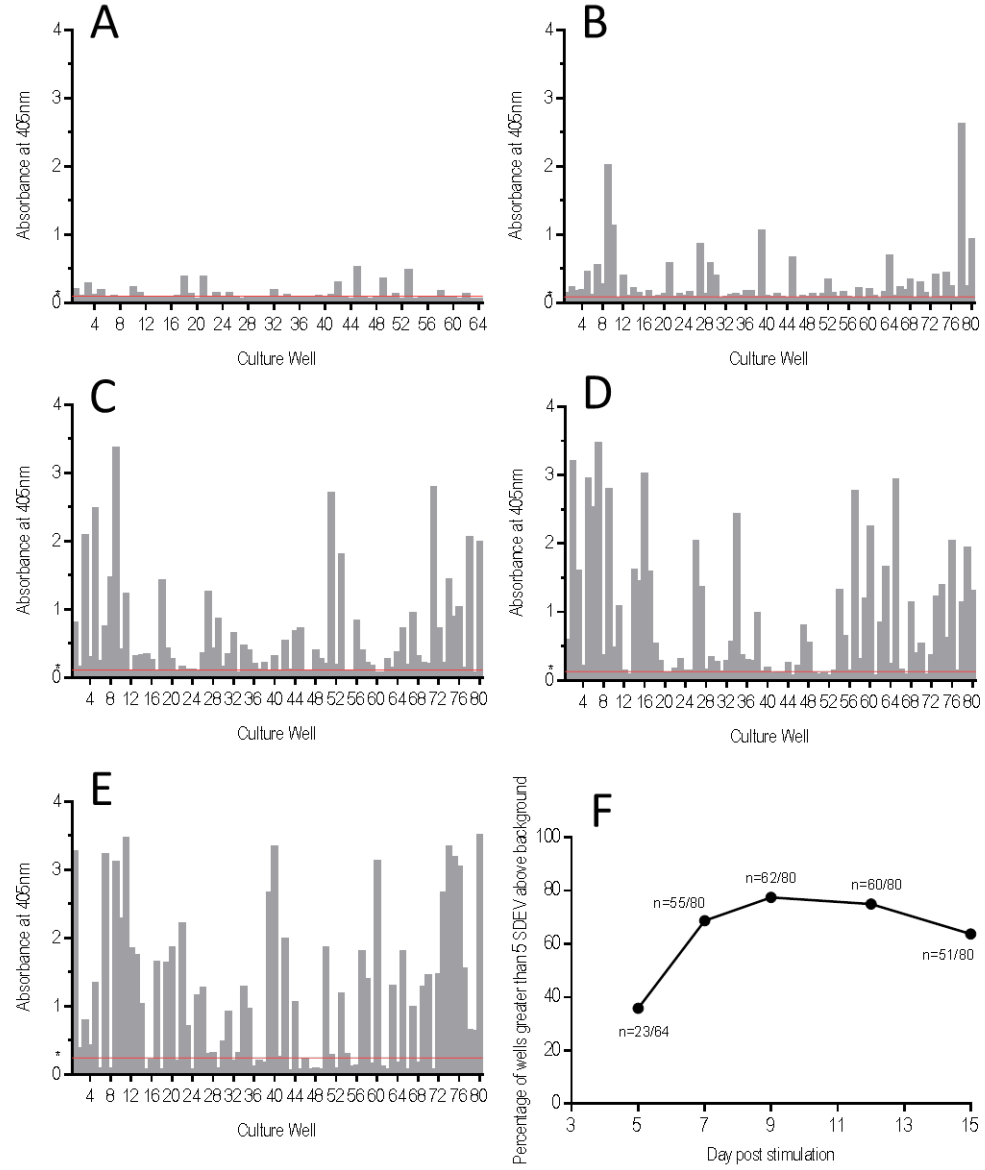


Figure 2-13| Time course experiment to measure IgG production from B-cell co-cultures during a 15 day culture period

The data were recorded for cultures seeded with an average of 1.3 memory B-cells per well and stimulated using 25ng/mL IL-4 and 100ng/mL IL-21. IgG secretion into the culture medium was measured using an IgG ELISA at 5 time points during the experiment. Positive wells were those recording an absorbance measurement for each condition producing IgG greater than 5 standard deviation units above background absorbance. Results were expressed as the number of positive wells as a percentage of the total number of wells tested. GraphPad Prism 6 was used to generate the graphs.

A) IgG ELISA data indicating antibody production from B-cell cultures measured on day 5. **B)** IgG ELISA data; day 7. **C)** IgG ELISA data; day 9. **D)** IgG ELISA data; day 12. **E)** IgG ELISA data; day 15. **F)** Summary of IgG production in B-cell cultures over a 15 day period.

from four wells (Figure 2-14, panel C). The results are summarised in Table 2-5.

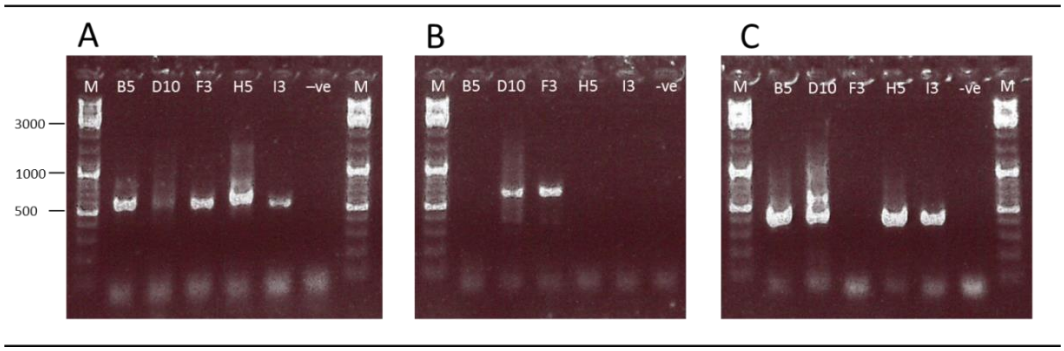


Figure 2-14| Amplification of IgG variable domains from B-cell cultures.

Messenger RNA transcripts were recovered on day 10 and reverse transcribed into cDNA using oligo$(dT)_{20}$ primers. The B-cell cultures were seeded with an average of 1.3 cells per well. PCR reactions were primed using the primers listed in tables 2-1 to 2-3. Negative control reactions omitted cDNA from the reaction mixture. The position of standards within the molecular weight marker (M) are shown in base pairs.
A) IgG variable domain amplification using heavy chain specific primers (Table 2-1). The expected size of a PCR product corresponding to heavy chain amplification was ~600bp. **B)** IgG variable domain amplification using kappa chain specific primers (Table 2-2). The expected size of a PCR product corresponding to kappa chain amplification was ~700bp. **C)** IgG variable domain amplification using lambda chain specific primers (Table 2-3). The expected size of a PCR product corresponding to lambda chain amplification was ~450bp.

Table 2-5| Summary of the results shown in Figure 2-14 of IgG variable domain amplification from B-cell cultures

Well	Heavy	Kappa	Lambda
B5	+	–	+
D10	+	+	+
F3	+	+	–
H5	+	–	+
I3	+	–	+
PCR –ve control	–	–	–

IgG^+ memory B-cell cultures, seeded with an average of 1.3 cells per well, were stimulated to produce IgG using 25ng/mL IL-4 and 100ng/mL IL-21. Variable domain PCR amplification was carried out for each culture well tested using primers specific for heavy, kappa and lambda chains in separate reactions.

PCR products for heavy chain and kappa chain variable domains were amplified from well F3, indicating that the well was seeded with a single B-cell. Wells B5, H5 and I3 produced PCR products for heavy chain and lambda chain variable domains, again suggesting a single cell seeding event. Well D10 however produced PCR products for all three variable domains, indicating a non-clonal seeding density.

Memory B-cell cultures were established using an average of 1.3 or 80 cells per well as previously described. Cell culture supernatants were harvested from between 6 – 10 wells for mRNA extraction and cDNA synthesis on culture day 6, 8, 10, 13 and 16. Gene specific PCR was carried out for the lambda chain variable domain. PCR products were analysed for the production of a band corresponding to variable domain amplification using agarose gel electrophoresis and visualised under UV radiation (Figure 2-15). For wells seeded with 80 memory B-cells: 100% (2/2) produced a band on day6; 50% (1/2) on day 8; 100% (2/2) on day 10; 100% (2/2) on day 13 (although these bands were very faint; 0% (0/1) on day 16 (Figure 2-15).

Wells seeded with an average of 1.3 memory B-cells yielded products for 25% (1/4) on day 6; 38% (3/8) on day 8; 88% (7/8) on day 10; 0% (0/6) on day 13; 33% (2/6) on day 16 (Figure. 2-15). The results are summarised in Table 2-6.

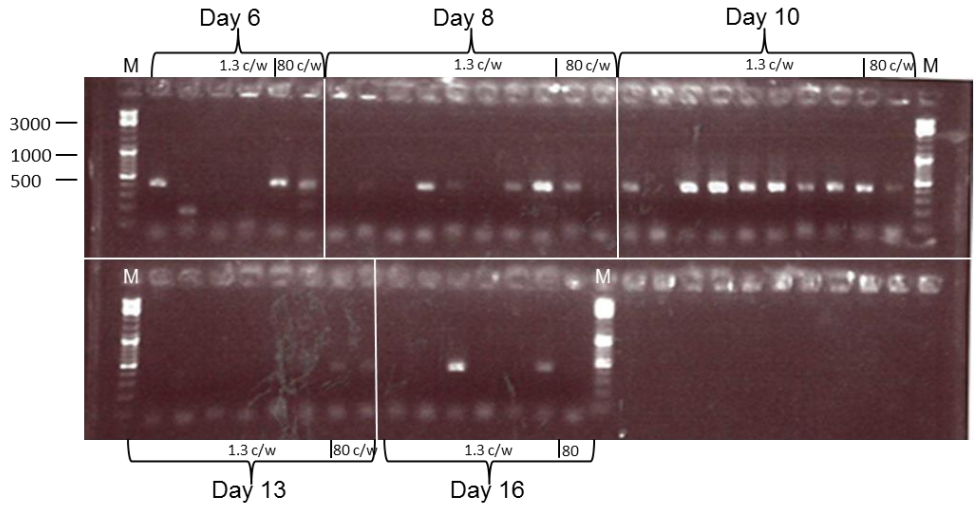


Figure 2-15| Amplification of lambda chain variable domains

The primers listed in Table 2-3 were used to amplify lambda chain variable domains from B-cell cultures seeded with either an average of 1.3 or 80 B-cells per well. All cultures were stimulated to produce antibody using 25ng/mL IL-4 and 100ng/mL IL-21 and incubated for 16 days. Messenger RNA transcripts were recovered on either day 6, 8, 10, 13 or 16 as indicated, and reverse transcribed into cDNA using oligo$(dT)_{20}$ primers. The position of standards within the molecular weight marker (M) are shown in base pairs. The expected size of a PCR product corresponding to lambda chain amplification was ~450bp.

Table 2-6| Summary of the results shown in Figure 2-15 of lambda chain variable domain amplification from mRNA harvested from B-cell cultures on different days

	Percent positive wells				
Number of B-cells per well	Day 6	Day 8	Day 10	Day 13	Day 16
80	100	50	100	100	0
1.3	25	38	88	0	33

IgG^+ memory B-cell cultures were seeded with either an average of 1.3 or 80 cells per well. The cultures were stimulated to produce IgG using 25ng/mL IL-4 and 100ng/mL IL-21. Lambda chain variable domain PCR amplification was carried out for each culture well tested on either day 6, 8, 10, 13 or 16. The lambda chain specific primers listed in Table 2-3 were used to prime the reactions. The results are expressed as the number of PCR positive wells for lambda chain amplification out of the total number of wells tested at each time point.

These results indicated that with multiple cells seeded, B-cells remain productive for mRNA extraction up until culture day 10, after which cell

viability reduces until reaching zero percent of wells producing mRNA by day 16. For wells seeded with a single B-cell, the viability of mRNA extraction increases up to a maximum of 88% on culture day 10. After this time point cell viability decreased indicating that culture day 10 was the optimum time for harvesting mRNA.

2.3.4 Sub-cloning IgG variable domains into an expression vector

Following on from variable domain PCR amplification, the next stage of the platform was to clone the PCR products into pORIgHIB huIgG1 and pORIgLIB huIgG1 expression vectors. In preparation for recombination cloning, the vectors were digested with restriction enzymes to linearize the plasmid DNA. pORIgHIB huIgG1 was digested using *HindIII* and *AfeI* and pORIgLIB huIgG1 with *BamHI* and *BsiWI.* Aliquots of digested and non-digested vector were analysed using agarose gel electrophoresis and visualised under UV radiation (Figure 2-16). Both parental vectors had been successfully digested (Figure 2-16, lanes 2 and 4).

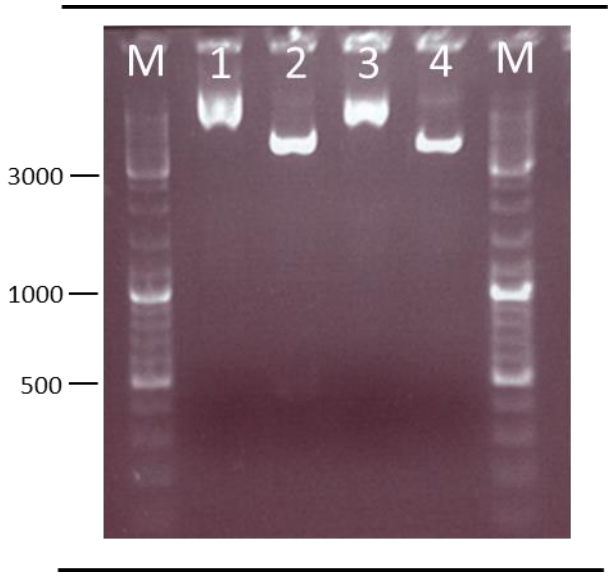


Figure 2-16| Agarose gel electrophoresis of linearised IgG expression vectors in preparation for homologous recombination cloning of IgG variable domains

The position of standards within the molecular weight marker (M) are shown in base pairs. Linearised plasmid DNA was expected to migrate further along the gel than supercoiled, undigested plasmid DNA.
Lane 1: pORIgHIB huIgG1 undigested. Lane 2: pORIgHIB huIgG1 digested using *HindIII* and *AfeI* restriction enzymes. Lane 3: pORIgLIB huIgG1 undigested. Lane 4: pORIgHIB huIgG1 digested using *BamHI* and *BsiWI* restriction enzymes.

In order to optimise the methods for cloning variable domains into the expression vectors, synthesised DNA encoding control mAbs, KZ52, 2G4, 4G7 and 13C6 heavy and light chain variable domains was chosen for sub-cloning rather than cell culture isolated PCR products corresponding to heavy and light chain variable domains. DNA for each control mAb was sub-cloned into the relevant pORIG expression vectors using homologous recombination and transformed into chemically competent *E.Coli* cells. Transformants were plated onto selective agar and incubated overnight at 37°C. Colonies were PCR screened for the presence of a band corresponding to the relevant IgG variable domain using primers that annealed at regions within the plasmid

vector that flanked the variable domain region. PCR products were analysed using agarose gel electrophoresis and visualised under UV radiation. An example for one control mAb, KZ52, is shown in (Figure 2-17). The results of variable domain specific PCR amplification showed that a variable domain had inserted into the expression vector for each colony tested.

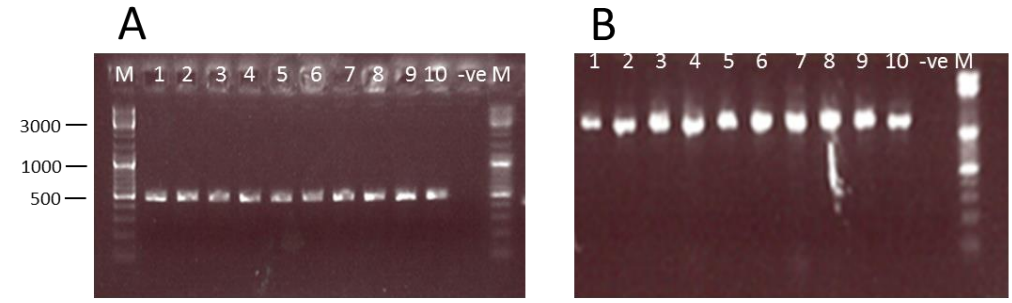


Figure 2-17| Cloning of KZ52 VH and VL domains into IgG expression vectors

DNA encoding each variable domain was synthesised commercially and sub-cloned into linearised pORIg expression vectors using homologous recombination. Plasmid vectors were transformed into chemically competent *E.* Coli cells, plated onto selective agar and incubated at 37°C overnight. The presence of an insert was indicated using PCR amplificationwith primers which spanned the variable region of the vector. The position of standards within the molecular weight marker (M) are shown in base pairs.

A) PCR amplification of DNA encoding the KZ52 V_H domain inserted within pORIgHIB huIgG1. The expected size of a band corresponding to a heavy chain insert was ~500bp. **B)** PCR amplification of DNA encoding the KZ52 V_L inserted within pORIgLIB huIgG1. The expected size of a band corresponding to a light chain insert was ~2000bp.

Bacterial colonies containing expression vector plasmids with the correct variable DNA sequence were identified using DNA sequence analysis. Plasmids were propagated and purified from bacterial cells and their DNA sequences re-analysed. Alignment of expression vector derived DNA sequence data with control mAb sequences verified the correct insertion of variable domains. An example, showing sequence alignment for the 4G7 heavy chain, is shown in Figure 2-18.

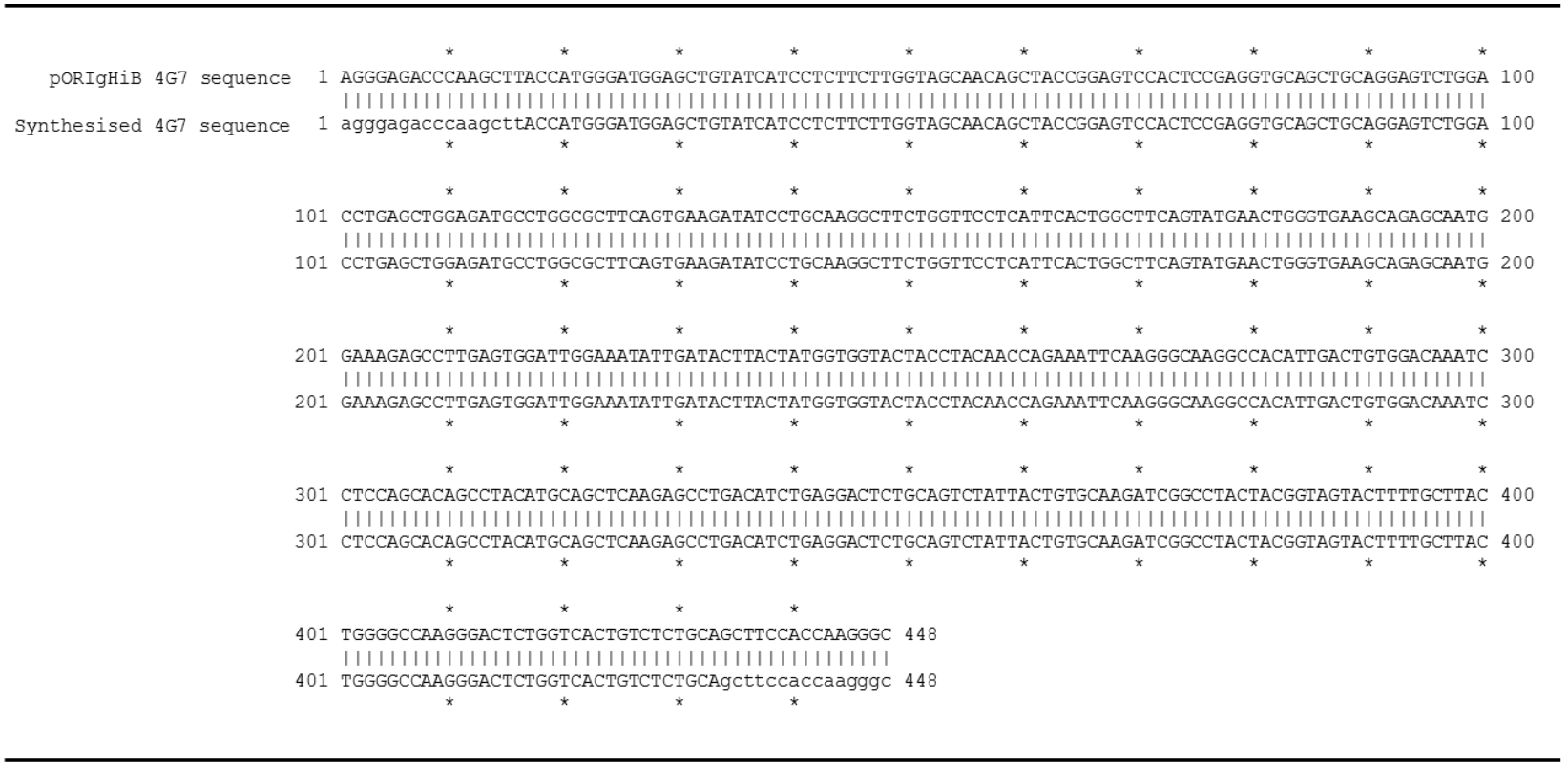


Figure 2-18| DNA sequence data alignment of the sub-cloned 4G7 variable domain with the corresponding synthesised DNA sequence

DNA encoding the heavy chain variable domain of control mAb 4G7 was sub-cloned into the pORIgHIB huIgG1 expression vector using homologous recombination. DNA sequence data derived directly from the expression plasmid was aligned with the 4G7 sequence that was synthesised in order to validate that the cloning procedure did not introduce error into the plasmid coding sequence.

Finally, plasmid derived DNA sequence data for all control mAb variable domains were analysed *in silico* using the International Immunogenetics (IMGT®) database (Brochet et al., 2008, Lefranc et al., 2009). IMGT/V-QUEST sequence alignments were carried out against known Ig genes deposited in the database. The results included highest percentage identity matches to germline V, J and D regions for heavy chain sequences and V and J regions for kappa and lambda chains. Complete sequence analysis was obtained, including annotation of framework and complementarity determining regions. Gene functionality was also identified; productive indicating that gene rearrangements contained no stop codons, had an in-frame junction and no defects in gene regulatory elements. Unproductive rearrangements result from; the presence of stop codons, frameshift mutations, out of frame junctions, and/or defects in gene regulatory elements. An example of 13C6 heavy chain analysis is shown in Figure. 2-19.

Result summary:	Productive IGH rearranged sequence: (no stop codon and in-frame junction) (a)		
V-GENE and allele	Homsap IGHV2-5*02 F	score = 802	identity = 75.26% (219/291 nt)
J-GENE and allele	Homsap IGHJ6*01 F (b)	score = 144	identity = 69.84% (44/63 nt)
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD5-18*01 F	D-REGION is in reading frame 3	
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.38.11]	[10.7.14]	CARRDPFGYDNAMGYW

```
                                  <---------------------------------- FR1-IMGT ----------------
13C6_VH                           caacttactctaaaagagtctggccct...gggatattgaagccctcacagaccctcagt
KF698731 Homsap IGHV2-5*02 F      --ga-c--ct-g--g--------t---...ac-c-gg----a---a------------cg
X62111 Homsap IGHV2-5*01 F        --ga-c--ct-g--g--------t---...ac-c-gg----a---a------------cg
L21964 Homsap IGHV2-5*05 F        --ga-c--ct-g--g--------t---...ac-c-gg----a---a------------cg
L21972 Homsap IGHV2-5*09 F        --gg-c--ct-g--g--------t---...ac-c-gg----a---a------------cg
L21969 Homsap IGHV2-70*01 F       --gg-c--ct-g-gg--------t---...-c-c-gg----a---a------------ca

                                  ----------------->_____________ CDR1-IMGT _____________<-----
13C6_VH                           ctgacttgttctttatctgggttttcactgagc......acttctggtgtgggtgtaggc
KF698731 Homsap IGHV2-5*02 F      -----c--ca-c--c--------c-----c---.........---ag---a--------g---
X62111 Homsap IGHV2-5*01 F        -----c--ca-c--c--------c-----c---.........---ag---a--------g---
L21964 Homsap IGHV2-5*05 F        -----c--ca-c--c--------c-----c---.........---ag---a--------g---
L21972 Homsap IGHV2-5*09 F        -----c--ca-c--c--------c-----c---.........---ag---a--------g---
L21969 Homsap IGHV2-70*01 F       -----c--ca-c--c--------c-----c---.........---ag---aa--t----ga--

                                  ---------- FR2-IMGT ------------------->_____________ CDR
13C6_VH                           tggtttcgtcagccttcagggaagggtctggagtggctggcactcatttggtgggat...
KF698731 Homsap IGHV2-5*02 F      ---a-c---------cc----a----cc-----------t----------at------...
X62111 Homsap IGHV2-5*01 F        ---a-c---------cc----a----cc-----------t----------at---a--...
L21964 Homsap IGHV2-5*05 F        ---a-c---------cc----a----cc-----------t----------at------...
L21972 Homsap IGHV2-5*09 F        ---a-c---------cc----a----cc-----------t----------at------...
L21969 Homsap IGHV2-70*01 F       ---a-c---------cc----------cc-----------t---------gat------...

                                  2-IMGT _________<-------------------------------------------
13C6_VH                           ......gatgataaatactataacccatccctgaag...agccaactctcaatctccaag
KF698731 Homsap IGHV2-5*02 F      ......---------gcg---c-g------t------...---agg---a-c---a-----
X62111 Homsap IGHV2-5*01 F        ......---------gcg---c-g------t------...---agg---a-c---a-----
L21964 Homsap IGHV2-5*05 F        ......---------gcg---cgg------t------...---agg---a-c---a-----
L21972 Homsap IGHV2-5*09 F        ......---------gcg---cgg------t------...---agg---a-c---a-----
L21969 Homsap IGHV2-70*01 F       ......---------------c-g-a----t------...-c-agg---a-c---------

                                  --------- FR3-IMGT -------------------------------------------
13C6_VH                           gatttttccagaaaccaggtattcctcaagatctccaatgtggacattgcagatactgcc
KF698731 Homsap IGHV2-5*02 F      --cacc----a---------gg----t-ca--ga----ca-----cc--tg--c--a---
X62111 Homsap IGHV2-5*01 F        --cacc----a---------gg----t-ca--ga----ca-----cc--tg--c--a---
L21964 Homsap IGHV2-5*05 F        --cacc----a---------gg----t-ca--ga----ca-----cc--tg--c--a---
L21972 Homsap IGHV2-5*09 F        --cacc----a---------gg----t-ca--ga----ca-----cc--tg--c--a---
L21969 Homsap IGHV2-70*01 F       --cacc----a---------gg----t-ca--ga----ca-----cc--tg--c--a---

                                  ----------->_________________ CDR3-IMGT _____________
13C6_VH                           acttactactgtgctcgaagagacccctttggttacgacaatgctatgggctattggggt
KF698731 Homsap IGHV2-5*02 F      --a--t--------a-ac---c
X62111 Homsap IGHV2-5*01 F        --a--t--------a-ac---c
L21964 Homsap IGHV2-5*05 F        --a--t--------a-ac---c
L21972 Homsap IGHV2-5*09 F        --a--t--------a-ac---c
L21969 Homsap IGHV2-70*01 F       --g--t--------a--g-t-c
```

Figure 2-19| Analysis of the 13C6 heavy chain variable domain using the Immunogenetics (IMGT®) Database

The IMGT/V-QUEST alignment software was used to align DNA sequence data derived from the cloned pORIgHIB huIgG1 expression to isolated sequences of known Ig genes deposited within the database. Productive gene rearrangements were recorded verifying that the sub-cloning procedure had not introduced errors into the expression vector.

2.3.5 Recombinant antibody expression

Following the cloning of control antibody variable domains into the expression vectors, the methods progressed into the next stage of the platform; to express the cloned variable domains as full length, functioning IgG molecules. HEK-293T cells were transfected with heavy and light chain expression vectors for each of the four control mAbs. Antibody expression was carried out for 48 hours before the culture supernatant was harvested and probed for antibody production using an IgG ELISA. A standard curve generated from standardised IgG concentrations in the ELISA allowed for the estimation of the concentration of each of the control mAbs (Figure 2-20).

All four antibodies were detected in cell culture supernatants although variable concentrations were indicated (Figure 2-20, panel C). Expression of mAb 13C6 produced the largest quantity of antibody at approximately 1.6μg/mL. KZ52 expression yielded approximately 1.2μg/mL. Concentrations of 2G4 and 4G7 were considerably lower at approximately 0.7μg/mL and 0.3μg/mL respectively.

As a means of platform development, these results demonstrated a proof of principle that single memory B-lymphocyte co-cultures can be suitably stimulated to produce measurable levels of antibody *in vitro.* The cell culture conditions maintain the production and viability of mRNA transcripts for IgG expression for up to 10 days of incubation, which was determined to be the optimum time point to harvest mRNA from the cultures for PCR amplification of variable domains. The cloning strategy was efficient and resulted in the production of specific IgG encoding expression vectors. Transient

transfections provided sufficient levels of recombinant IgG for *in vitro* analysis without the requirement for antibody purification.

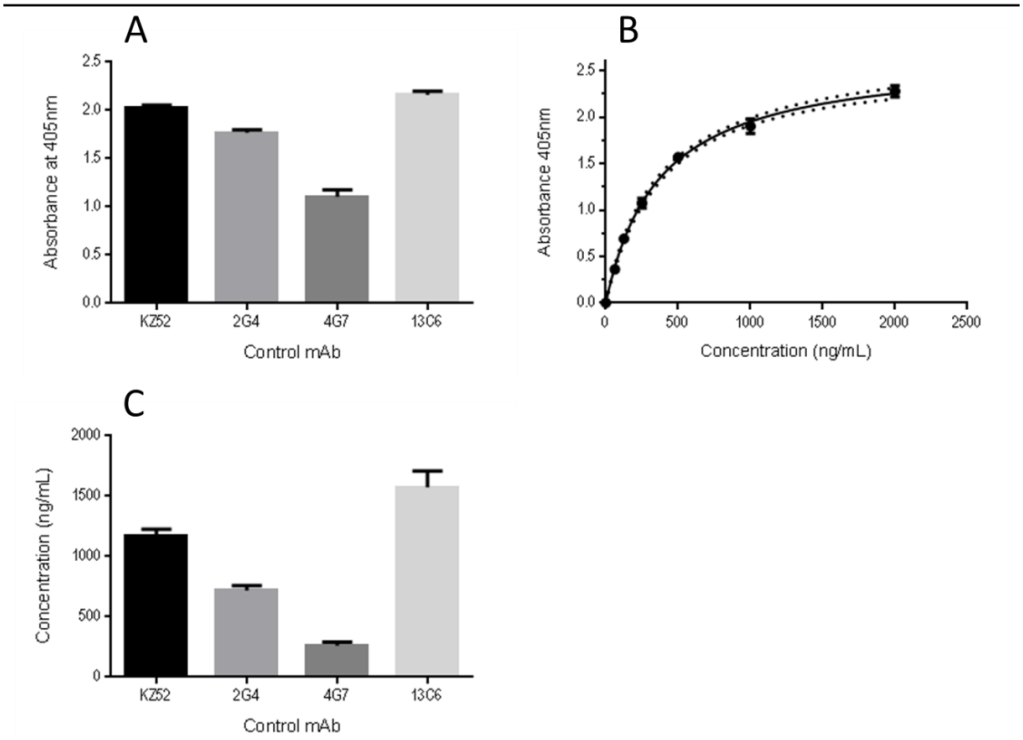


Figure 2-20| Recombinant IgG expression from transient transfections of HEK-293T cells with pDORIg expression vectors encoding mAbs KZ52, 2G4, 4G7 and 13C6

Transient co-transfections of HEK-293T cells were carried out using pORIgHIB huIg1 and pORIgLIB huIg1 vectors containing a heavy and light chain variable domain for each of the four control mAbs respectively. The cells were incubated for 48 hours following transfection and antibody secretion into the culture medium was measured using an IgG ELISA. In order to estimate the concentration of each antibody within the culture supernatant, a calibration curve was generated using a purified mAb dilution series to generate standard concentrations. Absorbance measurements for each cell culture expressed antibody were interpolated into the calibration curve data to produce concentration estimates.
A) IgG ELISA absorbance data for each of the four mAbs. **B)** Calibration curve data generated from a standardised concentration series of a purified monoclonal antibody. The concentrations of the standards were; 2000ng/mL, 1000ng/mL, 500ng/mL, 250ng/mL, 125ng/mL, 62.5ng/mL and 0ng/mL. **C)** Concentration estimates for each mAb derived from interpolation of IgG ELISA absorbance measurements.

2.3.6 EBOV GP binding interaction assay using control mAbs

Culture supernatants containing IgG from transient transfections of HEK-293T cells with KZ52, 2G4, 4G7 and 13C6 expression vectors were used in an EBOV GP binding assay to assess the interactions between the antibodies and immobilised EBOV GP_{1-2} derived from pseudo-virus producing HEK-293T cells (Figure 2-21). Each of the four control antibodies displayed binding to Ebolavirus GP_{1-2} but not to the vesicular stomatis virus glycoprotein control. The strongest interacting antibody was KZ52 (n=3; mean A_{405nm} = 3.4; SD=0.1). The non-neutralising mAb 13C6 demonstrated the next strongest binding interaction (n=3, mean A_{405nm} = 1.85; SD=0.54). Antibodies 2G4 and 4G7 exhibited very similar binding interactions (n=3, mean A_{405nm} = 1.0; SD=0.25 and 0.95; 0.2 respectively).

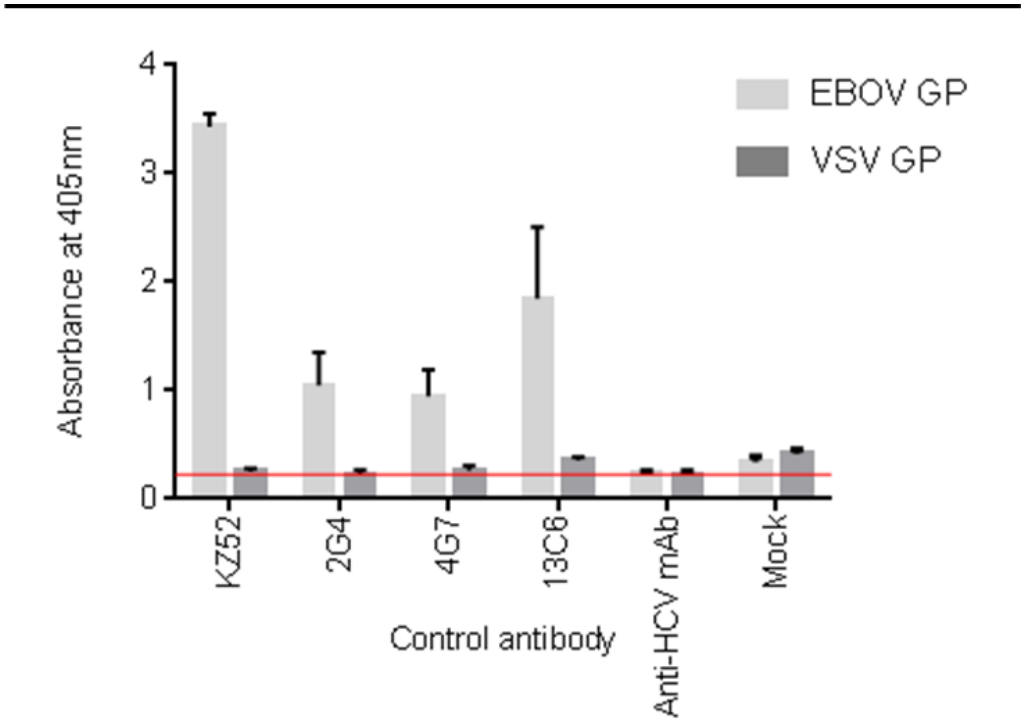


Figure 2-21 | EBOV GP binding assay using control mAbs produced from transient transfections of HEK-293T cells

The recombinant mAbs KZ52, 2G4, 4G7 and 13C6 were assessed for binding interactions with immobilised EBOV GP using a WGA ELISA. Ebolavirus pseudo-particle derived glycoproteins corresponding to the Mayinga EBOV GP GenBank accession NC_002549 were utilised during the assay. The anti-HCV negative control was AP33 used at 1μg/mL. The mock was provided by pseudo-particles devoid of EBOV GP.

2.4 Discussion

Historically, hybridoma cell lines and EBV immortalised B-cells have been the most frequently used technologies to produce human mAbs. Disadvantages with these techniques such as the requirement to generate stable cell lines and low transformation efficiencies of approximately 1-3%, (Stähli et al., 1980, Aman et al., 1984, Borrebaeck et al., 1988, Crain et al., 1989) have resulted in the development of alternative strategies for human mAb production; including cloning from single B-lymphocytes.

2.4.1 Feeder Cell Culture

Primary macrophages were the original cells investigated for establishing as co-culture feeder cells for memory B-lymphocytes. Several limitations were associated with using primary macrophages as feeder cells however. Firstly, a peripheral blood sample from a healthy donor was required from which to harvest primary monocytes to differentiate into macrophages and sample donation was not always available. Furthermore, yields of primary monocytes from PBMC were highly variable between donors, sometimes resulting in insufficient numbers of cells to attempt the differentiation into macrophage procedure (data not shown). Secondly, *in vitro* differentiation of monocytes into macrophages also resulted in variable yields of usable cells between experiments. The determination of a seeding density of $3x10^6$ irradiated macrophages per culture plate to achieve a monolayer with 95-100% confluence meant that the isolation of macrophages from a peripheral blood sample was a limiting factor in establishing memory B-lymphocyte cultures. A possible reason for the variability in differentiation efficiency could be the activation state of monocytes at the time of harvest (Arnold et al., 2015). Also,

carry through of activated T-lymphocytes from PBMC with the monocytes could result in inhibition of monocyte differentiation through the secretion of activation signals such as IL-4 or IL-13 (Ruppert et al., 1991, Hart et al., 1999). Coupled with this limitation was the tendency for primary macrophages to crash after a short incubation period without culture conditions changing between experiments, or indeed different culture plates (Figure 2-4). Due to these significant limitations, primary macrophages were not pursued as the source of feeder cells.

To circumvent the inherent limitations of using primary cells in co-culture with memory B-lymphocytes, an immortalised source of macrophages was investigated for its suitability as a feeder cell population. The cells chosen were THP-1 monocyte-like cells. Several procedures for differentiating these cells into macrophages were available in the literature (Tsuchiya et al., 1980, Auwerx, 1991, Park et al., 2007, Qin, 2012). A protocol for differentiation using the phorbol ester, PMA was adopted (Park et al., 2007), however reproducible *in vitro* differentiation was not achieved. Typical levels of differentiation in response to PMA are demonstrated in Figure 2-5. A number of studies have shown high degrees of variability in THP-1 differentiation using levels of surface CD-14 expression as an indicator of monocyte derived cell types. For example, a study by Aldo *et al*., (Aldo et al., 2013) demonstrated that THP-1 cells are highly sensitive to culture conditions in their response to PMA. The study showed that variation in factors such as cell concentration and the period of time at which THP-1 cells were maintained at a high cell density had a profound effect on the level of differentiation. Due to

the extremely low degree of inducible differentiation, THP-1 cells were not adopted as feeder cells for the memory B-lymphocytes.

Memory B-lymphocyte activation through ligation of surface expressed CD-40 receptors with CD-154 has been documented to stimulate B-cell proliferation and differentiation. A source of CD-154 presenting cells in the form of a stably transfected mouse fibroblast cell line, termed LL8 cells (Banchereau et al., 1994, Rancan et al., 2015), was kindly provided by Dr A Moosman. The cells were assessed for CD-154 expression using flow cytometry. It was established that within a given population of LL8 cells approximately 80% of fibroblasts maintained a surface population of CD-154 molecules for up to at least 12 passages in the absence of antibiotic selection. Following the twelfth passage for a population of LL-8 cells, antibiotic was introduced to the cultures to drive selection of CD-154 expressing cells.

The optimum seeding density of fibroblasts was established to be 31,250 cells per well in a 96 well culture plate, or $3x10^6$ cells per plate, to achieve a 95-100% confluent monolayer to provide a substrate on which memory B-lymphocytes could settle. The LL-8 cells grew readily in cell culture and could be maintained easily in 225mL culture flasks. The optimum seeding density of $3x10^6$ cells per plate allowed for enough cells to be generated in a 225mL culture flask to seed approximately 10 culture plates every 3-4 days. The level of gamma radiation required to drive the LL-8 cells into a quiescent state was taken from the literature (Wiesner et al., 2008) and did not require optimisation. Once plated, gamma irradiated LL-8 cells proved to be stable during incubation for at least 10 days with approximately 15% of cells expiring, as estimated using light microscopy.

In summary, LL-8 cells provided a stable source of CD-154 expressing cells that were easy to maintain in cell culture and proliferated rapidly to provide sufficient numbers of cells to seed 10 culture plates every 3-4 days if required. These characteristics contrasted with the inherent instability of primary monocyte derived macrophages. Although THP-1 cells would grow readily in cell culture, differentiation into macrophage like cells proved to be highly variable and inefficient. LL-8 fibroblasts were therefore selected as the source of feeder cells with which to establish memory B-lymphocyte co-cultures.

2.4.2 Memory B-lymphocyte Culture

Following the donation of a peripheral blood sample, PBMC were isolated and used as a source of memory B-lymphocytes. Non B-lineage lymphocytes were removed from PBMC by staining with commercially available magnetic beads conjugated to cluster of differentiation molecules typically expressed in surface membranes of non B-lymphocytes (Miltenyi Biotech, Bisley, UK). The two cell populations (non B-cell and B-cell) were separated using a column mounted within a magnetic field. Flow cytometric analysis of these cell populations showed that 99% of labelled non B-cells were removed during this purification step. The final purification step resulted in a pool of IgG expressing memory B-lymphocytes with a purity of 85%.

An alternative method of B-cell purification that has been described in the literature is the use of FACS to identify antigen specific memory B-lymphocytes expressing IgG as part of the B-cell receptor. For example, Weitkamp et al., (Weitkamp et al., 2003) utilised fluorescently labelled virus-like particles to sort rotavirus specific B-cells from a human donor into culture

plates before stimulating to induce antibody production. Amanna et al., (Amanna and Slifka, 2006) described a method to identify and quantify tetanus and diphtheria antigen specific B-lymphocytes from clinical samples, although a method of single cell sorting was not utilised. This method however can be hampered by nonspecific binding of some antigens (Doucett et al., 2005), although antigen specific labelling of memory B-lymphocytes can overcome this limitation. This in turn however limits B-cell selection to previously characterised antigens and removes the potential for identifying mAbs that bind to novel, undescribed epitopes.

The column purification procedures adopted during this study provided minimal levels of stress to the cells, increasing the level of cell viability at the end of the purification procedure compared to the use of flow cytometers (Mollet et al., 2008, Mollet et al., 2007, Dick, 2009, Xu et al., 2014a). The method could also be completed within approximately three hours from receipt of a blood donation, further reducing levels of stress to the memory B-lymphocytes. Another very important consideration in adopting the on-column purification method was that the cells were not pre-selected using a known target related antigen, for example a known antigen associated with a virus glycoprotein. This ensured that previously uncharacterised neutralising epitopes remained viable targets during B-cell cultures due to the non-discriminatory method of B-cell selection. This method required the use of a functional screen to characterise antibodies produced in B-cell cultures and will be described in the next Chapter. Also, labelling of the BCR with a magnetic bead could potentially result in up regulation of proliferation signals (reviewed in Xu *et al*., (Xu et al., 2014a)).

Following purification, memory B-lymphocytes were established into co-cultures with irradiated LL-8 feeder cells typically in 96 well culture plates. B-cells were seeded at clonal density in each well in order to produce monoclonal antibodies. For the purpose of method development however, because not all B-cells will be stimulated to produce detectable levels of antibody (Tiller et al., 2009), a percentage of culture wells were seeded with multiple memory B-cells in order to provide antibody production dependant only on the culture conditions. The use of a limiting dilution to seed culture wells was adopted based on the assumption that this would provide the least physical and mechanical stress to B-cells during the seeding process and also to maintain the lack of requirement for flow cytometry based techniques for B-cell isolation and seeding. Four culture conditions were assessed for their efficiency in inducing the production of IgG using combinations of three stimulants (in conjunction with LL-8 provided CD-154 stimulation): 1) IL2 + R-848 + IL-21. 2) IL-4 + R-848 + IL-21. 3) IL-2 + IL-21. 4) IL-4 + IL-21. The cocktails were assessed for levels of IgG production using an IgG ELISA after 9 days of culture. Wells were considered to be producers of antibody if the level of measurable IgG was greater than the absorbance equivalent to 5 standard deviation units above background. All culture conditions stimulated the production of IgG above threshold level in wells seeded with multiple B-cells indicating an apparent equivalency of induction for bulk cultures. Differences in stimulation efficiency were observed for cultures seeded with an average of 1.3 B-cells however. A reduced ability of some B-cells to expand and proliferate as they enter the memory pool (Pinna et al., 2009) suggests that not all memory B-cells will produce antibody in culture conditions. It was

therefore not expected to detect IgG in 100% of the wells tested. The optimum condition for producing the largest number of wells with detectable levels of antibody (70.8%) was the combination of IL-4 and IL-21. This level of stimulation was higher than has been reported for similar conditions. For example, Pinna et al., (Pinna et al., 2009) reported a 37% activation rate of single B-cells stimulated using a combination of IL-2 and R-848. In contrast to these results, inclusion of R-848 in the culture medium appeared to inhibit antibody production when combined with either IL-2 or IL-4. R-848 is a Toll-like receptor 7/8 agonist and has been shown to be a potent stimulator of nuclear factor –κB, (NF-κB) production in treated cells (Bishop et al., 2000). The effect could be due to TLR-7 activation through R848 ligation resulting in a dominating proliferation signal following NF-kB up regulation of proliferation inducing genes. R-848 has been shown to initiate both B-cell proliferation and antibody production *in vivo*, however the effect in *in vitro* single B-cell cultures could be to drive proliferation over differentiation.

The condition identified to generate the largest number of IgG producing wells was the combination of IL-4 and IL-21 administered together at the start of a culture period. The combination of IL-2 and IL-21 produced very similar results however, with a difference of only three wells above the threshold in the favour of IL-4. This suggests that the proliferative burst induced by the two cytokines is similar, however the number of IgG producing wells was greater with IL-4 in comparison with the equivalent conditions with IL-2 for three out of the four combinations tested.

Interestingly, driving single memory B-lymphocytes along the differentiation pathway though the administration of IL-21 in the absence of

IL-4, led to a larger number of wells producing IgG (25%) than when IL-4 was administered in the absence of IL-21 (6.5%). Presumably, because both culture conditions utilise CD-154 stimulation of the B-cells, CD-40 stimulation is sufficient to induce antibody secretion, however the level of antibody production is likely to be proportional to the initial stimulatory signal (Rush and Hodgkin, 2001).

Using the optimised combination of IL-4 and IL-21 added to the media at the establishment of the cultures, the time point at which maximum levels of IgG could be measured was determined to be day 9 (62/80 wells). The number of IgG producing wells decreased gradually to day 12 (60/80 wells) before dropping off more steeply on day 15 (51/80 wells). This trend was likely to be due to the memory B-cells expiring under the metabolic pressure exerted by the induction to secrete antibody and the optimal culture period that was determined was of similar duration to similar procedures reported in the literature (Walker et al., 2009, Huang et al., 2013a).

Taken together, these experiments identified the optimum culture conditions for single memory B-lymphocyte cultures to be the administration of IL-4 (25ng/mL) and IL-21 (100ng/mL) together at the start of a culture with the optimum time point for measuring IgG production being on day 9. These conditions provided detectable quantities of antibody produced in 71% of wells. This is comparable to levels of antibody produced using similar protocols documented in the literature. For example, Huang et al., (Huang et al., 2013a) report detecting antibody production in approximately 50% of wells using a similar stimulation conditions. A procedure utilising T-lymphocyte

culture conditioned media, reportedly produces antibody in approximately 73% of wells (Walker et al., 2009).

2.4.3 Polymerase Chain Reaction amplification of IgG variable domain sequences

IgG variable domain amplification using gene specific PCR was carried out for five memory B-lymphocyte culture wells, B5; D10; F3; H3; I3 seeded at clonal density using optimised stimulation medium. Cells were lysed on culture day 10 to liberate mRNA which was immediately reverse transcribed. The resulting cDNA was used as a template in the gene specific PCR. A 100% recovery rate of variable domain amplification was observed for the three primer sets. The sequences of the V_H primers were taken from those documented by Scheid et al., (Scheid et al., 2009a). The sense primers within the sets were designed to anneal further upstream within the leader region than those documented earlier (Wardemann et al., 2003, Tiller et al., 2008) which reported a much lower recovery rate. Although the primer sets used in this study displayed a high degree of coverage across different V-gene alleles, it should be noted that none of the combinations would provide complete coverage of all possible V-gene recombinations. These results however confirmed that all experimental methods from cell lysis through to gene specific PCR worked effectively for culture wells seeded with an average of 1.3 memory B-cells.

The combination of heavy and light chain variable domains that were amplified from culture wells B5, H5, I3 (heavy and lambda chain variable domains) and F3 (heavy and kappa chain variable domains) indicated that monoclonal antibody production was achieved in four out of the five wells

tested, resulting from single B-cell seeding event in these wells. PCR products corresponding to a heavy chain and both light chain variant variable domains were amplified from Well D10 however. This observation was only possible if multiple B-cells were seeded into this well. Without detailed characterisation of DNA sequence data (which was not undertaken) it was not possible to determine the number of B-cells seeded into well D10.

Lambda chain specific PCR was used to test the optimum time point for mRNA liberation from the B-cells in culture. It was clear from the results that culture day 10 was the optimum time to harvest mRNA from the cultures. Harvesting earlier than this time produced fewer amplified variable domains, presumably because a lower proportion of the cell population had not proliferated and/or differentiated. Later than this resulted in fewer amplified products because the cells had undergone necrosis under the high metabolic load exerted in the culture conditions. These results correlated with those observed for IgG production where the maximum antibody concentration was observed at day 9. The lower level of drop off in antibody concentration than that observed for PCR amplification of variable domains was due to the half-life of an IgG molecule being up to 11 days in culture (Mankarious et al., 1988) whereas the B-cells expired over a shorter time frame.

2.4.4 Sub-cloning IgG variable domains into an expression vector

Several studies have shown that rearranged heavy and light chain variable domains, isolated from single B-lymphocytes can be amplified using RT-PCR (Tiller et al., 2008, Volkheimer et al., 2007) to produce recombinant mAbs following cloning into a suitable expression system (Wardemann et al., 2003,

Tiller et al., 2008, Wrammert et al., 2008). In preparation for cloning, the two expression vectors pORIgHIB huIg1 and pORIgLIB huIg1 were linearised using restriction enzyme digestions. Each vector was digested with two enzymes (*HindIII* and *AfeI*; heavy chain and *BamHI* and *BsiWI*; light chain) in order to remove the existing variable domain from the IgG encoding region of the vectors. Digesting each vector with two enzymes ensured that sequences subsequently cloned into the vector would only be able to anneal with the correct polarity. Approximately 90-95% of total vector was digested, as assessed from the results of agarose gel electrophoresis.

All colonies tested using PCR for both KZ52 variable domains resulted in the amplification of a band of the correct size when visualised using agarose gel electrophoresis. Further verification of correct variable domain insertion into the expression vectors was achieved using DNA sequence analysis. The results confirmed the presence of the desired sequence by alignment with the original sequence. The final confirmation that a productive IgG variable domain had been inserted into the vector was achieved by analysing the cloned sequence using software developed by the International Immunogenetics Information System – IMGT/V-QUEST (Brochet et al., 2008, Lefranc et al., 2009). Analysis of DNA sequence data derived from sub-cloned expression vectors using the software, allowed for the sequences to be tested for productive rearrangements of antibody genes, the presence of premature stop codons, and that junction regions were in the correct reading frame. Framework and CDR sequences were also identified. Whilst this analysis was somewhat redundant for the control mAbs, it is invaluable for clinically derived sequences.

The results from sub-cloning the four control mAbs into the two expression vectors verified that the methods adopted worked efficiently and reproducibly and could be used for sub-cloning clinically derived IgG variable domains.

2.4.5 Recombinant antibody expression

The final method in the platform was to produce recombinant IgG molecules in mammalian cell culture in sufficient quantities to characterise for binding to target epitopes and ultimately neutralisation of virus particles. For the purpose of this method development chapter however, IgG characterisation experiments were not conducted. The expression vectors pORIgHIB huIgG1 and pORIgLIB huIgG1 were adopted because they allow for the expression of a complete IgG molecule from a mammalian cell culture system. The IgG_1 subclass is the most abundant in the serum (Schroeder and Cavacini, 2010). It can also form binding interactions with all three FcγR types and thereby possesses the ability to trigger effector functions such as ADCC and CDC (Jiang et al., 2011). It should be noted however, that in some cases, the initiation of effector functions proves to be undesirable. In these circumstances, the IgG_1 encoding region of the heavy chain vector could be replaced with one containing an IgG_4 constant domain, lowering the affinity of binding to FcγRI and FcγRII and completely abrogating interactions with FcγRIII (Jiang et al., 2011).

A mammalian cell line was chosen for IgG expression to ensure that the recombinant molecules were correctly glycosylated in order to induce correct folding of the IgG domains (Zheng et al., 2011). Glycosylation has also been implicated in IgG interactions with FcγR (Jiang et al., 2011). Transient co-

transfections with the heavy and light chain vectors were adopted in order to provide a method for IgG production in the shortest time-frame. The generation of stably transfected cells however would not be straightforward using separate pORIgHIB huIgG1 and pORIgLIB huIgG1 vectors due to only the heavy chain vector containing a mammalian cell suitable selection marker (conferring zeocin resistance). In the event of an IgG being identified and required for stable transfection, an expression vector containing both heavy and light chain constant domains and a zeocin resistance gene was available for use (Metheringham et al., 2009). Estimation of antibody concentration in cell culture supernatants using a standard curve and IgG ELISA demonstrated that antibodies could be produced between 300 and 1600 ng/mL. The variation in antibody concentration was likely due to variations during the transfection procedure rather than intrinsic differences in antibody sequences. This is because all IgG molecules shared the same transcription initiation signals and signal peptide sequences and therefore similar levels of expression within the cell and secretion into culture supernatant would be expected. The process of transient transfection to mAb harvesting and concentration estimates took 72 hours, thereby providing a rapid method of IgG production for characterisation.

All of the control antibodies, KZ52, 2G4, 4G7 and 13C6, produced from transient transfections of HEK-293T cells, demonstrated binding to immobilised Ebolavirus sGP in an ELISA binding assay. GP1-2 expressed in the context of a lipid bilayer derived from lysed pseudo-virus producer cells. All of the control antibodies used target conformational epitopes (Davidson et al., 2015) demonstrating that GP1-2 in the assay is folded with sufficient fidelity to present the conformational epitopes correctly.

The lower binding interaction of 2G4 and 4G7, which have been shown to bind the GP1-GP2 core region, could indicate that this area of the glycoprotein is somehow occluded slightly by the interaction of WGA used to anchor it to the assay plate. The stronger binding interaction of 13C6 suggests that the glycan cap region, containing the 13C6 epitope, adopts a more prone position for antibody binding. This would be somewhat counter intuitive however, due to the WGA lectin binding to the same glycosylated region. Furthermore, the strongest binding interaction was exhibited by KZ52. This antibody shares overlapping residues to 2G4 and 4G7 and has been shown to compete for binding due to the overlapping epitope footprint at the GP base. A simple steric occlusion effect is therefore unlikely to be the sole reason for the differences in apparent binding affinities. Non-specific binding encountered in the assay is unlikely however as none of the mAbs bound to the VSV-GP negative control. Binding affinities of the three antibodies KZ52, 2G4 and 4G7 could explain the differences in apparent binding in the assay. All three mAbs were used at the same standard concentration. Increasing the concentration of 2G4 and 4G7 could increase the binding interaction. This was not tested and would not have any relevance to binding assays carried out using clinically isolated mAb cocktails. Due to KZ52 showing the highest binding interactions, this antibody was used in all of the characterisation assays of clinically isolated mAbs described in Chapter 3.

3 Memory B-lymphocyte Platform Application: Neutralising antibodies against Ebolavirus

3.1 Introduction

The previous chapter described the optimised methods forming the basis of the platform for short term B-cell culture and recombinant antibody cloning and expression. In this chapter, an example of applying the methods to a specific target will be presented.

The results in Chapter 2 illustrated how the combination of methods lead to the isolation of recombinant monoclonal antibodies derived from the peripheral blood of a human donor. For the platform to have clinical relevance it was necessary to identify and isolate monoclonal antibodies with a particular therapeutic characteristic from a suitable donor. Following the outbreak of the West African Ebolavirus epidemic in December 2013, it was apparent that the technology developed in the memory B-lymphocyte platform could be applied in an attempt to isolate neutralising monoclonal antibodies against Ebolavirus from a suitable donor.

3.1.1 Source of blood sample

The source of blood sample from which to obtain human monoclonal antibodies against Ebolavirus was very important. A potential source of antibodies would have been a blood donation by a recent convalescent of Ebolavirus infection. Due to the requirement of Biosecurity Level (BSL) 4 facilities to work with potentially live Ebolavirus samples, coupled with the

logistical difficulties associated with the transport of convalescent blood samples from regions of the epidemic, an alternative source of anti-Ebolavirus antibodies was required.

A suitable alternative was provided in a blood donation kindly given by Professor Miles Carroll, a recipient of two trial anti-Ebolavirus vaccinations. The two vaccinations received by the donor were ChAd3 EBOZ (Stanley et al., 2014), co-developed by GSK and the NIH, and VSV-EBOV, developed by NewLink Genetics and Merck Vaccines USA in collaboration with the Public Health Agency of Canada (Marzi et al., 2011).

ChAd3 EBOZ utilises glycoproteins isolated from the Zaire strain of Ebolavirus responsible for the 2013-2015 outbreak to provoke an immune response in a vaccinated individual. The VSV-EBOV vaccination has been shown to produce a rapid immune response against Ebola virus glycoprotein derived from an isolate obtained from an outbreak in Kikwit, the Democratic Republic of the Congo in 1995 (Henao-Restrepo et al., 2015). The use of blood taken from a vaccinated donor entirely eliminated any possibility that the sample could contain live virus as well as removing the requirement to transport blood or blood products from areas affected by the epidemic and therefore the requirement for BSL 4 facilities. As described in previous chapters however, functional screening of the antibodies isolated using the platform is necessary to identify those with neutralisation potential. Using a live Ebolavirus functional screen would also require the work to be carried out at the highest biosecurity level negating any advantages of utilising a vaccinated donor. For this reason, an Ebolavirus pseudo-virus based neutralisation (entry inhibition assay), was employed to identify culture wells

containing IgG molecules with the ability to inhibit entry of particles into permissive cells. Psuedo-virus neutralisation assays have been described extensively in the literature and have been employed for a range of studies focused on enveloped viruses (Bartosch et al., 2003, Montefiori, 2009, Wright et al., 2009, Mohan et al., 2015, Urbanowicz et al., 2016) and reviewed by Carnell et al., (Carnell et al., 2015). The Ebolavirus pseudo-particles were constructed in producer HEK-293T cells following co-transfection with two expression vector plasmids. One plasmid encoded a HIV-1 core, comprising of retroviral *gag* and *pol* genes in conjunction with a luciferase reporter gene located between retroviral long terminal repeats (LTRs) and packaging signal (Ψ). The second expression plasmid encoded Ebolavirus envelope proteins (GP_{1-2}) corresponding to Zaire ebolavirus H.sapiens-tc/COD/1976/Yambuku-Mayinga 344 (Mayinga EBOV; GenBank accession NC_002549). The choice of envelope glycoprotein used to construct the pseudo-particles was made to reflect the glycoprotein delivered through VSV-EBOV vaccination (Marzi et al., 2011). This expression system allowed recombinant pseudo-typed particles to enter a permissive cell based on the envelope proteins employed, but prevented them from replicating within the cell. Cellular entry was measured by recording the relative luminescence provided by the reporter gene. The pseudo-typed particles therefore provided a suitable surrogate for wild type Ebolavirus as a method of characterising antibodies for neutralisation potential without the requirement to use live Ebolavirus.

This chapter describes the procedure of isolating memory B-lymphocytes from this donor and expanding them in cell culture to produce antibody

molecules that could be characterised *in vitro* for neutralisation of pseudo-typed Ebolavirus particles.

3.2 Materials and Methods

3.2.1 Memory B-lymphocyte culture

Cultures were established using memory B-lymphocytes purified from peripheral blood donated by a recipient of the ChAd3 EBOZ and VSV-EBOV trial vaccinations as described in Sections 2.2.1.1, and 2.2.2.1). For each experiment, eight 96 well culture plates were seeded with an average of 1.3 memory B-cells per well (88 wells/plate) and 13 memory B-cells per well (4 wells per plate) onto irradiated LL8 feeder cells (seeded with 31,250 cells per well) in a total of 200μL of RPMI 1640/FBS. Memory B-cells were stimulated to produce antibody using 25ng/mL IL-4 (R&D Systems, Abingdon, UK) and 100ng/mL IL-21 (R&D Systems, Abingdon, UK), which were added to the culture medium on day 0. Culture supernatants were assayed for IgG production (including an estimation of antibody concentration) on day 7 using the IgG ELISA method described in Section 2.2.2.4. The supernatants were also tested for neutralisation of Ebolavirus pseudo-typed particles on day 7 (see Section 3.2.5).

3.2.2 Amplification of IgG variable domains using IgG gene specific PCR

Messenger RNA transcripts were isolated and reverse transcribed using the methods described in Section 2.2.3.1. IgG variable domains were amplified using PCR as described in Section 2.2.3.2, using the primer sets listed in Tables 2-1 to 2-3. The amplification of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also included in the PCR to

indicate the effectiveness of mRNA extraction and RT-PCR. The primer sequences used for GAPDH amplification were;

Sense: 5' – CCGTATCGGTCGTATCGTTT – 3':

Anti-sense: 5' – TTTTGCGTTGCAGTAGTTGC – 3'.

PCR products were purified and analysed according to the methods described in Sections 2.2.3.3 to 2.2.3.6.

3.2.3 Cloning IgG variable domains into plasmid vectors

3.2.3.1 Sub-cloning IgG variable domains into pGEM-T Easy

When mixed DNA sequence reads were obtained from B-cell culture derived PCR products (arising from seeding B-cells at more than clonal density), the purified pools of mixed PCR products for heavy, kappa and lambda chain variable domains were ligated into pGEM-T Easy (Promega, Southampton, UK) at a 3:1 molar ratio of insert to vector in order to isolate individual PCR products. Reaction volumes of 10μL were assembled containing 3 units of T4 DNA ligase (Promega, Southampton, UK) and incubated at room temperature for 1 hour. Ligation products were transformed into *E. Coli* cells as described in Section 2.2.4.3, with the exception that agar plates contained 25μg/mL ampicillin to select for the pGEM-T Easy vector. After overnight incubation, colonies were screened by removing single colonies from the agar plates using a pipette tip. Each colony was used to inoculate a PCR mixture before transferring the remaining bacterial cells onto a labelled agar transfer plate containing 25μg/mL ampicillin. PCR mixtures were assembled in a total volume of 12.5μL containing 2.5pM of M13 sense and anti-sense primers, 2.5mM dNTPs (ThermoFisher, Loughborough, UK) and 0.3units HotStar Taq DNA Polymerase (Qiagen, Crawley, UK). Colonies were screened in a 3-step

cycling reaction consisting of 95°C for 15 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes. A final step of 72°C for 5 minutes completed the thermo-cycling. PCR products were detected using agarose gel electrophoresis as described in Section 2.2.3.3 and products of the desired size sequenced as per Section 2.2.3.5 (using an SP6 anti-sense primer). The resulting DNA sequence data were used to identify the different variable domains within the original pool. Each unique variable domain sequence was analysed using the Immunogentics Database as described in Section 2.2.3.6. DNA sequence data were translated to provide the amino acid sequence for each variable domain using the ExPASy translate tool, (Swiss Institute of Bioinformatics, Switzerland). Multiple amino acid sequences were aligned using PRALINE multiple sequence alignment software (Centre for integrated bioinformatics, the University of Amsterdam) (Heringa, 2002). Plasmid DNA was purified from bacterial colonies corresponding to each unique sequence using the methods described in Section 2.2.4.4.

3.2.3.2 Sub-cloning IgG variable domains into pORIg expression vectors

Each unique variable domain sequence was modified in preparation for sub-cloning into the pORIgHIB huIg1 and pORIgLIB huIg1 expression vectors. Second round PCR amplification was carried out using variable domain containing pGEM-T Easy vector DNA as a template using the methods described in Sections 2.2.4.1 to 2.2.4.4. Second round PCR primer sequences are displayed in Tables 3-1 and 3-2.

Table 3-1| Second round PCR primers for the modification of heavy, kappa and lambda variable domains in preparation for sub-cloning into pDORIg expression vectors

Sense	
5' HevZeb_Fcl_3	agggagacccaagctt**ACCATGGAC**TGGACCTGGAGGTTCCTCTTTGTGGTG
5’ HevZeb_Fcl_4	agggagacccaagctt**ACCATGGAC**TGGACCTGGAGGTTCCTCTTTGTGGTGGCAACATTACAAGGT
5' HevZeb_Fcl_5	agggagacccaagctt**ACCATGGAC**TGGACCTATGAAGCACCTGTGGTTC
5’ KapZeb_Fcl_4	taccgagctcggatcc**ACCATGAGG**GTCCCGGCTCAGCTGCTCTTCCTCCTGCTACTC
5’ LamZeb_Fcl_1	taccgagctcggatcc**ACCATGAGG**ATGGAGCTGTTCATCCTCTTCTTGGTAGCAACAGCTACCGGATCCTGGGCCCAGTCTGTGC
5’ LamZeb_Fcl_2	taccgagctcggatcc**ACCATGAGG**ATGGAGCTGTTCATCCTCTTCTTGGTAGCAACAGCTACCGGATCTCTCTCGCAGCTTGTGC
5’ LamZeb_Fcl_5	taccgagctcggatcc**ACCATGAGG**ATGGAGCTGTTCATCCTCTTCTTGGTAGCAACAGCTACCGGAGTGGATTCTCAGACTGTGG

Lower case sequences denote regions of homology with pDORIg expression vectors.
Sequences in bold font represent translation initiation signals.
All primers were synthesised commercially by Sigma Aldrich, Gillingham, UK.

Anti-sense	
3’ HevZeb_Rcl_1	gcccttggtggaggctGAGGAGACGGTGACC
3’ KapZeb_Rcl_1	gggccgctaccgtacgTTTGATTTCCACCTTGGTCCC
3’ LamZeb_Rcl_1	gggccgctaccgtacgCAGGACGGTGACTTTGGTCCC

Lower case sequences denote regions of homology with pDORIg expression vectors.
All primers were synthesised commercially by Sigma Aldrich, Gillingham, UK.

3.2.4 Recombinant antibody production and characterisation using a pseudo-virus based neutralisation assay

Recombinant antibody molecules were generated as described in Sections 2.2.5.1 and 2.2.5.2. Expression levels were analysed using an IgG ELISA according to the method in Section 2.2.2.4, with the exception that HEK-293T cell culture supernatant was screened in place of B-cell culture supernatant.

Recombinant antibodies were screened for their ability to neutralise Ebolavirus pseudo-particles. Neutralisation assays were carried out by Dr Richard Urbanowicz using the methods briefly described as follows;

HEK-293T cells were propagated as described in Section 2.2.5.1. Ebolavirus pseudo-particles were constructed following the co-transfection of plasmid DNA into HEK-293T cells. The plasmids used were:

i) pcDNA3.1, containing EBOV GP corresponding to Zaire ebolavirus H.sapiens-tc/COD/1976/Yambuku-Mayinga 344 (Mayinga EBOV; GenBank accession NC_002549),

ii) pTG126, containing a luciferase reporter gene,

iii) phCMV-5349, containing a MLV Gag/Pol-encoding packaging construct. Plasmids pTG126 and phCMV-5349 were kindly donated by Dr François-Loïc Cosset.

Transfections into HEK-293T cells were carried out using the procedure described in Section 2.2.5.2 with the exception that the cells were incubated for 72 hours.

Pseudo-particles were harvested by removing the supernatants and passing through a 0.45μm filter. Permissive HuH7 cells were seeded into 96

well white plates (Fisher Scientific, Loughborough, UK) at a density of 1 x 10^3 cells per well and infected using 100μL aliquots of pseudo-particle containing supernatants. Following infection, 200μL of DMEM/FBS were added to the cells which were incubated for 72 hours at 37°C with 5% CO_2. Following incubation, the medium was discarded from each well before cells were lysed using 50μL of Cell Lysis Buffer (Promega, Southhampton, UK) with gentle rocking for 15 minutes at room temperature. Luminescence was measured for each infection using a Fluorostar Optima plate reader (BMG Labtech, Aylesbury, UK). Typically, 50μL of luciferase substrate (Promega, Southampton, UK) were injected immediately before a 1 second luminescence measurement was recorded with a gain setting of 3600. Negative control readings were provided using pseudo-particles without surface expressed EBOV-GP.

3.3 Results

3.3.1 Memory B-lymphocyte cultures

The concentration of antibody produced in each culture well, seeded with memory B-cells at clonal density (total number of wells = 704), was estimated from a standard curve, generated using standardised concentrations of a monoclonal antibody, an example of which is presented in Figure 3-1, panel A. Absorbance measurements at 405nm were recorded for a 50μL aliquot of culture supernatant from each culture well using an IgG ELISA after 7 days of incubation. Standard curves were generated for each culture plate in order to estimate the concentration of IgG in each culture well. The data were presented as the total number of wells with an estimated antibody concentration falling within the ranges indicated in Figure 3-1, panel B. An inverse relationship between antibody concentration and number of wells was observed. The mean IgG concentration for all wells was 565ng/mL. A total of 56.3% (396 wells) of wells were estimated to produce IgG above the nominal concentration required for characterisation of 400ng/mL.

Following seven days of B-cell co-culture, a 50μL aliquot of culture supernatant was tested for the presence of neutralising antibodies using an Ebolavirus pseudo-particle neutralisation assay. Wells (seeded with an average of 1.3 B-lymphocytes: total number of wells = 704) were characterised for the presence of IgG molecules that inhibited Ebolavirus pseudo-particle entry into permissive HuH-7 cells following the incubation of pseudo-particles with B-cell culture supernatant. The entry of pseudo-particles into HuH-7 cells was reported by measuring cellular luminescence which was proportional to pseudo-particle entry.

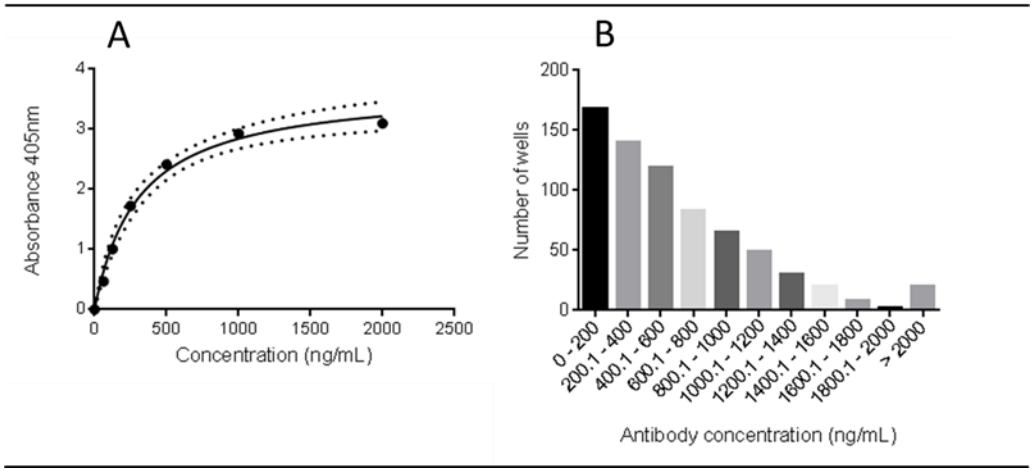


Figure 3-1| Summary of IgG expression from memory B-lymphocyte cultures seeded at clonal density.

Purified IgG^+ memory B-cells were seeded into wells of a 96 well culture plate containing irradiated LL8 feeder cells (31,250 cells per well) at an average of 1.3 cells per well. A total of 8 plates were used for the experiment. Antibody production was induced by the addition of 25ng/mL IL-4 and 100ng/mL IL-21 to the culture medium. The co-cultures were incubated for a total of 10 days and an IgG ELISA used to measure secretion of antibody into the culture supernatant on day 7. Absorbance measurements at 405nm were recorded to indicate the presence of IgG molecules. A calibration curve was generated for each 96 well culture plate using a dilution series of a purified mAb. Absorbance measurements for each B-cell culture well were interpolated into the calibration curve data to produce concentration estimates. Graphs were generated from the data using GraphPad Prism 6.
A) An example of a calibration for a 96 well culture plate. The concentrations of mAb standards were; 2000ng/mL, 1000ng/mL, 500ng/mL, 250ng/mL, 125ng/mL, 62.5ng/mL and 0ng/mL. **B)** Estimation of IgG concentration for each experimental memory B-cell culture well. The data were summarised as the total number of wells with an IgG concentration estimated to fall into the concentration ranges indicated.

Cellular entry data obtained for each culture well were expressed as a percentage of the cellular entry observed for an uninhibited control (Figure 3-2). The majority of antibody producing wells (97.7% (693/709)), fell into the range of 0-30% entry inhibition and were discounted. A small percentage of wells displayed entry inhibition within a range high enough to require further characterisation: In total, 1.4% (10/709) of wells fell into the range of 31-40% entry inhibition; 0.7% (5/709) in the range 41-50% and 0.1% (1/709) into the range of 51-60%.

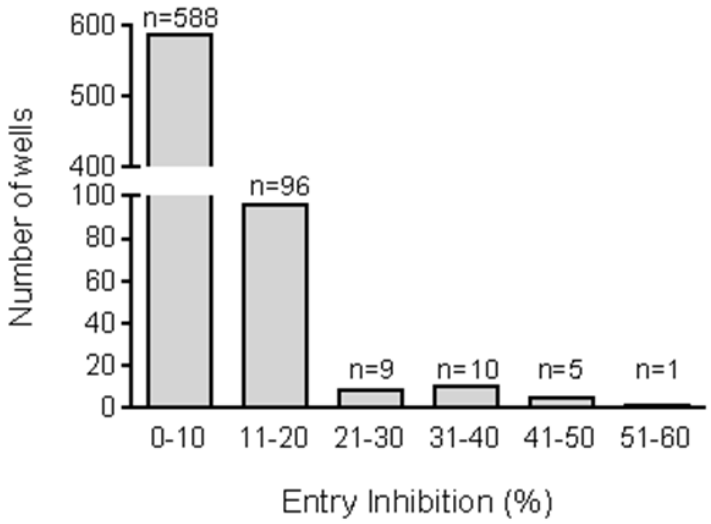


Figure 3-2| Summary of Ebolavirus pseudo-particle neutralisation data for IgG produced in memory B-cell cultures, expressed as percent entry inhibition.

An aliquot of B-cell culture supernatant was removed on day 7 and incubated with Ebolavirus pseudo-particles for 1 hour before transferring onto permissive HuH-7 cells. Pseudo-particle entry was quantified by measuring the luminescence of HuH-7 cells following the delivery of a luciferase gene encoded by the pseudo-particles. Entry inhibition was defined as the luminescence corresponding to each culture well expressed as a percentage of an uninhibited control. The data were plotted as the number of wells demonstrating a percentage entry inhibition within the groups displayed on the x axis. The graph was generated from the data using GraphPad Prism 6.

As demonstrated in Figure 3-1, a range of IgG concentrations were estimated for different culture wells, thereby introducing varying antibody concentrations into entry inhibition experiments. In order to assess the potency of neutralisation for IgG produced within each culture well, entry inhibition was calculated as a function of antibody concentration. Wells with an estimated concentration of less than 1ng/mL were omitted due to the low absorbance values corresponding to this concentration (A_{405} < 0.042). The results for each well were presented as total entry inhibition and entry

inhibition per nanogram of IgG (Figure 3-3). In order to assign values for entry inhibition per nanogram of IgG in this way, the assumption that a linear relationship existed between antibody concentration and the amount of neutralisation was made. This was not necessarily the case however and the values assigned must be considered as an estimate only. Although this did not provide a quantitative value, it did generate a second measurement of neutralisation for comparison of results between wells. Estimated values for entry inhibition per nanogram of IgG ranged from 0.1 to 4.4 (P7-G9).

From the neutralisation profiles shown in Figure 3-3, six wells; P1-B11, P1-E1, P4-H2, P4-H7, P7-A11, P7-G9, were targeted to take through to the next stage of the platform based on two selection criteria: 1) A percent entry inhibition per ng IgG greater than 1.0; and 2) A total entry inhibition value greater than 35% (due to the potential errors associated with estimating IgG concentration) and are indicated as red data points in Figure 3-3 and summarised in Table 3-1.

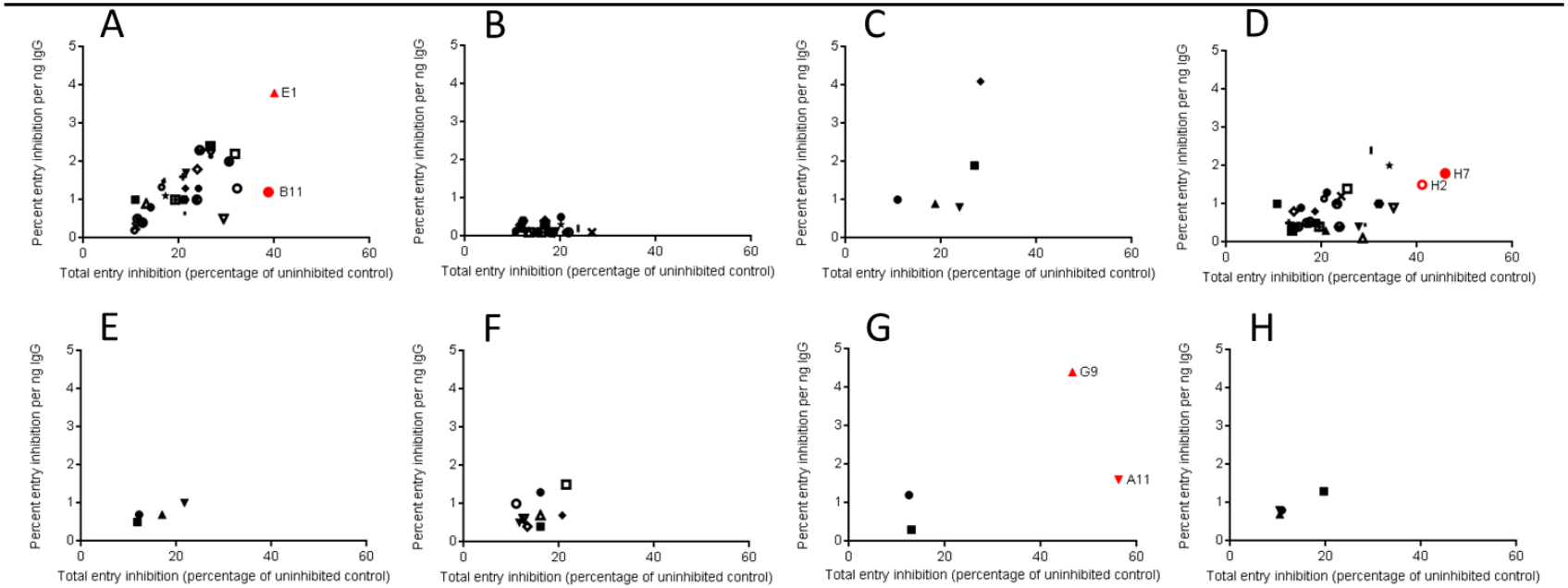


Figure 3-3| Ebolavirus pseudo-particle entry inhibition into permissive HuH-7 cells.

Pseudo-particles were incubated with memory B-cell culture supernatant for 1 hour at room temperature before adding to HuH-7 cells and incubating at 37°C with 5% CO_2 for 72 hours. Entry inhibition was recorded by measuring the luminescence exhibited by HuH-7 cells. The data were expressed as total entry inhibition and the percentage entry inhibition per nanogram of IgG as described in the text. The graphs were generated using GraphPad prism 6.

A) Memory B-cell culture plate P1. **B)** Memory B-cell culture plate P2. **C)** Memory B-cell culture plate P3. **D)** Memory B-cell culture plate P4. **E)** Memory B-cell culture plate P5. **F)** Memory B-cell culture plate P6. **G)** Memory B-cell culture plate P7. **H)** Memory B-cell culture plate P8.

Table 3-2| Summary of entry inhibition results for B-cell culture wells meeting the criteria for carry through to the next phase of the platform

B-lymphocyte Culture Well	Total Entry Inhibition (%)	Entry Inhibition per ng IgG (%)
P1-B11	38.8	1.2
P1-E1	40.0	3.8
P4-H2	41.1	1.5
P4-H7	45.9	1.8
P7-A11	56.2	1.6
P7-G9	46.6	4.4

A 50μL aliquot of memory B-cell culture supernatant was removed from the culture wells indicated. The antibody containing supernatant was tested for its ability to inhibit the entry of Ebolavirus pseudo-particles into permissive HuH-7 cells compared to an uninhibited control. The data were expressed as total entry inhibition as a percentage of the control. The same entry inhibition data were expressed as a function of the IgG concentration estimated for each well tested. The data were expressed as percent entry inhibition per nanogram of IgG.

3.3.2 Amplification of IgG variable domains

Variable domains for the heavy chain and both light chain variants were amplified in three separate reaction mixtures using gene specific PCR from cDNA isolated from the six culture wells (P1-B11; P1-E1; P4-H2; P4-H7; P7-A11; P7-G9) identified in Figure 3-3. A housekeeping gene (GAPDH) was also included as a control for mRNA extraction. PCR products were analysed using agarose gel electrophoresis and visualised using UV radiation. Positive controls for mRNA extraction/RT PCR were provided by including wells seeded with 13 memory B-lymphocytes (two per chain) in variable domain PCR amplification. PCR specific positive controls were provided by amplification of variable domains from plasmid DNA containing heavy and light chain variable domains. PCR products corresponding to the expected molecular weight were observed for variable domains for all three chain variants isolated from every well tested, with the exception of well P1-B11 kappa chain (Figure 3-4, panel C). It is not possible for clonal wells to produce

PCR products for both light chain variants, therefore this observation could only have arisen through either contamination of the PCR or from more than a single B-lymphocyte being introduced to the wells during seeding.

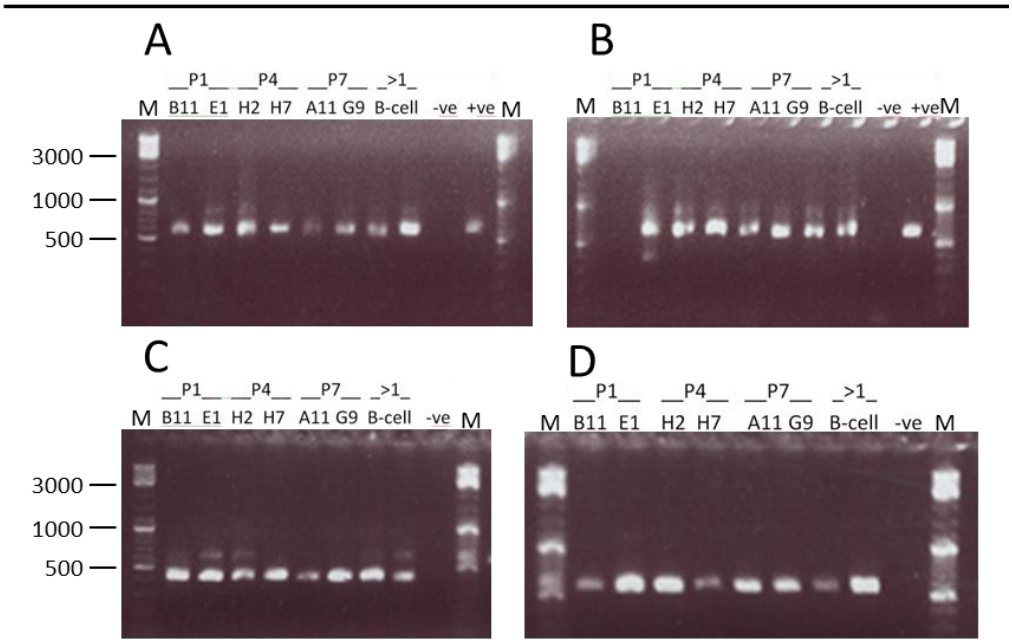


Figure 3-4| Amplification of IgG variable domains from B-cell cultures seeded with either an average of 1.3 or 80 cells per well.

Messenger RNA transcripts were isolated and reverse transcribed into cDNA using oligo$(dT)_{20}$ primers. on day 10. Variable domains were PCR amplified using the primers listed in tables 2-1 to 2-3. PCT amplification of GAPDH was included as a control for mRNA extraction/RT PCR. Positive control reactions used pORIg expression vector DNA containing KZ52 variable domains as template. Negative control reactions omitted cDNA from the reaction mixture. The position of the molecular weight standards within the marker (M) are shown in base pairs.
A) PCR amplification of V_H using the primer set in Table 2-1. The expected product size was ~600bp. **B)** PCR amplification of V_L kappa using the primer set in Table 2-2. The expected product size was ~700bp. **C)** PCR amplification of V_L lambda using the primer set in Table 2-3. The expected product size was ~450bp. **D)** PCR amplification of GAPDH. The expected product size was ~550bp

In order to identify whether the amplification of variable domains for all three chains was an artefact of contamination or seeding at greater than clonal density, all PCR products visualised in Figure 3-4 were submitted for DNA sequence analysis. Each PCR product returned a mixed sequence, but with varying degrees of apparent complexity. An example of the sequence data

obtained for a variable domain from each well is given in Figure 3-5. For the sake of clarity, only sequence data derived from V_H are shown. An arbitrary region of sequence data, corresponding to the beginning of FR1 to the end of CDR1, was selected to present for each heavy chain variable domain. All sequence data confirmed the presence of multiple sequences for each sample, however individual nucleotide sequences were different, showing that a polyclonal antibody mixture was present within the wells tested due to B-cells being seeded at greater than clonal density.

Individual variable domain encoding sequences were isolated for heavy, kappa and lambda chains. Well P1-E1 was chosen for further characterisation due to the apparent low complexity of the mixtures (Figure 3-5, panel B) coupled with relatively high neutralisation values (Table 3-1). Bulk PCR products, derived from first round PCR for each of the three chains (Figure 3-4) were cloned into the pGEM-T-Easy vector and transformed into chemically competent *E.Coli* cells. Individual colonies were selected as a template for PCR, thereby isolating individual variable domain encoding sequences. PCR products were analysed using agarose gel electrophoresis (Figure 3-6). In total, 94% (15/16: eg. H1) of colonies tested produced a PCR product of the correct size for a heavy chain variable domain (Figure 3-6, panel A). Kappa chain PCR was less efficient however, with only 50% (8/16: eg. K2) successful amplification (Figure 3-6, panel B). PCR using lambda chain specific primers produced variable domain encoding products in 94% (15/16: eg. L1) of colonies tested Figure 3-6, panel C).

Individual PCR products were sequenced in order to identify the total number of unique variable domains present. Nucleotide sequences for each unique variable domain were translated into the primary amino acid sequence.

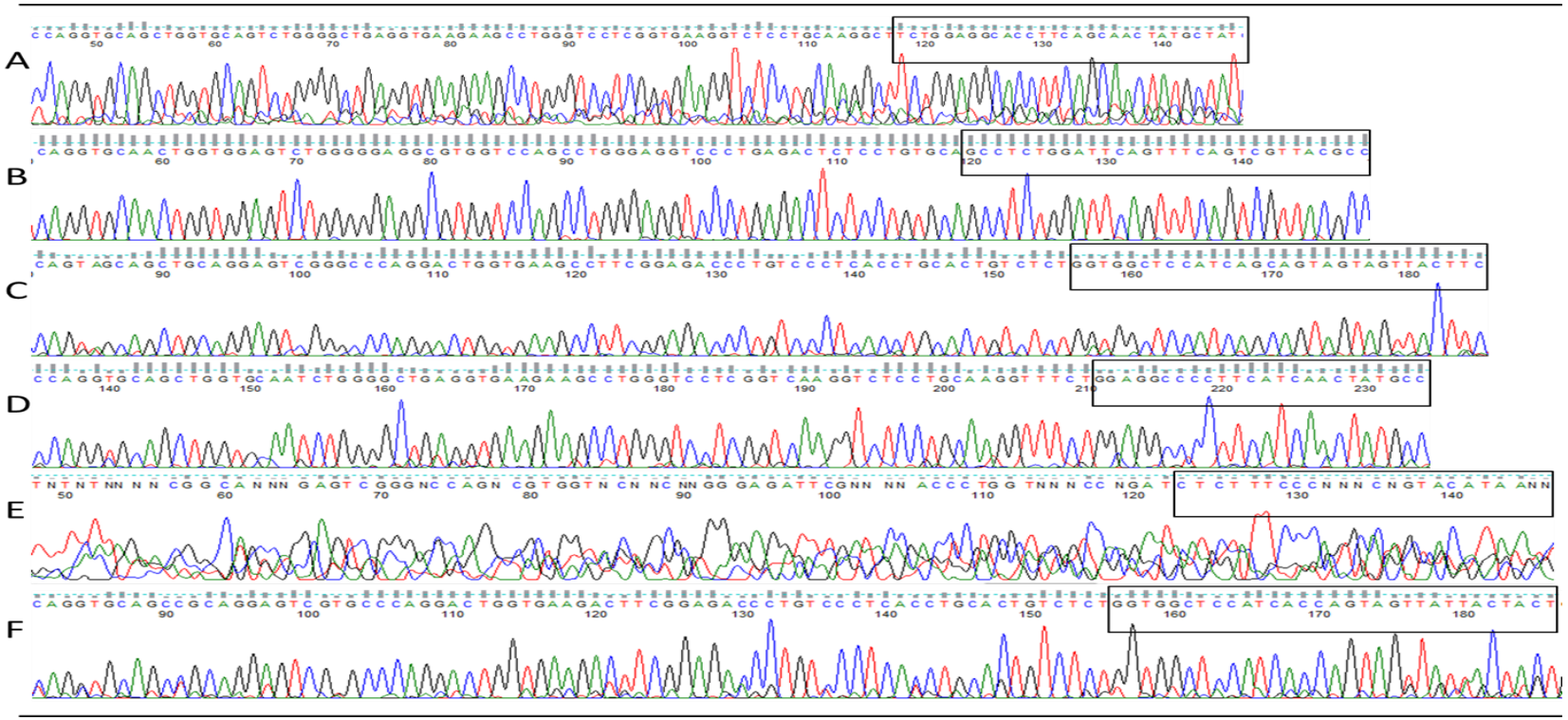


Figure 3-5| DNA sequence electropherograms of heavy chain variable domains

The sequence data span the region comprising FR1 to CDR1. CDR1 is indicated as a boxed region.
A) Sample P1-B11. **B)** Sample P1-E1. **C)** Sample P4-H2. **D)** Sample P4-H7. **E)** Sample P7-A11. **F)** Sample P7-G9.

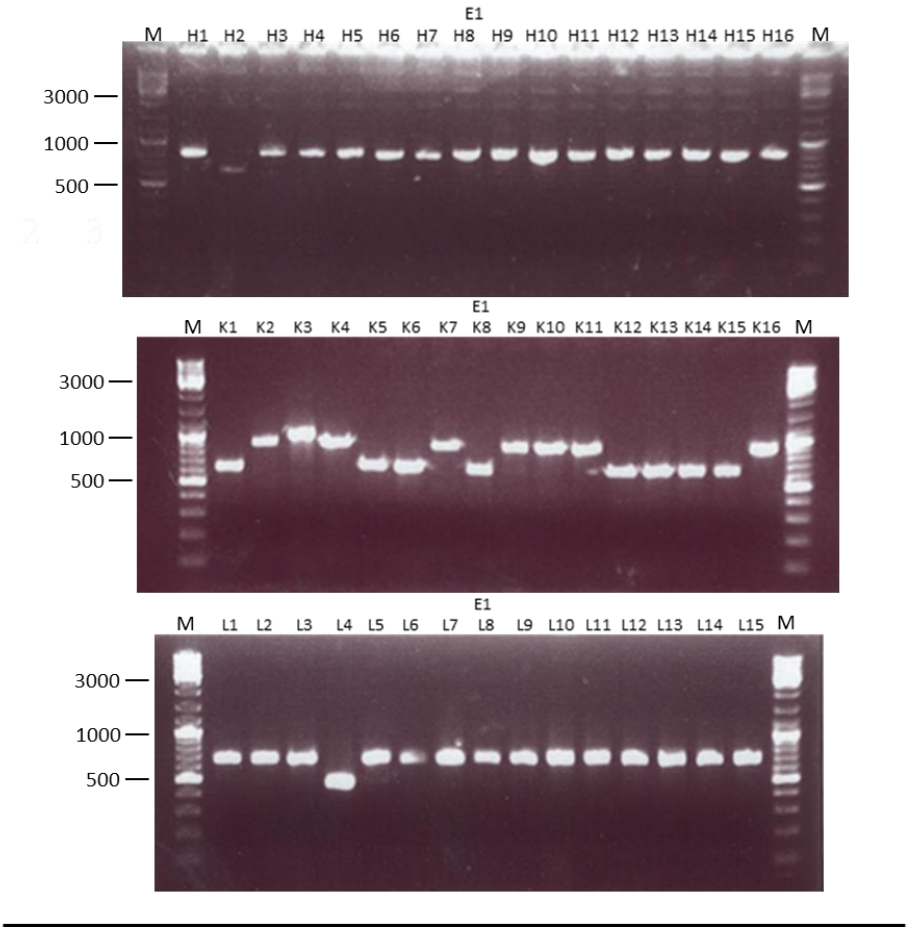


Figure 3-6| Amplification of IgG variable domains from PCR products derived from B-cell culture well P1-E1.

The mixture of variable domain encoding PCR products derived from B-cell culture well P1-E1 were sub-cloned individually into the pGEM-T Easy plasmid vector. Plasmid DNA was used as a template amplify individual sequences from the pool. pGEM-T Easy specific primers M13 were used in the reactions. The position of the molecular weight standards within the marker (M) are shown in base pairs.

A) PCR amplification of V_H. The expected product size was ~900bp. **B)** PCR amplification of V_L kappa. The expected product size was ~900bp. **C)** PCR amplification of V_L lambda. The expected product size was ~700bp.

Unique primary structures were then analysed for the degree of conservation between sequences within the sample pool for heavy (Figure 3-7), kappa (Figure 3-8) and lambda chains (Figure 3-9).

From the total of heavy chain samples, five unique sequences (H3; H4; H6; H7; H12) were obtained. Amino acid conservation between these five

sequences ranged from 59.3% (H3-H12; H3-H7) and 89.3% (H4-H6) (Figure 3-7).

Three unique kappa chain sequences were identified (K4; K10; K16). Amino acid sequence conservation for kappa chain variable domains was the highest out of the three variants, ranging from 90.1% (K4-K16) to 95.6% (K4-K10) (Figure 3-8).

The results obtained for the lambda chains were similar to those for heavy chains, with a total of five unique sequences (L2; L5; L6; L8; L9) being identified. Amino acid conservation ranged between 55.8% (L5-L8) and 90.7% (L2-L9) (Figure 3-9).

Despite the fact that the mixture of antibodies present in well P1-E1 could all potentially contribute to the neutralisation of Ebolavirus pseudo-particles observed during the initial screening, the variable domains isolated were cloned in order to determine whether the original neutralisation phenotype could be recreated by pairing V_H and V_L regions.

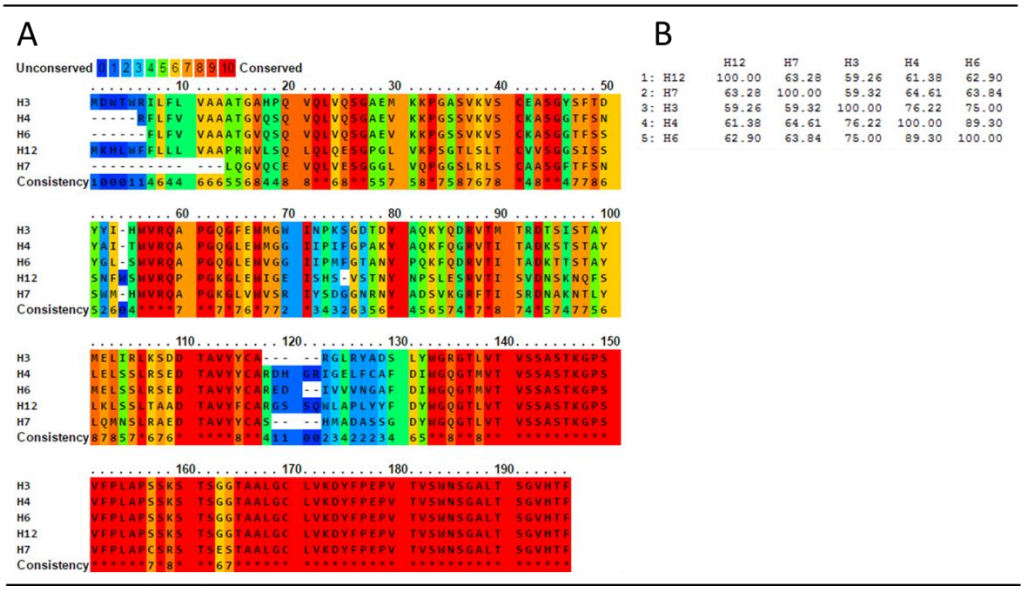


Figure 3-7| Amino acid sequence alignments for VH isolated from B-cell culture well P1-E1. DNA sequence data were translated using the ExPASy translate tool.

The resulting primary structures were aligned using PRALINE software to generate a heat map of sequence conservation. Percentage sequence identities were also calculated using the software

See text for references

A) Heat map of sequence conservation for the 5 unique heavy chain sequences.

B) Percentage identity matrix for the 5 sequences.

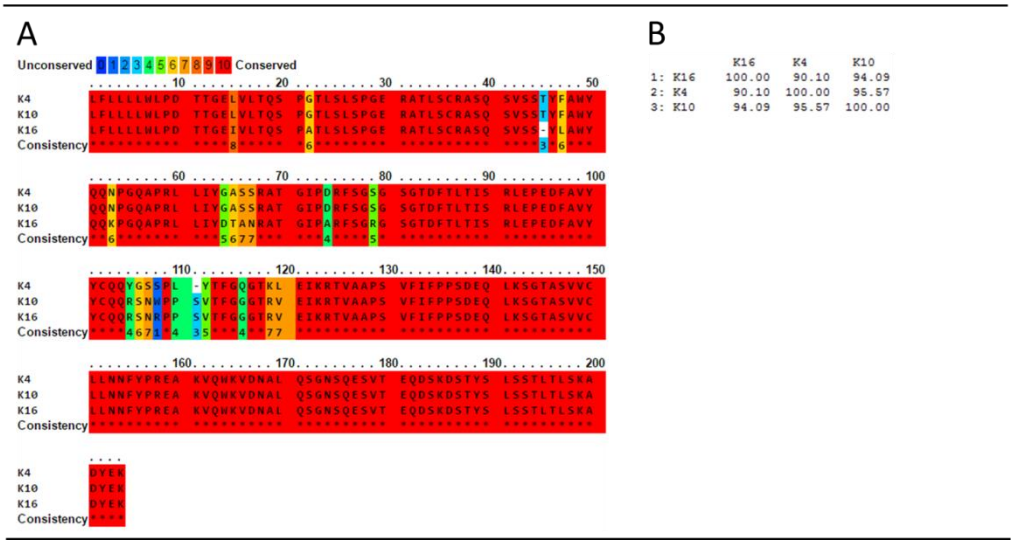


Figure 3-8| Amino acid sequence alignments for VL kappa isolated from B-cell culture well P1-E1.

DNA sequence data were translated using the ExPASy translate tool. The resulting primary structures were aligned using PRALINE software to generate a heat map of sequence conservation. Percentage sequence identities were also calculated using the software

See text for references

A) Heat map of sequence conservation for the 5 unique kappa chain sequences.

B) Percentage identity matrix for the 5 sequences.

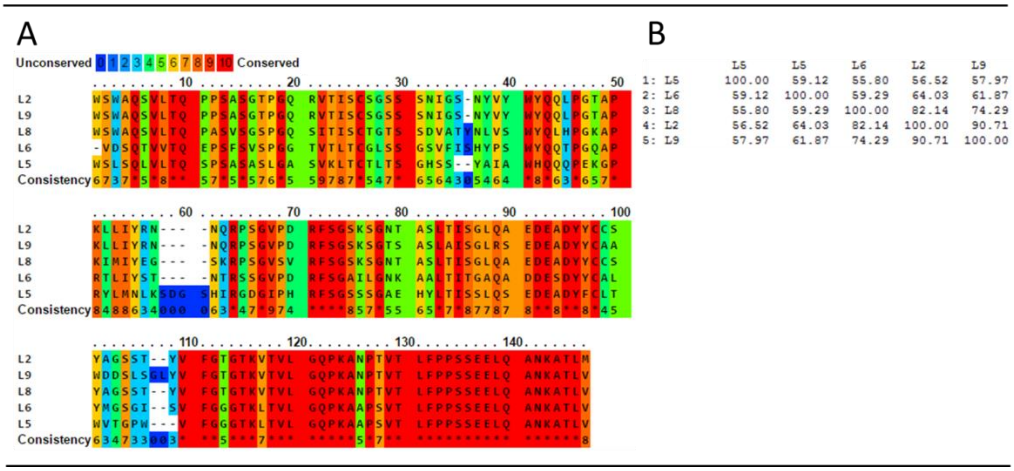


Figure 3-9| Amino acid sequence alignments VL lambda isolated from B-cell culture well P1-E1

The Expasy translate tool was used to translate DNA sequence data. The resulting primary structures were aligned using PRALINE software to generate a heat map of sequence conservation. Percentage sequence identities were also calculated using the software
See text for references
A) Heat map of sequence conservation for the 5 unique lambda chain sequences.
B) Percentage identity matrix for the 5 sequences.

3.3.3 Sub-cloning into expression vectors

In order to recreate the neutralisation observed in the initial functional screen, PCR products containing variable domain sequences identified in well P1-E1 mixtures were modified in preparation for sub-cloning into the pDORIg expression vectors. The modifications were required to insert a region of homology with the vector and a translation initiation/signal peptide sequence which was achieved using PCR (Figure 3-10). All products were successfully amplified with the exception of sample Lambda 5, which produced only very faint bands under UV illumination. The concentration of DNA produced for this sample was too low to allow for cloning and therefore sample L5 was not tested further.

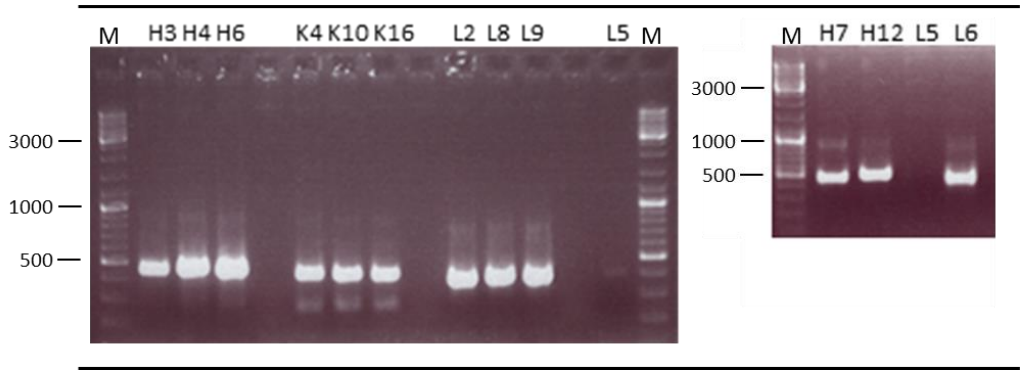


Figure 3-10| PCR modification of VH, and VL kappa and lambda in preparation for sub-cloning into expression vectors.

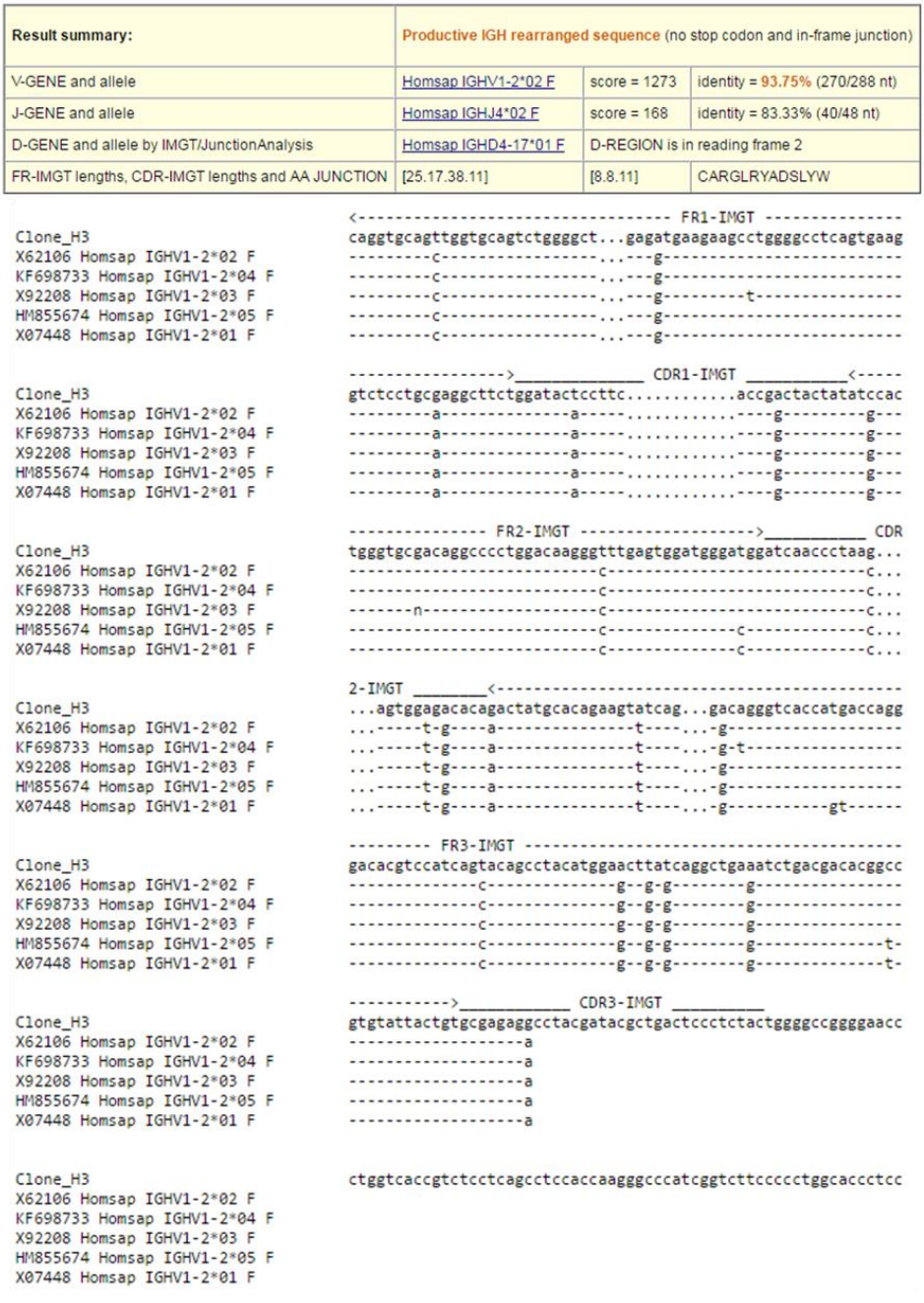
Immunoglobulin variable domains were PCR modified using the primers listed in Tables 3-1 and 3-2. Specifically, samples H3,H4,H6 were modified using HevZeb_Fcl3. H7 was modified using HevZeb_Fcl4 and H12 using HevZeb_Fcl5. The anti-sense primer HevZeb_Rcl1 was used for all products
Samples K4, K10 and K16 were modified using KapZeb_Fl4 and Kap_Zeb_Rcl1.
Lambda chain samples L3, L8 and L9 required LamZeb_Fcl_1. Sample L5 required LamZeb_Fcl2 and sample L6 required LamZeb_Fcl5. All lambda chain products were modified with the anti-sense primer LamZeb_Rcl1.
The position of standards within the molecular weight marker (M) are shown in base pairs.

Modified PCR products were cloned into the relevant expression vector. Three of the modified PCR products repeatedly failed to insert. These PCR products corresponded to clones; H7, H12, L6. Consequently these clones were unable to produce recombinant heavy or lambda chains.

To confirm that each variable domain had inserted into the expression vector with high fidelity, DNA sequence data were obtained from the vectors and aligned with the original sequence data. All variable domains aligned with 100% conservation to the original sequence.

Finally, sequence data obtained after sub-cloning into the expression vectors were tested for the presence of productive variable domains using the International Immunogenetics Information System IMGT/V-QUEST analysis program. As an example, a representative result (sample H3) is presented

showing productive VDJ rearrangement with in frame junctions and lacking premature stop codons and defects in regulatory elements (Figure 3-11). Similarly productive rearrangements were observed for all of the clones tested.



Result summary:	Productive IGH rearranged sequence (no stop codon and in-frame junction)		
V-GENE and allele	Homsap IGHV1-2*02 F	score = 1273	identity = 93.75% (270/288 nt)
J-GENE and allele	Homsap IGHJ4*02 F	score = 168	identity = 83.33% (40/48 nt)
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD4-17*01 F	D-REGION is in reading frame 2	
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.38.11]	[8.8.11]	CARGLRYADSLYW

```
                                <---------------------------------- FR1-IMGT ----------------
Clone_H3                        caggtgcagttggtgcagtctggggct...gagatgaagaagcctggggcctcagtgaag
X62106 Homsap IGHV1-2*02 F      ---------c------------------...---g--------------------------
KF698733 Homsap IGHV1-2*04 F    ---------c------------------...---g--------------------------
X92208 Homsap IGHV1-2*03 F      ---------c------------------...---g---------t----------------
HM855674 Homsap IGHV1-2*05 F    ---------c------------------...---g--------------------------
X07448 Homsap IGHV1-2*01 F      ---------c------------------...---g--------------------------

                                ----------------->_____________ CDR1-IMGT ___________<-----
Clone_H3                        gtctcctgcgaggcttctggatactccttc.............accgactactatatccac
X62106 Homsap IGHV1-2*02 F      ---------a--------------a-----.............----g---------g---
KF698733 Homsap IGHV1-2*04 F    ---------a--------------a-----.............----g---------g---
X92208 Homsap IGHV1-2*03 F      ---------a--------------a-----.............----g---------g---
HM855674 Homsap IGHV1-2*05 F    ---------a--------------a-----.............----g---------g---
X07448 Homsap IGHV1-2*01 F      ---------a--------------a-----.............----g---------g---

                                ---------------- FR2-IMGT ------------------->___________ CDR
Clone_H3                        tgggtgcgacaggcccctggacaagggtttgagtggatgggatggatcaaccctaag...
X62106 Homsap IGHV1-2*02 F      ---------------------------c------------------------------c...
KF698733 Homsap IGHV1-2*04 F    ---------------------------c------------------------------c...
X92208 Homsap IGHV1-2*03 F      -------n-------------------c------------------------------c...
HM855674 Homsap IGHV1-2*05 F    ---------------------------c--------------c-------------c...
X07448 Homsap IGHV1-2*01 F      ---------------------------c--------------c-------------c...

                                2-IMGT ________<-----------------------------------------------
Clone_H3                        ...agtggagacacagactatgcacagaagtatcag...gacagggtcaccatgaccagg
X62106 Homsap IGHV1-2*02 F      ...------t-g----a---------------t-----...-g------------------
KF698733 Homsap IGHV1-2*04 F    ...------t-g----a---------------t-----...-g-t----------------
X92208 Homsap IGHV1-2*03 F      ...------t-g----a---------------t-----...-g------------------
HM855674 Homsap IGHV1-2*05 F    ...------t-g----a---------------t-----...-g------------------
X07448 Homsap IGHV1-2*01 F      ...------t-g----a---------------t-----...-g----------gt------

                                --------- FR3-IMGT ----------------------------------------
Clone_H3                        gacacgtccatcagtacagcctacatggaacttatcaggctgaaatctgacgacacggcc
X62106 Homsap IGHV1-2*02 F      --------------c--------------g--g-g--------g----------------
KF698733 Homsap IGHV1-2*04 F    --------------c--------------g--g-g--------g----------------
X92208 Homsap IGHV1-2*03 F      --------------c--------------g--g-g--------g----------------
HM855674 Homsap IGHV1-2*05 F    --------------c--------------g--g-g--------g-------------t-
X07448 Homsap IGHV1-2*01 F      --------------c--------------g--g-g--------g-------------t-

                                ----------->____________ CDR3-IMGT __________
Clone_H3                        gtgtattactgtgcgagaggcctacgatacgctgactccctctactggggccggggaacc
X62106 Homsap IGHV1-2*02 F      -------------------a
KF698733 Homsap IGHV1-2*04 F    -------------------a
X92208 Homsap IGHV1-2*03 F      -------------------a
HM855674 Homsap IGHV1-2*05 F    -------------------a
X07448 Homsap IGHV1-2*01 F      -------------------a

Clone_H3                        ctggtcaccgtctcctcagcctccaccaagggcccatcggtcttcccccctggcaccctcc
X62106 Homsap IGHV1-2*02 F
KF698733 Homsap IGHV1-2*04 F
X92208 Homsap IGHV1-2*03 F
HM855674 Homsap IGHV1-2*05 F
X07448 Homsap IGHV1-2*01 F
```

Figure 3-11| Analysis of the heavy chain variable domain clone H3 using the Immugenetics (IMGT© Database).

DNA sequence data from pORIgHIB huIg1 with the H3 insert were aligned to known Ig sequences using the IMGT/V-QUEST software. Productive gene rearrangements were recorded.

3.3.4 Recombinant antibody production and characterisation for neutralisation potency

The variable domains that were successfully cloned into expression vectors were transiently transfected into HEK-293T cells to produce all possible heavy and light chain combinations. An aliquot of cell culture supernatant was taken to test antibody production using an IgG ELISA (Figure 3-12, panel A). Culture supernatants of transfections for all possible V_H/V_L pairings contained measurable levels of IgG. A standard curve, generated using standardised concentrations of mAbs, was used to estimate IgG concentration for each clone (Figure 3-12, panels B and C). Observed concentrations varied greatly from the highest, indicated at 2280ng/mL (H3L8) to the lowest at 57ng/mL (H4K4). Heavy chain 3 was expressed at greater than 1000ng/mL when combined with any light chain with the exception of kappa 4. Interestingly, heavy chains 4 and 6 produced low concentrations of IgG when combined with any kappa chain clone, whilst when paired with a lambda clone, the concentration more than doubled relative to the corresponding kappa clone (Figure 3-12, panel C).

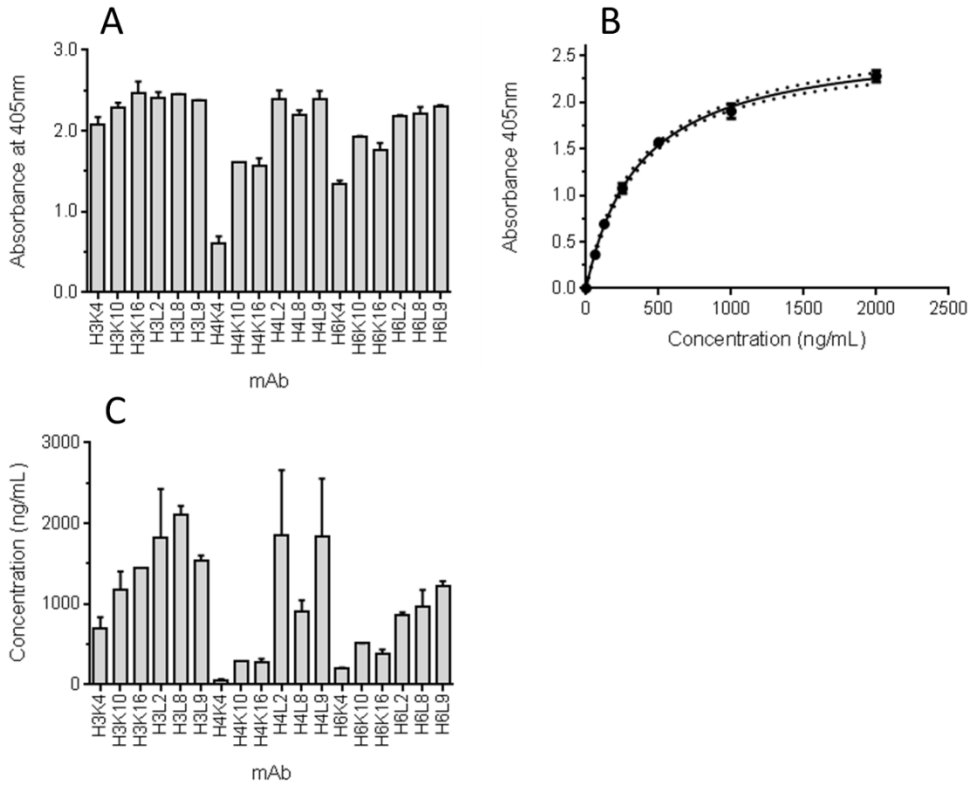


Figure 3-12| Expression of recombinant IgG from transient transfections of HEK-293T cells.

pORIgHIB huIg1 and pORIgLIB huIg1 vectors were transiently co-transfected with a heavy and light chain variable domain for each combination of cloned variable domains. Protein expression was carried out for 48 hours at 37°C with 5% CO_2. Antibody secretion into the culture medium was measured using an IgG ELISA. The concentration of each recombinant IgG was estimated from a calibration curve generated using a dilution series of purified mAbs. Absorbance measurements were interpolated into the calibration curve data to produce concentration estimates.
The graphs were generated using GrapPad Prism 6.
A) IgG ELISA absorbance data for each mAb expressed. **B)** Calibration curve data generated from a standardised concentration series of a purified monoclonal antibody. The concentrations of the standards were; 2000ng/mL, 1000ng/mL, 500ng/mL, 250ng/mL, 125ng/mL, 62.5ng/mL and 0ng/mL. **C)** Concentration estimates for each antibody derived from interpolation of IgG ELISA absorbance measurements.

Culture supernatants containing IgG from transient transfections were used in neutralisation assays to measure the entry inhibition potential for each antibody clone. Ebolavirus pseudo-typed particles were incubated with culture supernatants containing each IgG clone before incubating with permissive

cells. Infectivity was measured and plotted as a percentage of uninhibited control pseudo-virus (Figure 3-13, panel A). Four of the clones reduced infectivity to approximately 75% of the control (H3K4: 73.9%; H3K10: 72.9%; H3L8: 75.6%; H4K16: 73.7%;), corresponding to an entry inhibition of approximately 25%. The original entry inhibition observed for the parental B-cell culture well (P1-E1) was 40% (Figure 3-3, panel A and shown as a red line in Figure 3-13, panel A).

Entry inhibition as a factor of IgG concentration was calculated and plotted against the total entry inhibition measured for each clone (Figure 3-13, panel B). The highest entry inhibition per nanogram of IgG observed was 1.84 (clone H4K16). This clone recorded a total entry inhibition value of 23.4%. In comparison, the original neutralisation profile of the parental B-cell culture well; P1:E1 recorded an entry inhibition per ng IgG of 3.8% and a total inhibition of 40% (Figure 3-3, panel A).

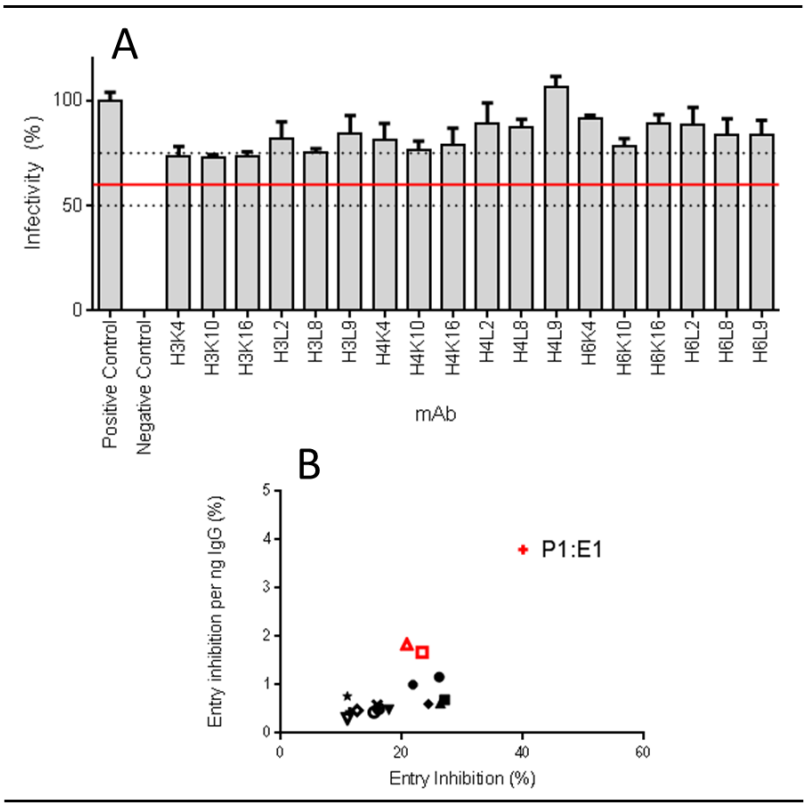


Figure 3-13| Ebolavirus pseudo-particle infectivity data.

Pseudo-particles were incubated with each recombinant mAb for 1 hour at room temperature before transferring to permissive HuH-7 cells. Entry of the pseudo-particles was quantified by measurement of HuH-7 intracellular luminescence following delivery of a pseudo-particle encoded luciferase gene. A positive control was provided by infecting HuH-7 cells with pseudo-particles in the absence of antibody and was used to normalise the data. The negative control was pseudo-particles incubated with 50ng KZ52. The graphs were generated from the data using GraphPad Prism 6.

A) Entry inhibition data for each recombinant mAb that was cloned. The data were expressed as percent infectivity of an uninhibited positive control (normalised to 100%). Broken lines indicate infectivity of 50% and 75%. The red line denotes the infectivity of Ebolavirus pseudo-particles when incubated with memory B-cell culture supernatant from the initial screening of culture well P1-E1, from which the panel of mAbs (H3K4 to H6L9) was derived. The original entry inhibition was 40%, corresponding to an inhibition of 60%. Error bars were calculated as the standard deviation of the mean. **B)** Percent entry inhibition for each mAb (H3K4 - H6L9) plotted against percent entry inhibition per ng of IgG. Recombinant mAb concentrations, estimated from HEK-293T transfections (Figure 3-12, panel C), were used to express percent entry inhibition of pseudo-particles per nanogram of IgG against the total entry inhibition recorded for each mAb. The mAbs which demonstrated the highest entry inhibition per nanogram of IgG are indicated on the graph; (H4K16, red triangle; H4K10, red open square). The position of the original culture well data is also indicated.

3.4 Discussion

3.4.1 Memory B-lymphocyte cultures

After six days of incubation, memory B-lymphocyte culture supernatants were assayed for IgG concentration. To provide a semi-quantitative method of assessing antibody concentration, a standard curve was produced using purified mAbs across a range of concentrations. The mean concentration for all 704 wells tested was 565ng/mL. The percentage of wells producing IgG at concentrations greater than 200ng/mL was 76.1%, whilst 18.3% of wells generated IgG at concentrations greater than 1000ng/mL. These results compare with those reported by Huang et al., (Huang et al., 2013a) who described a very similar method to the one used in this study to produce IgG from memory B-cells. They reported that approximately 55% of wells seeded with 2 to 5 B-cells produced antibody with a concentration greater than 200ng/mL and 20% of wells produced mAb greater than 1000ng/mL when measured after 13 days of culture. This demonstrates that the methods used in this study are as effective, or more so, than the results of similar methods described in the literature.

It was expected that not all memory B-lymphocytes would respond in exactly the same way to *in vitro* stimulation. Proliferative burst sizes were likely to vary and differences in the activation state of the lymphocytes would result in varying rates of differentiation into IgG secreting plasma cells (Kishimoto, 1985). These rates would be influenced by the condition of the B-cells prior to seeding. Also, the concentration of antibody produced within a culture well is not a predictor of neutralisation potency and therefore entry inhibition data should be considered in context of IgG concentration. The

mean IgG concentration (565ng/mL) recorded across all culture wells provided sufficient IgG for further characterisation using neutralisation assays.

The main test of neutralisation ability for IgG produced from B-cell cultures was entry inhibition experiments using pseudo-virus particles. The neutralisation assay provided a functional screen to characterise IgG as previously mentioned in Section 2.4.2.1. Cultured memory B-lymphocytes were deliberately screened without pre-selection for reactivity to a known Ebolavirus antigen. The rationale behind this decision was to provide the ability to identify previously uncharacterised neutralising epitopes by employing unbiased seeding of B-cells. For this approach to work, a functional screen was required that could identify the presence of IgG with the ability to impede Ebolavirus entry into permissive cells without prior knowledge of paratope-epitope interactions. This approach allows for the identification of antibodies targeting transient epitopes only present during virus particle–host cell interactions that mediate cellular entry. Several studies have identified Ebolavirus nAbs using known glycoprotein antigens however. For example, Qiu *et al.,* (Qiu et al., 2011) used EBOV GP to inoculate mice in order to elicit an immune response before harvesting spleen cells to generate hybridoma cells. Using this method they produced 8 mAbs that bound to EBOV GP. Furthermore, Zhang et al. (Zhang et al., 2016b) immunized rhesus macaques with recombinant EBOV GP derived from a Guinean patient identified in the 2014 outbreak. After isolating and culturing single B-cells from an IgG^{+} enriched pool of primate B-lymphocytes using FACS, the group identified three nAbs. None of the mAbs identified in these studies was derived directly from human survivors however.

Bornholdt et al., (Bornholdt et al., 2016) isolated and characterised 349 GP-specific mAbs from a survivor of the 2014 outbreak, 77% of which neutralised live Ebolavirus. Again, EBOV GP reactive memory B-cells were pre-selected using flow cytometry. The authors of the study determined that 3% of circulating IgG^+ B-cells were specific for the EBOV GP ectodomain for this individual. Incidentally this percentage of reactive circulating B-cells was similar to that observed for chronic HIV infection (Scheid et al., 2009a).

The majority of wells (97.8%) demonstrated entry inhibition of 30% or less than that observed for uninhibited control particles. These wells were discounted from further analysis due to this level of inhibition being too low to have any potential of clinical relevance. The remaining 2.2% of wells produced a neutralising phenotype of greater than 30% during screening. This number was comparable to the previously mentioned observation by Bornholdt et al., (Bornholdt et al., 2016) that approximately 3% of circulating IgG^+ B-cells showed reactivity to EBOV GP. Using the approach adopted in this study therefore presented the disadvantage that the majority of IgG^+ B-cells seeded into cultures were not producing antibody specific for Ebolavirus. It may prove worthwhile to adopt a pre-selection step using flow cytometry on the basis of a known antigen in conjunction with the non-selective approach that was used.

Functional screening of IgG produced using non pre-selected B-cells has resulted in the isolation of neutralising antibodies to other viruses however. For example, Huang et al., (Huang et al., 2012) produced the nAb, 10E8 which was shown to broadly neutralise HIV-1 infection using a pseudo-virus based functional screen. In a study by Walker et al., (Walker et al., 2009)

approximately 30,000 non pre-selected IgG^+ B-cells were isolated from an HIV-1 positive donor and cultured at near clonal density. Culture supernatants were interrogated for the presence of HIV-1 reactive antibodies using a binding assay incorporating envelope glycoproteins GP120 and GP41 and a neutralisation assay using primary HIV-1 isolates. Their results identified five antibodies that neutralised the HIV-1 strains tested. Of the 30,000 B-lymphocytes extracted, only 0.02% neutralised during screening. Also of interest was that 97.7% of culture supernatants that neutralised one of the HIV-1 strains tested did not bind to the corresponding monomeric GP. This observation demonstrates the advantages of functional screening to identify neutralising antibodies that do not necessarily demonstrate binding to immobilised glycoprotein in a binding assay.

As previously stated, 2.2% of the B-cell culture wells tested for neutralisation demonstrated entry inhibition greater than 30%. These wells were therefore selected for further characterisation. The highest entry inhibition value measured was 56.2%. These results were somewhat disappointing, but can be explained by considering the context of the donor.

The source of memory B-lymphocytes for these cultures was a blood sample donated by Professor Miles Carroll who had been vaccinated against Ebolavirus using the ChAd3 EBOZ vaccine as a prime and the VSV-EBOV boost 20 weeks later. Field trials using these vaccinations have shown promising initial indications of a protective immune response (Bornholdt et al., 2016, Ewer et al., 2016). A blood donation was given three weeks after the boost vaccination. According to the generalised response to prime boost vaccinations, a maximal antibody titre would have been present at the time of

blood donation. It should be noted however that there are inherent differences in an individuals' response to vaccination.

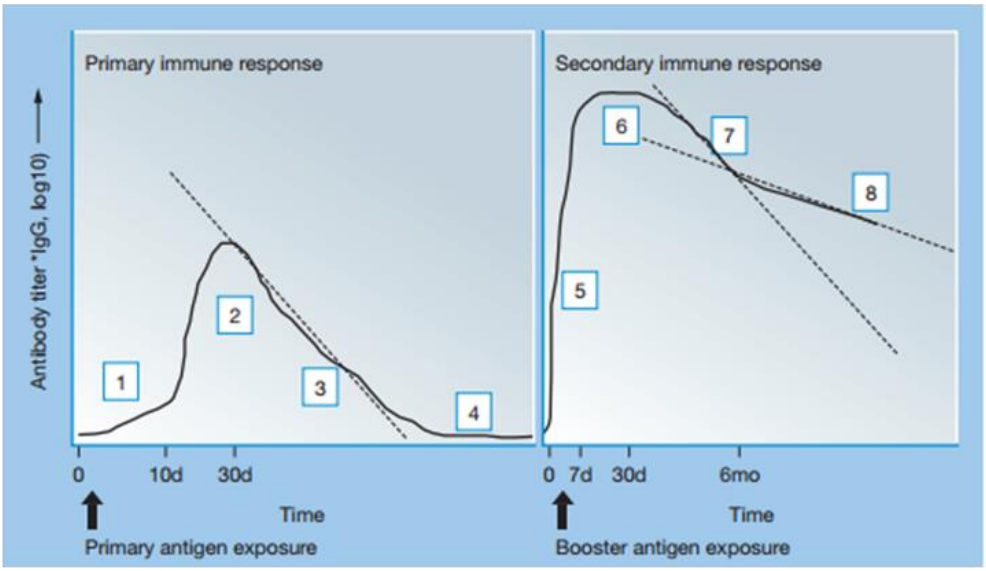


Figure 3-14| Correlation of antibody titers to the various phases of the vaccine response

1) The initial exposure to antigen (prime vaccination) elicits an extrafollicular B-cell response. **2)** Rapid expansion of B-cells in germinal centres and differentiation into plasma cells results in a rapid increase in IgG titre up to the peak value, approximately four weeks following immunisation. **3)** Antibody titre begins to decrease due to the short life-span of plasma cells. **4)** Baseline IgG titre is restored. **5)** The secondary response (boost vaccination) reactivates memory B-lymphocytes resulting in a rapid increase in IgG titre, usually less than seven days. **6)** Short-lived plasma cells maintain IgG levels at an increased peak value. **7)** Antibody titres decrease with similar kinetics to the primary response. **8)** A sub-population of long-lived plasma cells continue to produce antigen specific IgG which decline with slower kinetics than the primary response.
(Figure modified from Vaccines 6th Edition) (Plotkin, 2012).

A study investigating the frequency of Influenza specific antibodies in the blood of immunized donors, it was reported that antigen-specific B cells in human peripheral blood comprise less than 1% of the total B cell population 5 to 10 days following immunization (Kyu et al., 2009).

A recent study by Kennedy et al., (Kennedy et al., 2016) demonstrated varying antibody responses to seasonal Influenza vaccinations dependant largely on dendritic cell expression of HLA-DR. Their results highlighted

meaningful differences in antibody production in response to seasonal Influenza vaccination. It is not unreasonable to assume that similar responses would be observed for the trial Ebolavirus vaccinations. Indeed, the immune response elicited to the vaccinations by the donor used in this study was unknown and the relatively low entry inhibition that was observed was possibly due to the vaccinations failing to produce a potent neutralising response, or through the vaccinations failing to stimulate and maintain sufficiently high numbers of circulating Ebolavirus specific memory B-cells to screen efficiently using the methods of this research.

A potentially more fruitful source of potently neutralising antibodies would have been memory B-cells isolated from survivors of the 2014-2015 Ebolavirus epidemic. These individuals were able to mount a sufficiently rapid and targeted immune response to infection with Ebolavirus to be able to keep the virus titre below a critical threshold before a fatal infection could be established. It is also likely that survivors harbour long lived Ebolavirus infections due to reservoirs of virus becoming established within regions of the body undetected by the immune system (Deen et al., 2015, Varkey et al., 2015, McElroy et al., 2015, Corti et al., 2016, Jacobs et al., 2016). In order to maintain the infection, virus particles leach back into the blood stream periodically to re-stimulate an immune response leading to sustained and robust immunity. Earlier studies have described Ebolavirus infection to be immunosuppressive through inhibition of the interferon response (Basler et al., 2000, Basler and Amarasinghe, 2009), maturation and activation inhibition of infected antigen presenting cells (Bosio et al., 2003) and through the progression to a general lymphopenia (Sanchez et al., 2004). More recent

studies conducted since the 2014 outbreak, have demonstrated more robust adaptive immune responses (McElroy et al., 2015, Ruibal et al., 2016). Indeed, as described previously, Bornholdt et al., (Bornholdt et al., 2016) isolated 349 EBOV-GP specific mAbs, including 269 nAbs from a convalescent donor who survived the 2014 Ebolavirus outbreak highlighting the significant potential of convalescent donors as a source of neutralising antibodies. The unprecedented scale of the 2014 outbreak tragically resulted in more than 11,000 fatal infections during the course of the epidemic (W. H. O. Ebola Response Team, 2014). The magnitude of the total number of infections also resulted in more than 17,000 cases of people surviving infection and subsequent EVD, providing huge potential to understand the immune response to Ebolavirus infection as well as a resource for isolating neutralising antibodies. Unfortunately a source of Ebolavirus convalescent memory B-lymphocytes was not secured during the period of this research.

Entry inhibition experiments were carried out using a standard 50μL volume of culture supernatant. Due to the variation in IgG concentrations estimated across culture wells, different amounts of IgG would have been introduced into entry inhibition assays for each culture well. In order to account for varying IgG concentrations, entry inhibition was calculated as a function of IgG concentration estimates thus providing a value for percent entry inhibition per nanogram of IgG. This value provided an estimate of the potency of neutralisation for antibodies produced within a given culture well. Entry inhibition experiments with an indicated IgG concentration of 0 – 10ng were not included in the analysis due to the error associated with the corresponding absorbance measurements. Wells carried forward to the next

phase met the criteria of demonstrating a percent inhibition per ng IgG greater than 1.0 and a total entry inhibition of 70% or greater of the highest observed inhibition of 56.2%.

3.4.2 Amplification of IgG variable domains

Culture wells that met the criteria described in the previous section were progressed to the next stage of the platform. In order to provide an internal control for mRNA extraction and cDNA synthesis, first round PCR for a housekeeping gene was carried out concurrently with variable domain amplification. Housekeeping genes are required for the maintenance of basal cellular functions and are actively expressed during normal cellular maintenance and metabolism. The gene chosen was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has been shown to be actively expressed in B-lymphocytes (Garcia-Vallejo et al., 2004). Other potential housekeeping genes suitable as controls using B-lymphocytes are the human proteasome subunit Y and phosphomanomutase (PPMM) (Garcia-Vallejo et al., 2004).

Amplification of a PCR product corresponding to GAPDH expression from all wells analysed indicated that mRNA recovery and RT-PCR worked efficiently for all wells. Analysis of PCR products derived from first round amplification of variable domains revealed bands corresponding to all three chains, with the exception of well B1, from which only heavy chain and lambda chain products were amplified. Two possibilities exist for these observations; either that the PCR products were derived from contamination or that the wells were seeded with more than a single memory B-lymphocyte. No template negative control lanes produced a band suggesting that contamination

was not the source of the PCR products. The most likely cause of the amplification of both light chains per well, was if several memory B-lymphocytes were introduced into each culture well during the seeding process. This would potentially result in PCR amplification for each chain. The ratio of kappa to lambda light chains in human serum has been reported to be 3:1 (Leffel et al., 1997), therefore on average, at least four memory B-cells would have been seeded.

In order to confirm that the multiple products were an artefact of non-clonal seeding densities, DNA sequence data were obtained for each PCR product. All PCR products that were analysed returned multiple sequence strings across the variable domains thereby showing that PCR amplification of heavy, kappa and lambda chain variable domains from each culture well was due to non-clonal seeding events. Well B1, which did not amplify a product for a kappa chain, returned a mixed read for the heavy chain and a single read for the lambda chain. This observation showed that at least one light chain sequence was missing for this well.

DNA sequence data clearly identified that multiple memory B-lymphocytes were introduced into culture wells during the seeding process. Multiple seeding events were likely to have arisen from inaccurate cell counting prior to dilution. This error was introduced intrinsically due to cell counts being carried out using a haemocytometer. Accurate cell counts are achievable using this method if the cell concentration is in the range of $1x10^6$ to $8x10^6$ cells per mL (Cadena-Herrera et al., 2015). The concentration of memory B-cells following the purification process however was typically in

the range of $7x10^4$ to 10^5 cells per mL. Accurate dilutions following cell counts would therefore still result in inaccurate seeding densities.

Seeding the cells at anything greater than clonal density increases the number of heavy and light chain combinations required to recreate any desirable phenotype observed during the culture phase and is therefore to be avoided. Limiting dilutions were adopted in this study as a method for seeding single B-cells into culture wells. Other methods may have provided more reproducible seeding densities however. For example, several studies have utilised fluorescently labelled surface markers to stain B-cells in order to enrich and sort individual cells into culture wells using flow cytometry (Scheid et al., 2011, Huang et al., 2012, Huang et al., 2013b, Jones et al., 2013, Zhang et al., 2016a). A potential disadvantage of staining the B-cells is that it may result in activation of the cells causing a decrease in cell viability during the culture phase. An alternative method would be to incorporate a negative selection process through staining of IgG^- B-cells in order to gate a population of un-stained IgG^+ B-cells, which can be subsequently sorted into culture wells (Huang et al., 2013a).

In order to identify each variable domain sequence from within the mixtures, a sample well was chosen for deconvolution through sub cloning individual PCR products into an intermediate vector and transforming into competent *E.Coli* cells to isolate individual domains. Well P1-E1 was selected because it demonstrated a relatively high neutralisation profile (total entry inhibition of 40%, percent entry inhibition per nanogram IgG of 1.4). A second factor in the selection of this well was that the mixture of variable domains for each chain appeared to be relatively low from analysis of DNA

sequence data derived from bulk PCR products. PCR products corresponding to individual variable domains were cloned into the pGEM T-easy vector via artefactual terminal deoxythymidine and deoxyadenosine residues arising from amplification using Taq polymerase. These residues were allowed to anneal with complementary residues of the linearised vector. PCR amplification of variable domains derived from individual colonies provided material for DNA sequence analysis. A total of 15 samples were analysed for heavy and lambda chains and eight for the kappa chain. DNA sequence data were translated into amino acid sequences and aligned using PRALINE software to generate heat maps displaying the degree of sequence identity between each sample (Heringa, 2002). Heavy and lambda chains returned five individual sequences and the kappa chain three unique sequences, indicating that complete sample recovery was not achieved. Similar degrees of sequence identity were recorded for heavy and lambda chains (heavy chain mean identity = 61.4%; lambda chain mean identity = 67.3%). The low level of conservation between the sequences indicated that they had arisen from immunoglobulin gene rearrangements in maturing B-cells. Kappa chain sequences demonstrated a much higher degree of sequence conservation however, with a mean identity of 9.1% between the three sequences. This degree of sequence identity could have arisen through template swapping during amplification with Taq polymerase (Odelberg et al., 1995)

3.4.3 Sub-cloning into expression vectors

Modification of variable domains in preparation for sub-cloning was carried out using an identical translation initiation/signal peptide sequence as described in Section 2.4.2.1. All samples were successfully modified with the exception

of lambda 5. A unique forward primer was used for PCR modification of lambda 5 that was not used for any other sample. Analysis of the primer sequence (Oligo analyser 3.1, Integrated DNA Technologies) revealed the potential for two forward primers to hybridize to form a homodimer, reducing the efficiency of the primer to hybridize to the target strand (Hyndman et al 2003). The Gibbs free energy for this reaction was calculated to be -22.52 kcal/mole. In contrast, the Gibbs free energy calculated for homodimerization of the forward primer that was used to successfully amplify the other lambda sequences was -10.11 kcal/mole. The higher propensity for homodimerization of the lambda 5 primer was therefore likely to have reduced the efficiency of the PCR resulting in the failure to amplify a modified product for this sample.

During homologous recombination, three modified PCR products corresponding to samples Heavy 7, Heavy 12 and Lambda 6 repeatedly failed to insert into the expression vector. The two heavy chain samples were modified using the same forward primer, unique to these two samples. Similarly, the Lambda 6 sample was modified using a unique forward primer. It is reasonable to assume that the 5' region of the samples that failed to insert contained hair-pin elements preventing the regions of homology from annealing (Hyndman et al 2003). Unfortunately, due to time constraints these technical issues were not overcome.

The variable domains that did insert into the expression vectors were aligned to the original DNA sequences to confirm that faithful PCR amplification occurred. The sequences were also confirmed to contain productive *V(D)J* recombinations using the International Immunogenetics

Information System IMGT/V-QUEST analysis program as previously described.

3.4.4 Recombinant antibody production and characterisation for neutralisation potency

Expression plasmids containing clinically isolated variable domains were co-transfected into HEK-293T cells to recreate all possible heavy and light chain combinations in order to express functional recombinant IgG1 molecules. Culture supernatants were harvested and assessed for antibody production using an IgG ELISA. A standard curve provided a method of estimating IgG concentration for each mAb. A variation in concentrations across the series of mAbs was observed. All heavy chain constructs and all light chain constructs were generated using identical translation initiation/signal peptide sequences and should theoretically be translated and trafficked from the producer cells to the same level. The variation in concentrations observed for different mAbs is therefore hard to rationalise. It is not clear for example why heavy chain 4 and 6 combined with any kappa chain, produced low concentrations of mAb, whilst in combination with a lambda light chain produced relatively large concentrations. It is possible that the variations arose from differences in the purity of the expression plasmids, however this would not explain why kappa chains in combination with heavy chain 3 produced relatively large concentrations of mAbs, but in combination with heavy chain 4 or 6 produced low mAb concentrations.

Cell culture supernatants containing recombinant mAbs were used to assess the potential entry inhibition using Ebolavirus pseudo-typed particles. Glycoproteins derived from the Mayinga strain of Ebolavirus (GenBank

accession NC_002549), isolated from the original Zaire outbreak in 1976 (Johnson, 1977), were used to construct the particles. When expressed as percent infectivity of an uninhibited control, several mAbs reduced infectivity by approximately 25%. These combinations were H3K4 (26.1%); H3K10 (27.1%); H3L8 (24.4%) and H4K16 (26.3%). Total entry inhibition measured for the original culture well P1-E1 was 40%, therefore the entry inhibition data for these four mAbs represent between 61% (H3L8) and 68% (H3K10) of the original entry inhibition. To compensate for variations in mAb concentration, entry inhibition values were expressed as a function of IgG concentration. Clone H4K16 recorded the highest value, of 1.84% in comparison to 3.8% for the original well. Although these observations identify mAbs with neutralising potential against Ebolavirus pseudo-typed particles, they do not recreate the entry inhibition observed for the original culture well. It could be possible that polyclonal antibodies were acting synergistically in the culture well to produce a greater degree of entry inhibition as has been described for nAb cocktails against HIV-1 (Mascola et al., 1997), Herpes simplex virus (Sanna et al., 2000), Botulinum neurotoxins (Diamant et al., 2014). To test this theory, mixtures of mAbs could be tested in entry inhibition assays to determine any combinations with an increased degree of inhibition. The production of synergistic mixtures of neutralising antibodies in the original culture wells however would have required the random seeding of numerous memory B-cells producing neutralising antibody against Ebolavirus. Considering that the percentage of circulating cells with the specific reactivity required to achieve this was likely to be less than 1% (Walker et al., 2009) this possibility was somewhat unlikely.

Considering total entry inhibition values alone, heavy chain 3 consistently reduced pseudo-virus entry by the largest levels, regardless of its light chain pairing. The overall lowest value was recorded for heavy chain 3 paired with kappa chain 10 (27.1% inhibition). The combination of Kappa chain 10 with heavy chain 4 and 6 demonstrated total entry inhibition of 23.4% and 21.8% respectively; which was the greatest reduction in infectivity observed for both of these heavy chains. Taken together, these observations suggest that, if a single antibody was responsible for the observed entry inhibition, the combination of heavy chain 3 and kappa chain 10 may replicate the original culture well mAb composition.

Percent entry inhibition per nanogram of IgG data however, conflict with this conclusion. As previously stated, the highest recorded value was for clone H4K16 (1.84). Clone H4K10 however, recorded a very similar value of 1.67 suggesting that kappa chain 10 was contributing to the neutralising phenotype. Further entry inhibition assays are required with standardised mAb concentrations to better understand the neutralising phenotype of each clone. Unfortunately, these experiments fell outside of the time frame of this body of research.

Regardless of whether the original culture well antibody composition was recreated as recombinant antibody, a number of mAbs were produced that reduced the infectivity of Ebolavirus pseudo-typed particles by around 25%. Although this level of inhibition is not of the magnitude to be clinically relevant it does demonstrate that application of the experimental methods forming the platform have the potential to identify, isolate and recreate monoclonal antibodies with a neutralising phenotype against Ebolavirus.

Further memory B-cell cultures using vaccinated donor blood samples may provide mAbs with higher neutralising potential however, the most likely source of sample to provide the most potently neutralising anti-Ebola mAbs would be derived from survivors of the 2014-2015 Ebola epidemic as previously discussed.

4 Conclusions and Future Work

The threat posed by emerging or re-emerging pathogenic enveloped RNA viruses to the global human population is unquestionably a significant one. Recent outbreaks of SARS-CoV, Lujo virus, Zika virus and Ebolavirus for example, have resulted in significant negative consequences to the regions affected but in different ways. For example, the symptoms caused by primary Zika virus infection are typically mild, manifesting as low grade fever, localised rash, joint discomfort and conjunctivitis. Recent outbreaks of Zika virus infections in South America however have identified that infections sustained during pregnancy can result in severe malformations and neurological disorders to the developing foetus, whilst causing only mild symptoms from the primary infection (Johansson et al., 2007, Rasmussen et al., 2016).

In stark contrast to Zika virus infection, the 2014 Ebolavirus epidemic centred in West Africa, resulted in 11,232 deaths as a direct result of a confirmed infection. The CFR for the outbreak was 39.5% (from a total of 28,646 confirmed cases). Although this CFR was relatively low compared to previous EBOV outbreaks (Muyembe-Tamfum et al., 2012) the unprecedented scale of the epidemic resulted in a devastating impact for the affected regions.

These are just two examples from recent years of the potential threat posed by emerging or re-emerging viruses and of course say nothing of what the future holds in terms of outbreaks of novel strains of enveloped RNA viruses.

The rapid production of potent nAbs in response to exposure to an emerging or re-emerging virus is one mechanism which allows certain

individuals to survive an infection. The subsequent identification and isolation of these nAbs provides a potentially effective mechanism for controlling disease progression within affected areas, minimising the capacity of an outbreak to spread into naïve populations. The same nAbs could also provide prophylactic measures to protect healthcare workers and contacts, further minimising transmission. Furthermore, nAbs also allow the potential to aid in the design of vaccines.

The aims of this project were to develop a novel platform of methods to isolate human mAbs directly from the peripheral blood of a donor with the ability to identify nAbs to a particular virus. The resulting platform would thereby provide the required technology to produce nAbs with the ability to restrict the transmission of pathogenic viruses. The period of this research coincided with the outbreak of the Ebolavirus epidemic in West Africa and so this virus was chosen as the target of the platform. The modular nature of the platform allowed for the sequential optimisation of the methods comprising each phase. The development of methods required to co-culture IgG^+ memory B-lymphocytes resulted in an efficient procedure for the *in vitro* production of IgG using CD-154, IL-4 and IL-21 stimulation. An optimal 10 day culture period provided the ability to produce antibody within a short timeframe as the temporal separation of cytokines into the cultures did not increase the overall antibody production. Typical results in terms of the amount of antibody secreted into culture supernatant were at least equal, if not greater than similar methods reported elsewhere (Walker et al., 2009, Huang et al., 2013a) with the majority of culture wells producing sufficient IgG concentrations to allow for functional screening of cultures using a pseudo-virus based neutralisation

assay. A single, yet significant limitation existed with the B-cell co-cultures however. In order to produce monoclonal antibodies within the culture system, it was essential that clonal expansion of IgG^+ memory B-lymphocytes in each culture well followed seeding of the cells at clonal density. The method used to seed memory B-cells was a limiting dilution of a bulk pool of cells. Assuming a Poisson distribution, the dilution was calculated to transfer an average of 1.3 cells to each culture well in order to achieve a clonal seeding density (Jones et al., 2013). The results presented in Chapter 3 however demonstrated that this was not always achieved. As previously discussed, several alternative methods exist that utilise FACS to pre-select B-cells with a specific reactivity before seeding into individual culture wells (Weitkamp et al., 2003, Scheid et al., 2009b, Huang et al., 2012, Huang et al., 2013a, Jones et al., 2013, Zhang et al., 2016b). This method was not adopted however, as an unbiased method of seeding the B-cells was preferred. Two alternative FACS procedures exist that could be used to increase the accuracy of seeding events without introducing bias towards a particular antigen. The first would be to isolate IgG^+ B-cells following the depletion of IgG^- cells using cell surface staining as described in Huang et al (Huang et al., 2013a). In this way, unlabelled IgG^+ B-cells could be seeded at clonal density without exposing them to pre-selection bias. Alternatively, a pre-purified pool of un-labelled IgG^+ B-cells could be sorted individually into culture wells based on forward and side scatter only, thereby avoiding any selection bias (Watson et al., 2004). Indeed, this method was tested during this research, however the results were inconclusive in the timeframe of the project. Further examination of this method is required. Future iterations of the platform therefore could utilise one

of these alternative methods of single B-cell sorting during the establishment of cultures to improve the accuracy of seeding.

Following optimisation, the subsequent stages of the platform provided an efficient method of cloning individual V_H and V_L regions derived from cultured B-cells, into plasmid expression vectors. The efficiency of this stage of the platform could be increased by modification of the pORIgHIB huIg1 and pORIgLIB huIg1 vectors by inserting a functional translation initiation and secretion signal in-frame with the constant domain encoding regions. This would remove the requirement to modify isolated PCR products containing V_H and V_L regions prior to homologous recombination with the expression vectors to include these signals. As described in Chapter 3, this process was not always successful, especially in the case of lambda chain products which required a longer forward primer due to the initial PCR primers annealing into template sequences at a more distal location to the translation and secretory signals than heavy and kappa chains. Although modification of the vectors in this way would not completely eliminate the requirement for variable domain encoding PCR products to be prepared for cloning, (due to the requirement of a region of homology between the vector and the variable domain) it would significantly decrease the length of the sense primer thereby increasing the efficiency of the PCR through a reduction in the propensity of secondary structure formation (Dieffenbach et al., 1993).

Transient co-transfections of the two expression vectors into HEK-293T cells provided sufficient concentrations of IgG to allow culture supernatants to be screened using a pseudo-virus neutralisation assay. The advantage of transient co-transfections was two-fold. Firstly, cloning V_H and V_L domains

into separate expression vectors provided flexibility in combining different heavy and light chains to form functioning IgG molecules. Secondly, it presented an efficient method of screening as the producer cells required only 48 hours to secrete sufficient quantities of antibody into the culture supernatant to use for characterisation. In the event of identifying a V_H, V_L domain combination for further characterisation, it would be possible to clone each domain into a single expression vector to produce a fully functioning recombinant IgG molecule from a single transfection (Metheringham et al., 2009). This would allow for the generation of a stable mammalian cell line producing the mAb of interest (for a review of this process see (Wurm, 2004)).

Unfortunately, during the period covered by this project, a potently neutralising antibody to Ebolavirus was not identified. The most likely reason for this was that the source of the blood sample was from a donor vaccinated with two trial vaccines (Stanley et al., 2014, Henao-Restrepo et al., 2015). Although the results in Chapter 3 identify the presence of nAbs in B-cell culture supernatants, the magnitude of the donor's humeral immune response to vaccination was not identified. It is possible that it was insufficient to stimulate a sufficiently robust increase in memory B-cells producing potently neutralising antibodies to be isolated using the platform. As described previously, blood derived from a survivor of Ebolavirus infection would have been more likely to yield one or more nAbs. Indeed, it was reported by McElroy (McElroy et al., 2015) that frequencies of IgG^+ plasmablasts isolated from the blood of convalescent individuals comprised between 10-50% of B-cells, coinciding with the presence of Ebolavirus-specific IgG within serum samples. Furthermore, the level of EBOV GP specific T-cell activity observed

for survivors appears to be on average an order of magnitude higher than for vaccinated individuals (Miles Carroll, personal communication). Coupled with the observation that a latent reservoir of virus appears to persist in convalescent individuals maintaining an immune response of a larger magnitude than would be expected in the case of complete clearance, adds further potential to isolate nAbs from blood donations made by survivors.

The results presented in this body of work demonstrate that the platform can be used to successfully identify, isolate and reproduce nAbs against Ebolavirus. Furthermore, the methods used, including the utilisation of a pseudo-virus based functional screen, provide the potential for the platform to be adapted to other emerging or re-emerging enveloped RNA viruses within a short time frame.

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