

Development and characterisation of human monoclonal antibodies against the Ebola virus glycoprotein

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Abstract

The current Ebola virus outbreak in West Africa has infected over 27,000 people and claimed over 11,000 lives, making it the deadliest and most widespread Ebola epidemic in history. The high caseload and rapid geographic spread has been due in part to crumbling healthcare infrastructure, critical shortages of healthcare workers, paucity of resources, and local traditional practices. In addition, although supportive care is available at treatment centres, there are no effective therapies available in the affected countries. A number of potential Ebola virus therapeutic agents are currently being tested in accelerated clinical trials, but there is still no drug with proven efficacy in humans.

In this dissertation I describe the isolation of the first fully human Ebola-specific monoclonal antibodies (mAb) from vaccinees during phase 1 of the Oxford Ebola vaccine trial. This was achieved through single cell sorting of antibody secreting cells (ASC) at day seven post boost, isolation of the immunoglobulin (Ig) heavy and light chain genes, expression vector cloning and transfection of human cell lines to produce mAbs. I also attempt to characterise the Ebola-specific mAb's binding affinity, binding site, and cross-reactivity with other historic Ebola virus strains by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assays (IFA). I have also tested their neutralising ability with an *in vitro* pseudotyped influenza microneutralisation (MN) assay. I present a diverse panel of twelve mAbs with a range of binding characteristics and neutralising abilities, four of which are cross-reactive with both the Zaire and Sudan Ebola viruses. Once fully characterised, these antibodies may potentially be used as part of a mAb cocktail for pre- or post-exposure prophylaxis and cure for Ebola virus disease.

List of Abbreviations

ASC	Antibody secreting cell
BCR	B cell receptor
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
ChAd	Chimpanzee adenovirus vector
DMEM	Dulbecco's Modified Eagles Media
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein Isothiocyanate
GP	Glycoprotein
GPΔmuc	Mucin-deficient glycoprotein
IFA	Immunofluorescence assay
Ig	Immunoglobulin
LB	Lysogeny broth
mAb	Monoclonal antibody
MLD	Mucin-like domain
MN	Microneutralisation
MVA	Modified vaccinia virus Ankara
NHP	Non-human primate
NP	Nucleoprotein
PBMC	Peripheral blood mononucleocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
RNA	Ribonucleic acid
rSAP	Recombinant shrimp alkaline phosphatase
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNA	Small interfering ribonucleic acid
SOC	Super optimal broth
VGM	Viral growth medium
VP	Viral protein
WIMM	Weatherall Institute of Molecular Medicine

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1. Introduction

1.1. Ebola virus outbreak in West Africa

The scale of the current Ebola outbreak in West Africa far exceeds previous Ebola epidemics, with over 27,000 reported cases and more than 11,000 deaths (WHO, 2015). The high caseload and rapid geographic spread has been due in part to crumbling healthcare infrastructure, critical shortages of healthcare workers, paucity of resources, and local cultural beliefs and practices. The high mortality rate exceeding 40% is also a consequence of the lack of a licenced vaccine or effective treatment.

The *Ebolavirus* belongs to the *Filoviridae* family of enveloped, non-segmented, negative sense single-stranded RNA viruses. The *Ebolavirus* genus includes four species that are pathogenic in humans: Zaire, Sudan, Tai Forest (Ivory Coast), and Bundibugyo (Uganda). The viral genomes from the current Guinea strain are on average 97% similar to the genomes from the 1976 outbreak in Zaire (Baize et al., 2014; Jun et al., 2015). The genome contains seven genes each encoding functional proteins: the membrane-associated viral protein (VP) VP24, matrix proteins VP30, VP35 and VP40, RNA polymerase (L), nucleoprotein (NP), and glycoprotein (GP) (Muhlberger, 2007). While all the Ebola virus genomes are similar, inter-strain variation is most common in regions encoding the NP, RNA polymerase, and GP (Jun et al., 2015). Analysis of blood from Ebola survivors has found that antibodies primarily react to four functional proteins: NP, VP30, VP40, and GP (Sobarzo et al., 2013).

1.2. The Ebola virus GP

The Ebola virus GP is the only expressed protein on the virus surface and most important protein in Ebola virus disease pathogenesis. It is the primary protein responsible for cellular tropism, viral entry, cytotoxicity and vascular permeability. The Ebola virus GP is also the target for effective neutralising mAbs and efforts to develop vaccines and mAb treatment have thus far focussed on this protein.

Structurally, the GP is a 676-residue membrane-anchored protein consisting of a trimer of heterodimers. The protein undergoes post-translational furin-mediated cleavage to form GP1 and GP2 subunits which are linked by disulphide bonds to form the heterodimers (Sanchez et al., 1998). Three GP2 subunits form a cradle around the three GP1 subunits in the trimer (Lee et al., 2008). The receptor binding site on GP1, masked by a glycan cap and mucin-like domain (MLD), is responsible for attachment to the host cell-surface receptor (Lee and Saphire, 2009a). However, a critical host receptor has yet to be identified. The GP2 protein is responsible for fusion with the host cell membrane and receptor-mediated endocytosis (Sanchez, 2007). Despite the glycocalyx shield, there are regions at the base of the GP chalice and within the MLD which are exposed to antibody binding (Lee and Saphire, 2009c). The MLD and glycan cap are cleaved by cathepsin before receptor binding, and during entry the GP undergoes a conformational change (Lee and Saphire, 2009a). A study looking at the binding of a human survivor antibody, KZ52, to the Ebola GP showed that GP1 was needed to maintain the correct conformation of GP2 for the antibody to bind (Lee et al., 2008). KZ52 was shown to bind both GP1 and GP2, preventing the conformational change of the GP necessary for membrane fusion, thereby inhibiting viral entry.

A truncated homo-dimeric isoform of GP that lacks the MLD, secreted GP (sGP), is thought to play a role in immune evasion. This sGP isoform is able to bind anti-GP antibodies, directly competing with the membrane-bound GP as a “decoy” antigen (Ito et al., 2001). It has also been reported that sGP biases the host humoral response toward the production of antibodies against GP epitopes it shares with the membrane-bound GP, thereby subverting the host humoral immune response (Mohan et al., 2012).

1.3. Human mAb therapy

Ebola virus disease has a long asymptomatic incubation period but is rapidly fatal during the symptomatic phase and as such, targeted treatment needs to be effective at a late stage of infection in the absence of early diagnosis. Current treatment of infected patients is predominantly supportive: replacement fluid therapy, antivirals, and management of Ebola virus disease symptoms and sequelae (Clark et al., 2012). Several experimental therapies have been tried including passive immunotherapy

using convalescent plasma or immunoglobulin transfusions, antisense morpholino oligomer therapy (Warren et al., 2010), and a slew of other antiviral drugs (Kilgore et al., 2015). Recently, small interfering ribonucleic acid, siRNA (Geisbert et al., 2010), therapy was shown to be 100% protective in rhesus monkeys (Thi et al., 2015). The only other candidate that has shown efficacy in non-human primates (NHP) is mAb therapy (Marzi et al., 2012; Olinger et al., 2012; Pettitt et al., 2013; Qiu et al., 2014).

There is mixed evidence pointing towards the importance of the humoral response in survival from Ebola virus disease. One murine study used transgenic mice to selectively knockout either the CD8 cytotoxic adaptive immune response or the B cell-mediated humoral response (Wong et al., 2012). The mice were vaccinated with a recombinant replication-deficient Adenovirus type 5 vaccine expressing the Zaire Ebola virus GP and then challenged four weeks later with a lethal dose of mouse-adapted Zaire Ebola virus. All the vaccinated CD4 T cell$^{-/-}$ and B cell$^{-/-}$ (CD8 T cell$^{+/+}$) mice died, whereas all the vaccinated CD8 T cell$^{-/-}$ (CD4 T cell$^{+/+}$ and B cell$^{+/+}$) mice survived the challenge. This suggests that the humoral immune response plays a major role in protection from Ebola virus. However, Sullivan et al. (2011) found by selectively depleting CD8^{+} T lymphocytes that the protective effect was largely mediated by these cells in NHPs vaccinated with the same vaccine. In another study passive immunisation of macaques with purified equine IgG from vaccinated horses was found to not be protective (Jahrling et al., 1999). In humans, blood transfusions from Ebola survivors containing Ebola-specific IgG were protective during the 1995 outbreak in Kikwit, Democratic Republic of the Congo (Mupapa et al., 1999). Mortality rate amongst the eight patients transfused was only 12.5%, as opposed to 80% in the general population. These transfused patients also received better quality of care later in the outbreak, which may in part account for these findings. In samples taken during the Ebola virus outbreak in Gabon in 1996, specific IgG was present in increasing titres in survivors, but absent in fatal cases (Baize et al., 1999). This again points to the importance of humoral immunity in protection from Ebola virus.

There are several benefits to treating patients with purified fully human mAbs instead of pooled human serum. Purified antibody is safer: it removes the risks of transmitting blood-borne infections such as HIV and Hepatitis B from donor serum, and the risk of allergic and anaphylactic reactions is diminished. It is also possible to titrate the dose

of the mAbs and provide the antibodies in therapeutic cocktails. In terms of public health interventions and planning, it is possible to stockpile mAbs and upscale production in case of an outbreak. Pooled human serum relies on the availability of survivors and healthcare infrastructure to screen, prepare and safely store the pooled serum.

Therapy with a single neutralising mAb, such as KZ52, has not been shown to provide protection in NHPs (Oswald et al., 2007). However, there are several cocktails of Ebola GP-specific mAbs that have had some success in NHP trials, including MB-003 from Mapp Biopharmaceutical (Olinger et al., 2012) and ZMab produced by Defyrus (Qiu et al., 2012). ZMapp (Mapp Biopharmaceutical) is a combination of three chimeric mAbs (c13C6, c2G4, and c4G7) raised from vaccinated mice. When this cocktail was given to macaques on day three post challenge with a lethal dose of Ebola virus, there was 100% survival (Qiu et al., 2014). The antibody c13C6 binds within the glycan cap, while the other two mAbs bind the GP base (Murin et al., 2014).

Antibodies directed against the MLD and glycan cap appear to be non-neutralising as their epitopes are cleaved from the viral particles by cathepsin prior to receptor binding (Murin et al., 2014). However, when these mAbs are tested in an Ebola virus neutralisation assay in the presence of complement, there is significant neutralisation of the virus (Wilson et al., 2000). Even if it is non-neutralising, c13C6 could improve presentation of the constant regions of c2G4 and c4G7, thus improving immune effector function (Murin et al., 2014). c13C6 is also cross-reactive with sGP, which acts as a decoy mopping up the antibody (Qiu et al., 2011). Neutralising antibodies including KZ52, c2G4 and c4G7, prevent the conformational change of the GP necessary for viral entry (Murin et al., 2014). However, the two ZMapp mAbs that bind the GP core, c2G4 and c4G7, have been shown to cross-inhibit each other *in vitro*, recognising the same epitope on the Ebola GP (Audet et al., 2014). The cross-species neutralising efficacy of these Zmapp mAbs (Zaire Ebola-specific) is also not known. Theoretically, future mAb cocktails would be more effective if they contained a combination of neutralising mAbs recognising a selection of epitopes that are distinct from epitopes on sGP.

ZMapp is currently undergoing a clinical trial in humans in Liberia and the United States. The mAbs are produced in a *Nicotiana benthamiana*-based rapid antibody-manufacturing platform (Giritch et al., 2006). This plant-derived mAb production process is inefficient, and as a result ZMapp is in short supply (Li et al., 2015). A cocktail of mAbs that is more rapidly scalable in order to meet global demand during a pandemic is therefore needed.

Another consideration when developing mAb therapy for Ebola is the use of chimeric antibodies (such as Zmapp) or humanised mAbs, versus fully human antibodies. It is controversial whether fully human antibodies hold advantage over chimeric and humanised mAbs. In theory, because these mAbs are generated in humans, their safety, immunogenicity and efficacy in human disease are better than antibodies produced in mice or other species. In reality, humanised and even fully human sequence-derived antibody molecules still carry immunological risk. Human derived antigen-specific mAbs usually contain somatic hypermutations. They are therefore not germline and may be recognised as foreign eliciting an immune response. Chimeric and humanised mAbs may also elicit anti-drug antibody responses, limiting the efficacy of repeat dosing.

1.4. Isolation of mAbs

In past mAbs were developed through the production of hybridomas from immortalised B cells, and chimerisation or humanisation of murine antibodies (Weiner, 2006). A more recent approach to produce fully human antigen-specific mAbs is the isolation of antibodies from ASCs and other B cells sorted from peripheral blood mononucleocytes (PBMCs) (Smith et al., 2009; Tiller et al., 2008; Wrammert et al., 2008). This method is more efficient and has a higher throughput. It has been successfully used to isolate and clone, amongst others, influenza- (Wrammert et al., 2008) and HIV- (Scheid et al., 2009a) antibodies, and could potentially be used to generate Ebola virus-specific mAbs from Ebola vaccine recipients.

There are currently seven Ebola virus vaccine candidates in clinical trials. One of the leading vaccine candidates currently being tested in Oxford is a replication deficient recombinant chimpanzee adenovirus (ChAd) type 3 vector vaccine encoding the Zaire

Ebola virus surface GP. A single-shot vaccination with ChAd3 encoding the Zaire Ebola virus surface GP has been shown to provide 50% protection after challenge with Ebola virus at 10 months in NHPs (Stanley et al., 2014).

In 2014, 60 volunteers were vaccinated with a replication deficient recombinant ChAd3 vector encoding the Zaire Ebola virus surface GP in the phase 1 clinical Ebola vaccine trial in Oxford (Rampling et al., 2015). The volunteers in the human trial were divided into three dose groups—low (1×10^{10} viral particles), medium (2.5×10^{10} viral particles), and high (5×10^{10} viral particles). The vaccine induced both $CD4^+$ and CD8 T^+ cells, with the best response in the high dose group. This finding was also consistent with animals immunised against Ebola (Sullivan et al., 2011).

In NHP studies it has been shown that ChAd3 with modified vaccinia virus Ankara (MVA) boost is better than the homologous ChAd3 prime-boost regimen, with 100% and 33% survival respectively (Stanley et al., 2014). In line with this evidence, ten volunteers from each group of the Oxford vaccine trial were given a quadrivalent replication deficient MVA booster vaccination containing Zaire and Sudan Ebola GP, Tai Forest Ebola NP, and Marburg virus GP. A significant increase in titres of Ebola GP-specific antibody was detected day seven post-MVA boost (Ewer et al., submitted).

Naïve B lymphocytes in the germinal centres of lymph nodes post vaccination are antigenically stimulated and activated through the B cell receptor (BCR) with T cell help (Crotty, 2015). The cells undergo affinity maturation in the secondary lymphoid organs through somatic hypermutation and clonal selection (McHeyzer-Williams and McHeyzer-Williams, 2005). This process results in B cell proliferation and differentiation into memory B cells. In response to a repeat exposure to the same antigen, such as the Ebola MVA quadrivalent boost, the memory B cells rapidly differentiate into plasmablasts capable of secreting large volumes of antibody (Tarlinton and Good-Jacobson, 2013). Typically, there is a transient increase in these antigen-specific plasmablasts in the blood on day 5-8 post boost vaccination (Bernasconi et al., 2002; Wrammert et al., 2008), when they transit between the lymph nodes and bone marrow or spleen. The activated plasmablasts express CD19 (B cell marker), CD27 (memory B cell phenotype) and CD38 (plasma cell marker).

They also have down-regulated expression of CD20 (marking late stage B cell differentiation) and CD21 (absence marks B cell activation). Once in the bone marrow or spleen, the plasmablasts terminally differentiate into non-dividing plasma cells, which are the main source of secreted antibody.

ASCs can be isolated from fresh vaccinee PBMC by fluorescence-activated cell sorting (FACS) when they peak in the blood at day 7 post boost. The Ig RNA is isolated, reverse transcribed to complementary DNA (cDNA), and the specific heavy and light chain genes are amplified. These Ig chains can then be cloned into expression vectors and used to transfect human cell lines to produce mAbs. The mAbs are then screened by ELISA for antigen specificity. Once isolated, we can characterise the Ebola-specific mAbs by their binding affinity, binding site and cross-reactivity with other strains of *Ebolavirus* by ELISA and indirect immunofluorescence. We can also test their *in vitro* neutralising ability with pseudotyped influenza MN assays, determine their *in vivo* potency through animal models, and map their epitopes with X-ray crystallography, competition assays using well defined mAbs, target-antigen mutagenesis, and escape mutant-analysis (Wilson and Andrews, 2012).

1.5. Dissertation aims

The aims of this project are to isolate and then characterise fully human mAbs against the Ebola virus GP from vaccinees, which could potentially be used as therapy for Ebola disease.

2. Materials and Methods

2.1. Materials

2.1.1. Reagents and cell lines

Reagent	Company
2X ReddyMix PCR master mix	Thermo Scientific
4-nitrophenylphosphate-pNPP tablet	Sigma
Agarose	Sigma
Azide	Thermo Scientific
BCIP/NBT development buffer	Europa Bioproducts
Bovine serum albumin (BSA) 30%	Pierce
Carbenicillin	Sigma
Casein blocking buffer	Pierce
CASYton counting buffer	Sedna Scientific
Diethanolamine 5x buffer	Fisher Scientific
Dimethyl sulphoxide (DMSO) 2.5%)	Sigma
Diphtheria toxoid	NIBSC
Dulbecco's Modified Eagle Medium (DMEM)	Sigma
FastDigest buffer 10x	Fisher Scientific
FastDigest enzymes: Age1/ BshT1, Bsiw1/Pfl23II, Sal1, and Xho1	Fisher Scientific
Fetal calf serum (FCS)	Biotest Biosera
Formaldehyde 4%	Sigma
Gene ruler DNA ladder	Thermo Scientific
Glycerol	Sigma
Goat anti-human IgG (γ chain specific) -alkaline phosphatase conjugated	Sigma
Goat anti-human IgG γ-chain-HRP conjugate	Sigma
Goat anti-human IgG-Fluorescein Isothiocyanate (FITC) conjugate	Invitrogen
Goat anti-human IgG (Polyvalent)	Invitrogen
HEK293A cell line	In house (non-commercial)
HEK293E cell line	In house (non-commercial)
HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Life Technologies
Expi293 complete expression media	Life Technologies
Freestyle media	Life Technologies
L-glutamine	Gibco
Loading dye 6x	New England Biolabs
Lysogeny broth (LB)	Sigma

MDCK-SIAT	In house (non-commercial)
MDCK-SIAT-1 (E-SIAT)	In house (non-commercial)
MDCK-SIAT-1 (expressing GPΔmuc)	In house (non-commercial)
Nuclease-free water	Qiagen
Phosphate buffered saline (PBS)	Sigma
PBS with Tween-20 (PBS/Tween)	Sigma
Penicillin/Streptomycin	Gibco
Phusion polymerase	New England BioLabs
Protein G IgG elution buffer	Thermo Scientific
Polyethylenimine (PEI), Linear (MW 25,000)	Alfa Aesar
Qiagen Sensiscript RT kit	Qiagen
Qiagen Plasmid Plus Midi Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
Quick ligase	New England BioLabs
Quick ligase buffer 10x	New England BioLabs
RNase Zap	Sigma
RNasin ribonuclease inhibitor	Promega
RPMI-1640	Sigma
Recombinant shrimp alkaline phosphatase (rSAP)	New England BioLabs
Super optimal broth (SOC)	Invitrogen
Stellar competent cells	Takara Bio Europe SAS
Sybr Safe DNA gel stain	Invitrogen
1M Tris-HCl pH 8.0	Sigma
TrypLE Express (trypsin)	Invitrogen
Tryphan blue	Sigma
Zaire and Sudan Ebola GP (monomer and trimer)	In house (non-commercial)

2.1.2. Primers

RT-PCR primers		
#	**Name**	**Sequence 5' - 3'**
1	5' L VH1	ACAGGTGCCCACTCCCAGGTGCAG
2	5' L VH3	AAGGTGTCCAGTGTGARGTGCAG
3	5' L VH4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
4	5' L VH5	CAAGGAGTCTGTTCCGAGGTGCAG
5	3' Cγ CH1	GGAAGGTGTGCACGCCGCTGGTC
6	5' L VK1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
7	5' L VK3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
8	5' L VK4	ATTTCTCTGTTGCTCTGGATCTCTG
9	3' CK 543	GTTTCTCGTAGTCTGCTTTGCTCA

10	5' L Vλ1	GGTCCTGGGCCCAGTCTGTGCTG
11	5' L Vλ2	GGTCCTGGGCCCAGTCTGCCCTG
12	5' L Vλ3	GCTCTGTGACCTCCTATGAGCTG
13	5' L Vλ4/5	GGTCTCTCTCSCAGCYTGTGCTG
14	5' L Vλ6	GTTCTTGGGCCAATTTTATGCTG
15	5' L Vλ7	GGTCCAATTCYCAGGCTGTGGTG
16	5' L Vλ8	GAGTGGATTCTCAGACTGTGGTG
17	3' Cλ	CACCAGTGTGGCCTTGTTGGCTTG
Screening/sequencing primers		
54	5'Ab sense	GCTTCGTTAGAACGCGGCTAC
55	AbVec IgG Rv	GGAAGTAGTCCTTGACCAGGC
56	AbVec Igκ Rv	GTTATTCAGCAGGCACACAACAG
57	AbVec Igλ Rv	TATCTGCCTTCCAGGCCAC
CPEC (nested PCR) primers		
58	CPEC VH1/5/7	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
59	CPEC VH3	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG
60	CPEC VH4	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG
61	CPEC VH3-23	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG
62	CPEC VH4-34	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG
63	CPEC VH1–18	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTTCAGCTGGTGCAG
64	CPEC VH1–24	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG
65	CPEC VH3–9/30/33	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG
66	CPEC VH6–1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG
67	CPEC VH4–39	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG
68	CPEC VH3–33	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG
69	CPEC JH1/2/4/5	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACCAG
70	CPEC JH3	GATGGGCCCTTGGTCGACGCTGAAGAGACGGTGACCATTG
71	CPEC JH6	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACCGTG
72	IPCR AbVec GAMMA Fw	GTCGACCAAGGGCCCA
73	CPEC VK1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC
74	CPEC VK1-9/1-13	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT
75	CPEC VK1D-43/1-8	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC
76	CPEC VK2	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC
77	CPEC VK2-28/2-30	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC
78	CPEC VK3-11/3D-11	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC
79	CPEC VK3-15/3D-15	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC
80	CPEC VK3-20/3D-20	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT
81	CPEC VK4-1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC
82	CPEC JK1/2/4	ATGGTGCAGCCACCGTACGTTTGATYTCCACCTTGGTC
83	CPEC JK3	ATGGTGCAGCCACCGTACGTTTGATATCCACTTTGGTC
84	CPEC JK5	ATGGTGCAGCCACCGTACGTTTAATCTCCAGTCGTGTC
85	CPEC JK150/3	ATGGTGCAGCCACCGTACGTCTGATTTCCACCTTGGTC
86	IPCR AbVec Kappa Fw	CGTACGGTGGCTGCAC

87	CPEC VL1	CTTTTTCTAGTAGCAACTGCAACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG
88	CPEC VL2	CTTTTTCTAGTAGCAACTGCAACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG
89	CPEC VL3	CTTTTTCTAGTAGCAACTGCAACCGGTTCTGTGACCTCCTATGAGCTGACWCAG
90	CPEC VL4/5	CTTTTTCTAGTAGCAACTGCAACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA
91	CPEC VL6	CTTTTTCTAGTAGCAACTGCAACCGGTTCTTGGGCCAATTTTATGCTGACTCAG
92	CPEC VL7/8	CTTTTTCTAGTAGCAACTGCAACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG
93	CPEC CL reverse	GGCTTGAAGCTCCTCACTCGAGGGYGGGAACAGAGTG
94	IPCR AbVec Lambda Fw	CTCGAGTGAGGAGCTTCAAG
95	IPCR AbVec Lambda Rv	ACCGGTTGCAGTTGCTACTA
mAb expression cassette primers		
96	J2620-mabK7Fw	GAGTCAGTGAGCGAGGAAG
97	J2621-mabK7Rev	GTTGCTGACTAATTGAGATGC

2.1.3. Growth media, buffers and solutions

Name	Constituents
D0	500mL **DMEM** 5mL Pen/Strep (100U Penicillin, 100µg Strep/mL final concentration) 10mL L-glutamine (4mM final concentration)
D10	500mL **DMEM** 50mL **FCS** (10% final concentration) 5mL Pen/Strep (100U Penicillin, 100µg Strep/mL final concentration) 10mL L-glutamine (4mM final concentration)
R10	500mL **RPMI**-1640 media 50mL **FCS** (10% final concentration) 5mL Pen/Strep (100U Penicillin, 100µg Strep/mL final concentration) 5mL L-glutamine (4mM final concentration)
Development buffer	16mL deionised water 4mL 5x diethanolamine buffer 20mg 4-nitrophenylphosphate-pNPP tablet
Viral growth medium (VGM)	500mL DMEM 1.66mL 30% BSA (0.1% final concentration) 5mL HEPES (10mM final concentration) 5mL Pen/Strep (100U Penicillin, 100µg Strep/mL final concentration) 5mL L-glutamine (2mM final concentration)
Wash buffer	500mL PBS 1.66mL 30% BSA (0.1% final concentration) 0.1mL Azide (0.02% final concentration)
Fax-fix	18mL Wash buffer 2mL 4% Formaldehyde (0.4% final concentration)

2.1.4. Laboratory consumables

Consumable	**Company**
0.22µm syringe filter	Millipore
96-well U-bottom plate	Corning
96-well flat bottom cell culture plate	Corning
96-well PCR plate, non-skirted	Star Lab
Amicon Ultra 15mL centrifuge filters	Millipore
CASYton tubes	Sedna Scientific
Centrifuge tubes, 150mL	Corning
F96 Maxisorp Nunc Immuno-Plate	Nunc
HiTrap Protein G HP 5mL column	GE Healthcare Life Sciences
ELIspot Plates	Millipore
Erlenmeyer conical flasks, 250mL	Corning
Erlenmeyer conical flasks, 1000mL	Corning
PCR tubes strip domed 12 cap	Bio-Rad
T75 flat-bottom tissue culture flasks	Corning
Stericup 0.22µm vacuum filter	Millipore

2.1.5. Electronic equipment

Equipment	**Company**	**Software**
Axiovert S100 inverted fluorescent microscope	Carl Zeiss	Axiovision digital microphotography software
AID ELIspot plate reader	AID	AID ELIspot Reader v5
ÄKTA Pure FPLC machine	GE Healthcare Bio-Sciences	
ÄKTA start FPLC machine	GE Healthcare Bio-Sciences	
CASY cell Counter	Schärfe System	
CLARIOstar plate reader	BMG Labtech	CLARIOstar software
G Storm PCR Machine	Lab Tech	
Gel tank	Bi-Rad	
Infinite F50 Absorbance Microplate Reader	Tecan	Magellan reader
MoFlo cell sorter	Dako Cytomation	
Nanodrop ND-1000 Spectrophotometer	Labtech	ND-1000

2.2. Selection of volunteers: Enzyme-linked immunospot (ELISPOT)

The *ex-vivo* ASC ELISPOT assay was used to determine the immunological response to the Ebola vaccination in human volunteers. The PBMCs used in this experiment were taken from samples seven days post MVA boost, which was the time point when the highest levels of Ebola GP-specific ASCs and antibody were detected. Usually ASCs are detected from 14 to 28 days post boost. The volunteer samples with the highest percentage Ebola GP-specific ASC ELISPOT responses were selected for isolation of antigen-specific mAbs. ELISPOT responses are reported in raw numbers of Ebola GP-specific ASC, and percentage of Ebola GP-specific ASCs in the IgG^+ PBMC population.

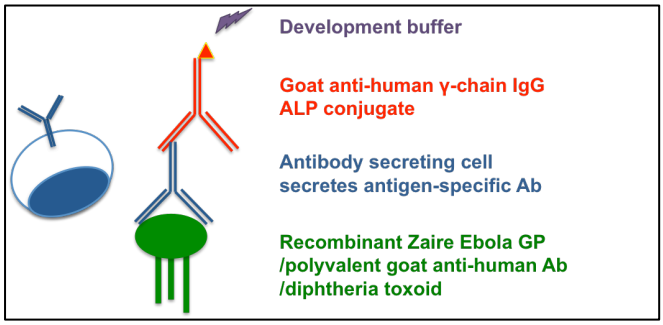


Figure 1. The enzyme-linked immunospot

ELISPOT plates were coated with 50µL Zaire Ebola GP at 10µg/mL. For the detection of total IgG secreting PBMCs, wells were coated with 50µL polyvalent goat anti-human IgG at 50 µg/mL in PBS. Control wells were coated with PBS or diphtheria toxoid at 10µg/mL. Plates were left overnight at 4ºC. The following day, plates were washed twice with 100µl/well sterile PBS and blocked with 100µl/well R10 media for 1 hour at 37°C.

PBMCs were thawed from frozen, washed, suspended in R10 media and counted in CASYton counting buffer with the CASY cell counter. PBMCs were then resuspended at $5x10^6$ cells/mL in R10 media and plated at $2.5x10^5$ cells/well. Additional dilutions were made of 1:2 for the antigen-specific wells, and 1:5 and 1:25 dilutions for the wells coated with the anti-human IgG. Plates were incubated for 18 hours at 37°C and 5% CO_2, and then washed six times with PBS containing 0.05% Tween. 50µL/well of goat anti-human IgG (γ-chain) conjugated to alkaline

phosphatase diluted 1:5000 in PBS was added and incubated at room temperature (RT) for 4 hours. Plates were again washed six times with PBS/Tween and 50µl/well phosphate substrate BCIP/NBT development buffer was added. When spots appeared after 3-5 minutes, plates were washed with tap water and left to dry overnight. Spots in each well were counted by an AID automated plate reader and results were obtained from the average of duplicate wells after subtracting background.

2.3. Isolation of human mAbs from ASCs sorted from vaccinated volunteers PBMCs

The protocol for the isolation of mAbs from ASCs is based on established methodologies (Smith et al., 2009; Tiller et al., 2008).

2.3.1. Cell sorting

ASCs, defined as $CD19^{+}$, $CD27^{+}$, $CD20^{-}$, $CD21^{-}$, $CD38^{+}$, and IgG^{+}, were isolated from fresh vaccinee PBMC by FACS using fluorescently labelled marker-specific antibodies and a MoFlo cell sorter. Cells were single sorted into 48 wells of a 96-well plate containing RNasin catch buffer, a ribonuclease inhibitor that preserves the RNA transcripts coding for the antigen-specific BCR within the plasmablasts. Plates were stored at -80°C prior to RT-PCR.

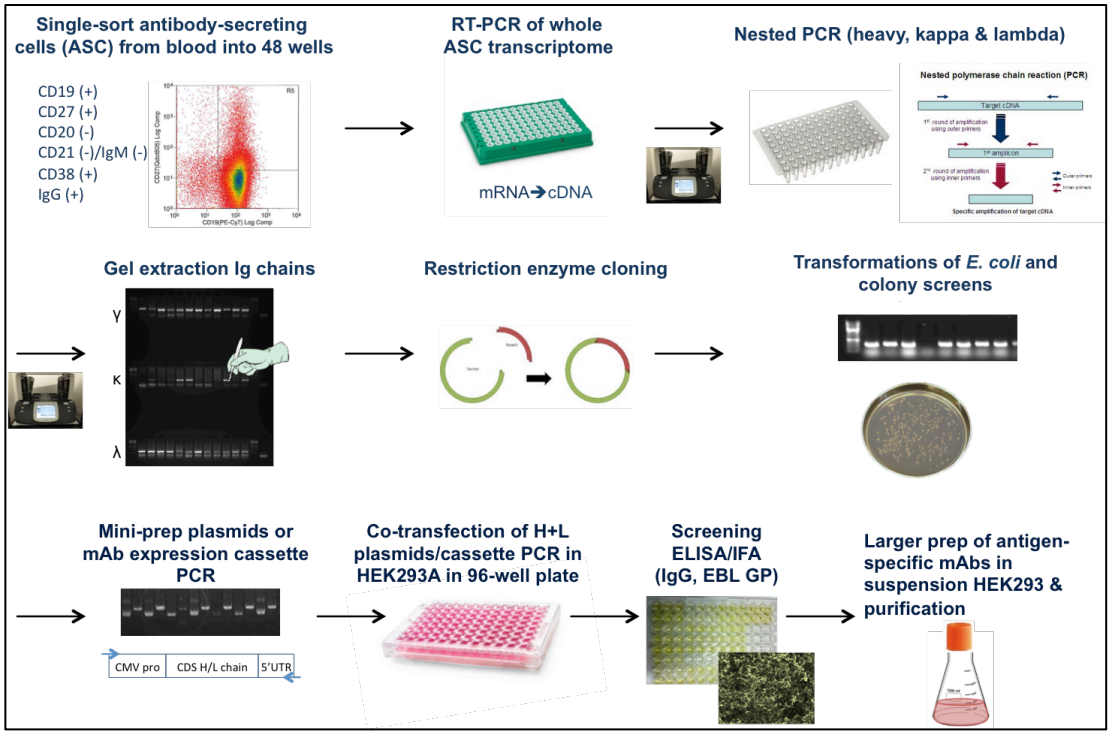


Figure 2. Workflow from single-cell sort of ASCs through to purification of antigen-specific mAbs

2.3.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Plates were thawed on ice and spun briefly to prevent cross-contamination of wells when removing lids. A primer mix (Table 1), containing 14 forward and 3 reverse primers was constituted. This was added to a RT-PCR mix (Table 2). 10µL of the RT-PCR mix was added to each well containing approximately 10µL of catch buffer and the single-sorted ASC. Wells were sealed with cap strips. All PCR programmes were run on a G Storm PCR Machine. The RT-PCR programme was as follows: 40 minutes at 37°C; 10 minutes at 43°C; 10 minutes at 50°C, with a holding temperature of 4°C. The resulting cDNA product was frozen for later use.

Table 1. RT primer mix

	Total:	
40µL each FWD primer 100µM stock	560µL	(3.57µM FWD primers in primer mix)
56µL each REV primer 100µM stock	168µL	(5.00µM REV primers in primer mix)
392µL nuclease-free water (Qiagen)	392µL	
	1120µL	

Table 2. RT-PCR reaction (Qiagen Sensiscript RT kit)

Nuclease-free water	3µL	
10x buffer	2µL	
RT primer mix	2µL	(0.357µM FWD primers and 0.5µM REV primers in Sensiscript RT-PCR reaction)
dNTP	2µL	
Sensiscript RT enzyme	1µL	
Well containing catch buffer and ASC	10µL	
	20µL reaction	

2.3.3. First PCR (cDNA amplification) and nested PCR

The first PCR reaction was a cDNA amplification step followed by an Ig chain-specific nested PCR. 19µL of a PCR master mix (Table 3) was added to each well of a clean 96-well PCR plate and 1µL of cDNA was added using a multichannel pipette. Wells were sealed with cap strips and the following PCR programme was run: 98°C for 30 seconds; then 15 cycles of alternating 98°C for 10 seconds and 72°C for 1 minute; 15 cycles of 98°C for 10 seconds alternating with 68°C for 30 seconds and 72°C for 1 minute; and finally 72°C for 10min, with a holding temperature of 4°C.

Table 3. First PCR reaction

Nuclease-free water	7.5µL	
RT primer mix	1µL	(0.357µM FWD primers and 0.5µM REV primers in PCR reaction)
DMSO (2.5%)	0.5µL	
Phusion polymerase (New England BioLabs)	10µL	
cDNA	1µL	
	20µL reaction	

The PCR product was diluted 1:100 by dispensing 180µL of nuclease-free water in each well prior to nested PCR. PCR master mixes containing phusion polymerase, DMSO and chain-specific primers were prepared for each Ig chain (Table 4-Table 6). 19µL was added to each well of a 96-well PCR plates (one plate for each of the heavy, kappa and lambda chains). 1µL of the 1:100 dilution of the first PCR product was added using a multichannel pipette. Wells were sealed with cap strips and the following PCR programme was run: 98°C for 30 seconds; 40 cycles of alternating 98°C for 10 seconds and 72°C for 1 minute; then 72°C for 10min, with a holding temperature of 4°C.

Table 4. Nested PCR reaction (heavy chain)

Heavy chain primer mix: 14 CPEC heavy primers, No. 58-71		
	Total:	
7µL each heavy primer (100µM stock)	98µL	(2.41µM each primers in primer mix)
42µL nuclease-free water	42µL	
	140µL	
Nested PCR Reaction:		
Nuclease-free water	8.25µL	
Heavy chain primer mix	0.25µL	(0.06µM of each primers)
DMSO (2.5%)	0.5µL	
Phusion polymerase	10µL	
cDNA (1:100 dilution in water)	1µL	
	20µL reaction	

Table 5. Nested PCR reaction (kappa chain)

Kappa chain primer mix: 13 CPEC kappa primers, No. 73-85		
	Total:	
7µL each kappa primer (100µM stock)	91µL	(2.41µM each primers in primer mix)
49µL nuclease-free water	49µL	
	140µL	
Nested PCR Reaction:		
Nuclease-free water	8.25µL	
Kappa chain primer mix	0.25µL	(0.06µM of each primers)
DMSO (2.5%)	0.5µL	
Phusion polymerase	10µL	
cDNA (1:100 dilution in water)	1µL	
	20µL reaction	

Table 6. Nested PCR reaction (lambda chain)

Lambda chain primer mix: 7 CPEC lambda primers, No. 87-93		
	Total:	
21µL each lambda primer (100µM stock)	147µL	(1.86µM each primers in primer mix)
273µL nuclease-free water	273µL	
	420µL	
Nested PCR Reaction:		
Nuclease-free water	8.25µL	
Lambda chain primer mix	0.75µL	(0.19µM of each primers)
Phusion polymerase	10µL	
cDNA (1:100 dilution in water)	1µL	
	20µL reaction	

2.3.4. Gel extraction/purification

The entire PCR product (around 20µL) with 3µL of 6x loading dye was run on a 1.8% agarose gel and screened for positive bands around 440bp. The positive bands were cut out, purified using the Qiagen QIAquick gel extraction kit, and eluted in 30µL of nuclease-free water into 1.5mL Eppendorf tubes. The cDNA concentrations were then measured using the NanoDrop 1000 spectrophotometer.

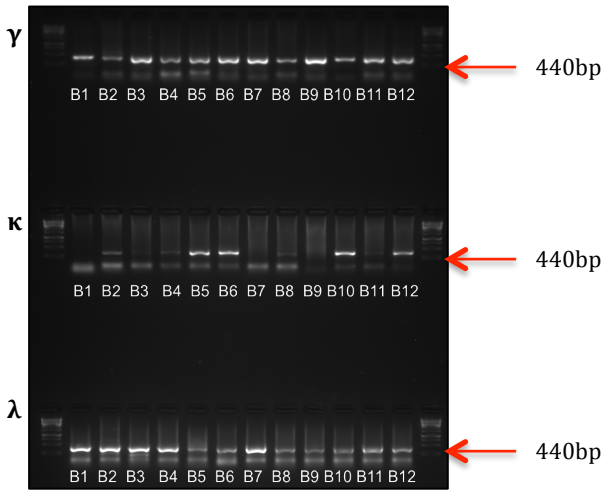


Figure 3. Protein gel electrophoresis, Row B Plate 136 (γ, κ, and λ chains)

2.3.5. Restriction enzyme cloning

Insert Digestion

30µL (approximately 300-600ng) of each cDNA insert was digested for 1 hour at 37°C in Eppendorf tubes using FastDigest enzymes and buffer from Fisher Scientific (Table 7). The reactions were then heat inactivated at 80°C for 10 minutes.

Table 7. Insert digestion reaction

Ig chain	Chain-specific enzyme	Reaction
Heavy	0.5µL Sal1 FastDigest	0.5µL Age1/ BshT1 FastDigest enzyme + 0.5µL Sal1/ Bsiw1/Pfl23II/ Xho1 FastDigest enzyme + 3.4µL FastDigest buffer + 30 µL gel extraction
Kappa	0.5µL Bsiw1/Pfl23II FastDigest	
Lambda	0.5µL Xho1 FastDigest	

Backbone Digestion

Ig chain-specific vectors (Figure 4) were digested in 40 µL aliquots using the same enzyme combinations (Table 8) as the insert digestions (FastDigest). The reaction also contained rSAP. The backbones were digested at 37°C for 1 hour and then heat inactivated at 80°C for 10 minutes.

Table 8. Backbone digestion reaction

Ig chain	Chain-specific enzyme	Reaction
Heavy	1µL Sal1 FastDigest	1µL Age1/ BshT1 FastDigest enzyme + 1µL Sal1/ Bsiw1/Pfl23II/ Xho1 FastDigest enzyme + 4µL FastDigest buffer + 31µL water + 2µL rSAP + 1µg (1µL) H/κ/ λ plasmid
Kappa	1µL Bsiw1/Pfl23II FastDigest	
Lambda	1µL Xho1 FastDigest	

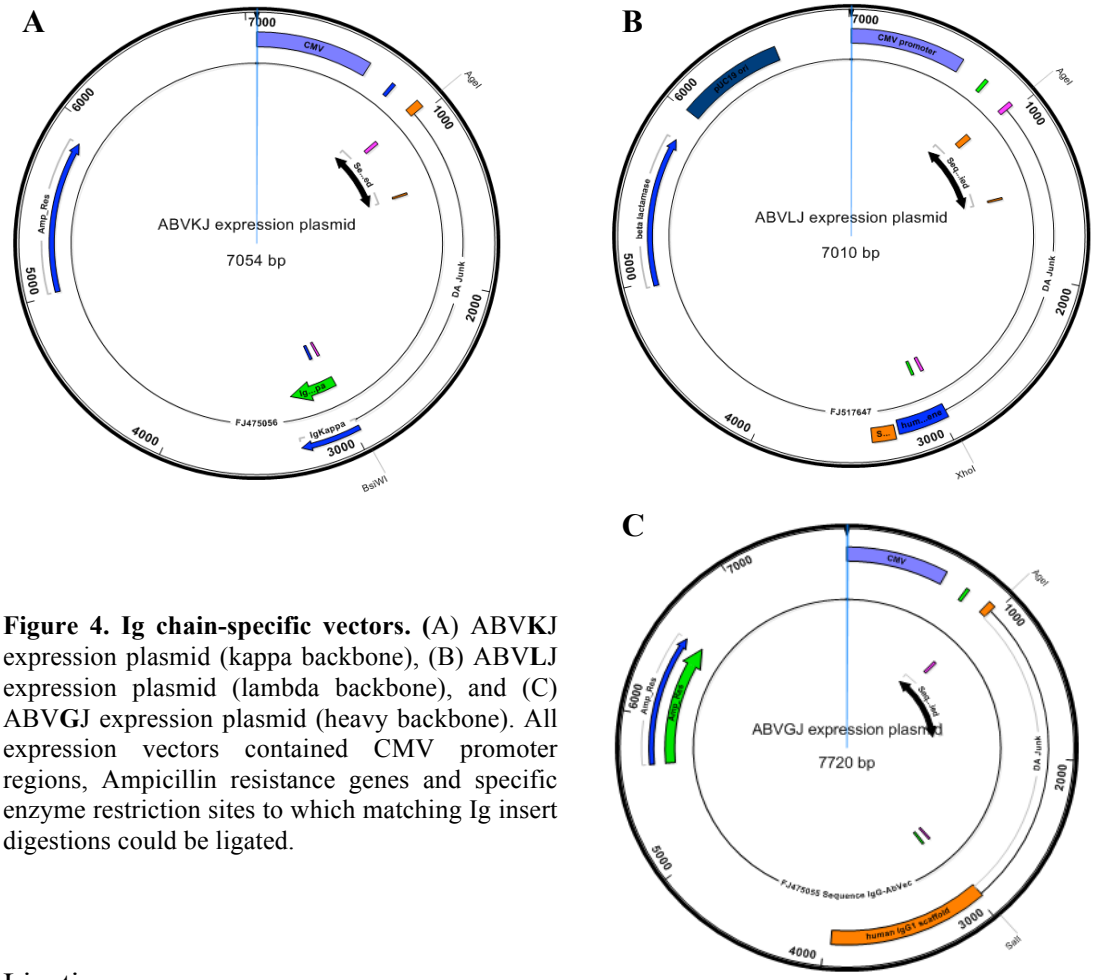


Figure 4. Ig chain-specific vectors. (A) ABV**K**J expression plasmid (kappa backbone), (B) ABV**L**J expression plasmid (lambda backbone), and (C) ABV**G**J expression plasmid (heavy backbone). All expression vectors contained CMV promoter regions, Ampicillin resistance genes and specific enzyme restriction sites to which matching Ig insert digestions could be ligated.

Ligation

Ligations of insert digestions into chain-specific backbones were performed using Quick Ligase. 1-3µL (approximately 20ng) of digested insert was added to 2µL of digested insert-specific backbone, 15µL of nuclease-free water, 1.5µL Quick Ligase buffer and finally 0.5µL Quick Ligase. The 20µL ligation reaction took 10 minutes at RT and was used to transform *E. coli.*

2.3.6. Transformation

2µL ligation was combined with 10µL Stellar competent cells in a 96-well PCR plate and left on ice for 30 minutes. The samples were heat shocked for 30 seconds in a 42°C water bath and then placed on ice for a further 2 minutes. 50µL of SOC medium was added and samples were incubated for 10 minutes at 37°C. 30µL of the cell mixture was spread on warmed 0.1% Carbenicillin agar plates and left overnight in a 37°C incubator.

2.3.7. Colony screening

Four colonies were randomly selected from each agar plate and screened with a PCR mix (Table 9) containing 2X ReddyMix PCR Master Mix (Taq polymerase), and heavy/kappa/lambda chain-specific (forward and reverse) plasmid screening/ sequencing primers. 10µL/well of the chain-specific master mix was added to a 96-well PCR plate and the wells were inoculated using a pipette-tip from each colony. Wells were sealed with cap strips and the following PCR programme was run: 98°C for 6 minutes; 10 cycles of alternating 95°C for 25 seconds, 68°C for 1 minute, 72°C for 90 seconds; 25 cycles of 95°C for 25 seconds, 68°C for 30 seconds, 72°C for 1 minute; 72°C for 5 minutes, with a holding temperature of 4°C.

Table 9. Colony screen reaction

Nuclease-free water	4µL
ReddyMix 2x PCR Master Mix	5µL
5'AbSense primer 10uM	0.5µL
AbVec Ig**G**/**K**/**L** Rev primer 10uM	0.5µL
	10µL per reaction

The total volume of the PCR reaction was run on a 1% agarose gel and screened for positive bands. Positive bands were around 600-700bp for heavy and 550bp for light chains.

2.3.8. mAb expression cassette

Initially all positive colonies were cultured overnight in 4mL of 0.1% Carbenicillin LB in a 37°C shaking incubator and plasmids were purified using the QIAprep Spin Miniprep Kit. Matching pairs of purified plasmids were quantified by spectrophotometry and used to co-transfect HEK293A cells. This method proved time consuming; instead a mAb expression cassette step was added.

The mAb expression cassette step amplifies the whole expression cassette of the plasmid. Positive clones were cultured in a 96-well flat-bottom tissue culture plate containing 80µL 0.1% Carbenicillin LB inoculated with the positive colonies. Plates were placed in a shaking incubator at 180RPM at 37°C for 2 hours.

A 50µL mix (Table 10) containing phusion polymerase, forward (J2620-mabK7) and reverse (J2621-mabK7 Rev) primer was added to the wells of a 96-well PCR plate.

The mAb expression cassette mix was then inoculated with 0.3µL of bacterial culture. Wells were sealed with cap strips and the following PCR programme run: 98°C for 6 minutes; 35 cycles of alternating 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 2 minutes; 72°C for 10 minutes, followed by a 4°C holding temperature.

Table 10. mAb cassette reaction

Nuclease-free water	23µL
Primer J2620-mabK7 Fwd (10uM)	1µL
Primer J2621-mabK7 Rev (10uM)	1µL
Phusion	25µL
	50µL per reaction

The PCR product was then screened by gel electrophoresis: 1µL of 6x loading dye was added to 5µL of the PCR product and run on a 1.8% agarose gel. Positive bands were detected around 800-900bp for heavy and 600-700bp for light chains (Figure 5). Matching pairs of mAb expression cassette product were used to co-transfect HEK293A cells (in house, non-commercial cell line).

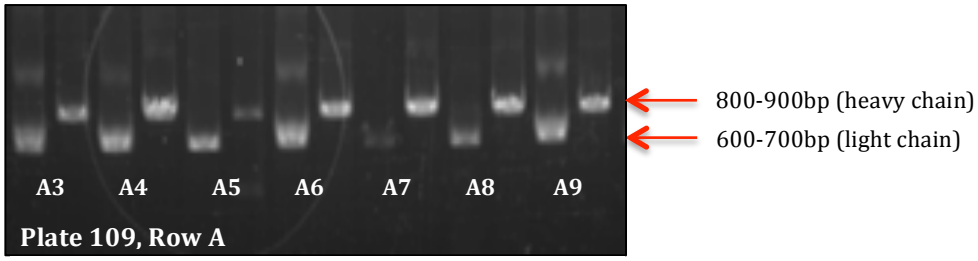


Figure 5. mAb cassette screening on a 1.8% agarose gel

2.3.9. Transfection of HEK293A cells

HEK293A cells were maintained as a rolling stock in D10 media in T75 flat-bottom tissue culture flasks and split every 3-5 days depending on confluency. In preparation for transfection, cells were washed with PBS and trypsinised with 5mL TrypLE Express for 5 minutes at 37°C. After detachment, live cells were counted with tryphan blue and a haemocytometer, and re-suspended at $2x10^5$/mL in D10 media. 100µL of cells was added to each well of a 96-well flat-bottom tissue culture plate and left overnight in a humidified 37°C incubator (5% CO_2). For routine passage, a new T75 flask was seeded with $5x10^5$ cells in 10mL D10 media.

Transfections were performed in triplicate: 100ng of plasmid (or 2µL of mAb Cassette PCR product) from each of the matching heavy and light chains was added to 50µL warm (37°C) D0 media in a 96-well U-bottom plate. 50µL of a 60µg/mL PEI solution diluted in D0 media was added slowly to the plasmid DNA. The U-bottom plate was then vortexed on the lowest setting for 1 minute and incubated at RT for 15 minutes. 100µL of the PEI/DNA mix was added to corresponding wells of HEK293A cells in the 96-well flat-bottom tissue culture plate and topped up with 50µL/well of warm D10 media. Plates were incubated for 3 days in a humidified 37°C incubator (5% CO_2).

2.3.10. Screening ELISA

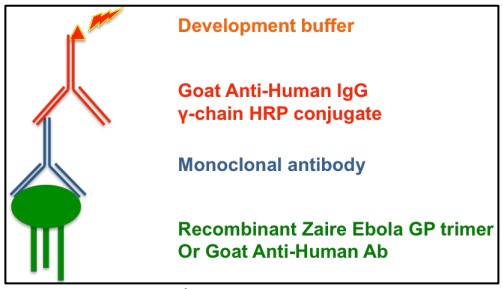


Figure 6. Screening ELISA

After incubation, supernatant from the triplicate wells was screened by ELISA (Figure 6) for IgG and antigen (Zaire Ebola GP trimer) specificity. F96 Maxisorp Nunc Immuno-Plate were coated with 1/1000 polyvalent goat anti-human IgG or 2µg/mL Ebola GP trimer diluted in PBS, covered and left overnight at RT. The following day, the plates were washed twice with PBS/Tween, blocked with 200µl/well Casein blocking buffer, and incubated for 1 hour at RT. Plates were then washed twice with PBS/Tween and 50µL HEK293A transfection culture supernatant was added as the primary antibody. Control wells contained PBS (negative control) and 3CSY (=KZ52, positive control). Plates were incubated for 1 hour at RT and then washed six times with PBS/Tween. The secondary antibody, a 1:1000 dilution of goat anti-human IgG γ-chain-HRP conjugate in PBS was added and the plate was incubated for 1 hour at RT. Plates were washed a further six times with PBS/Tween and 100µL development buffer was added. ELISA plates were read at 405nm after approximately 10 minutes using an Infinite F50 Absorbance Microplate Reader with Magellan reader and data analysis software.

2.3.11. Miniprep, sequencing of plasmids, and glycerol stock

Once a mAb was shown to be Ebola GP-specific, positive clones of matching heavy and light chains were inoculated from either the initial agar plates (see section 2.3.7) or mini-culture (see section 2.3.8) into 4mL 0.1% Carbenicillin LB. This was left overnight in the shaking incubator (225RPM, 37°C). 1.5mL of the culture was purified using the QIAprep Spin Miniprep Kit according to the manufacturer's guidelines. The plasmid was eluted in 50µL water and quantified by spectrophotometry. 7.5µL of plasmid DNA miniprep and 2.5µL 5'AbSense primer (10µM) was added to a 1.5mL Eppendorf tube and sent for sequencing by GATC-Biotech. Insert nucleotide sequences were analysed using the online analysis program IGBLAST. Glycerol stocks of both heavy and light chain cultures were made by adding 1mL of 70% glycerol to 1mL of the overnight culture in a 2mL cryovial and stored at -80°C.

2.3.12. Transfection of HEK293E cells in suspension

Positive clones of matching heavy and light chains were inoculated into 50mL 0.1% Carbenicillin LB in 250mL Erlenmeyer conical flasks and placed in the shaking incubator (225RPM, 37°C) overnight. Plasmid was extracted and purified using the Qiagen Plasmid Plus Midi Kit according to the manufacturer's guidelines. The plasmid was eluted in 200µL water and quantified by spectrophotometry.

Antigen-specific mAbs were bulked up by doing a large-scale transfection of HEK293E cells in suspension (in house, non-commercial cell line). HEK293E cells, maintained as a communal rolling stock, were re-suspended at $1x10^6$ cells/mL in 300mL of Expi293 complete expression media and added to a 1L Erlenmeyer conical flask. 1µg/mL of total plasmid DNA (150µg heavy and 150µg light chain) was combined with 12mL (4mL/100mL cell culture) Freestyle media at 37°C. 600µg of PEI (2µg/mL cell culture) was added to the tube, which was then vortexed for 1 minute and left at RT for 15 minutes. The PEI/DNA mix was slowly added by pipette while swirling the conical flask. The flask was incubated for 6 days in a humidified 37°C shaking incubator (5% CO_2).

2.3.13. mAb purification

The supernatant was harvested after 6 days by centrifuging at 2500xg for 10 minutes in 150mL Corning centrifuge tubes. The supernatant was then passed through a 0.22µm vacuum filter. Samples were refrigerated at 4°C until purification. mAb supernatant was purified and fractionated with a 5mL HiTrap Protein G HP column using the ÄKTA Start and ÄKTA Pure. Antibody fractions were eluted in 15mL Protein G IgG buffer, neutralised with 1M Tris-HCl pH 8.0 and then buffered exchanged into PBS using Amicon Ultra 15mL centrifuge filters following the manufacturer's guidelines. The purified, filtered mAbs were resuspended in 5mL PBS, filtered using a 0.22µm syringe filter, vortexed and aliquotted into 0.5mL cryovials. Concentrations were measured using the NanoDrop 1000 spectrophotometer.

2.4. Characterisation of mAbs

2.4.1. Indirect ELISA of purified mAbs

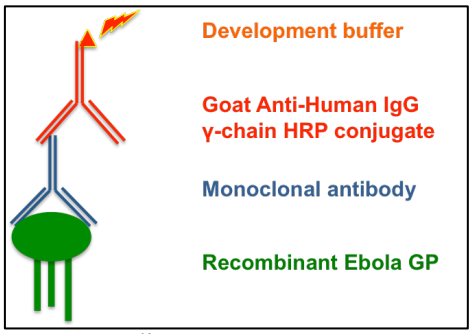


Figure 7. Indirect ELISA

To characterise the Ebola GP-specific antibodies, the purified mAbs were screened against Zaire and Sudan Ebola GPs in monomeric and trimeric form, as well as a crude unfractionated Zaire Ebola GP provided by a collaborating group at the WIMM. The method for the ELISA (Figure 7) has already been described in section 2.3.10. For this indirect ELISA, plates were coated with the above-mentioned Ebola GP antigens at 2µg/mL diluted in PBS. 50µL purified mAb diluted in PBS at 10µg/mL was added as the primary antibody (instead of the HEK293A transfection culture supernatant). Control wells contained AMA 1 and PBS (negative controls), 3CSY (positive control), and mAb 040 (positive control). mAb 040 is a fully human Ebola-specific mAb produced by collaborators in the same group prior to this project. In addition, a standardised dilution curve was created for the Zaire Ebola GP trimer antigen (2µg/mL) using 10-fold serial dilutions of the mAbs in PBS with a starting concentration of 10µg/mL. Plates were read at 405nm after 5 minutes for the Zaire Ebola GPs and 10 minutes for the Sudan Ebola GPs.

2.4.2. Indirect immunofluorescence assays

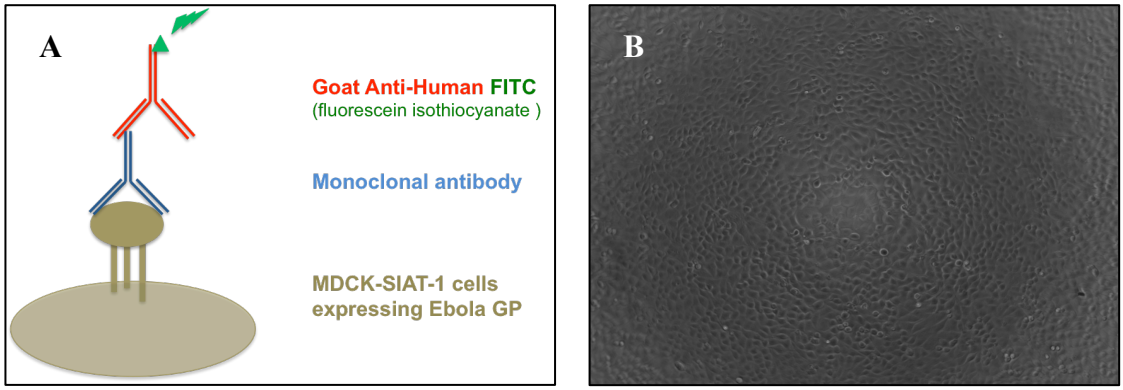


Figure 8. (A) Indirect IFA. (B) A confluent monolayer of MDCK-SIAT-1 cells.

The Ebola GP-specific mAbs were screened by indirect immunofluorescence (Figure 8) on canine MDCK-SIAT-1 cells stably expressing Zaire Ebola GP (called E-SIAT cells; in-house) and Zaire Ebola GPΔmuc (Mucin-deficient Zaire Ebola GP; in-house). Both cell lines were maintained as a rolling stock in D10 media in T75 flat-bottom flasks and split every 3 days. In preparation for the assay, cells were washed with PBS and trypsinised with 5mL TrypLE Express for 15 minutes at 37°C. After detachment, cells were counted with tryphan blue and a haemocytometer, and re-suspended at $3x10^5$/mL in D10 media. BSA (30%) was used instead of FCS in this assay. 100µL of cells was added to each well of a 96-well flat-bottom tissue culture plate and left overnight in a humidified 37°C incubator (5% CO_2). For routine passage, a new T75 flask was seeded with $5x10^5$ cells in 10mL D10 media.

The following day, the supernatant was discarded and the wells were washed twice with 200µL PBS. 50µL purified mAb at 10µg/mL (diluted in PBS) was added to each well in duplicates. Control wells contained PBS (negative control), mAb P6 (positive control), mAb 040 (positive control), and a control antibody known to bind the MLD of the Zaire Ebola GP. Plates were incubated for 1 hour at 37°C. The wells were washed twice with 200µL PBS and the secondary antibody, a 1:160 dilution of goat anti-human IgG-Fluorescein Isothiocyanate (FITC) conjugate in wash buffer was added. Plates were incubated for 1 hour at 4°C, washed twice with 200µL/well wash buffer, and 100µL/well fax-fix was added. Wells were viewed under the Axiovert S100 inverted fluorescent microscope and images were captured with the Axiovision digital microphotography software.

2.4.3. In vitro pseudotyped influenza microneutralisation assay

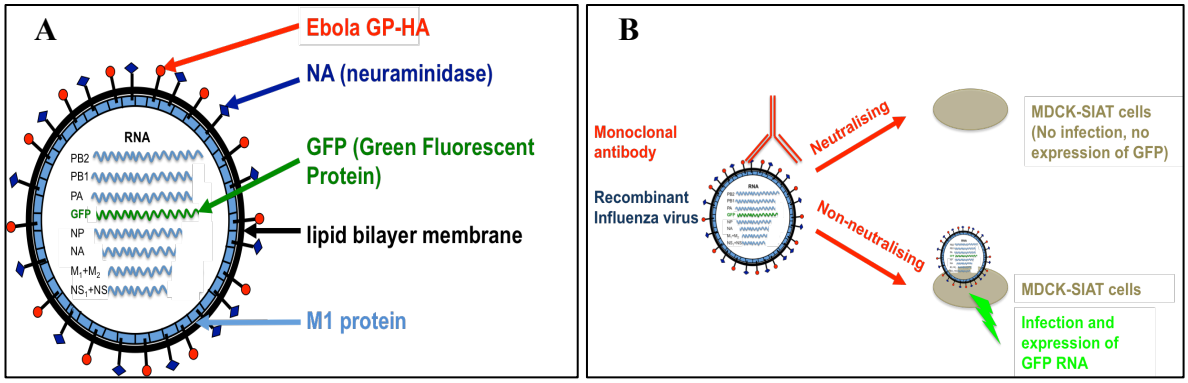


Figure 9. (A) Recombinant influenza virus expressing Zaire Ebola GP on a hemagglutinin cytoplasmic domain (S-Flu-EH) containing green fluorescent protein (GFP) RNA, and (B) influenza MN assay

The *in vitro* pseudotyped influenza MN assay (Figure 9) was developed by a collaborating group at the WIMM, based on paper by Rowe et al. (1999). The MDCK-SIAT-1 cell line used in this assay was maintained as a rolling stock in D10 media in T75 flat-bottom flask and split every 3 days. Recombinant replication deficient influenza virus expressing Zaire Ebola GP with a hemagglutinin cytoplasmic domain (S-Flu-EH) containing green fluorescent protein (GFP) RNA was provided by a collaborating group at the WIMM. This virus can infect cells and express the viral core proteins (including GFP) and neuraminidase, but is replication incompetent. For the assay, 50µL/well viral growth medium (VGM) was added to a 96-well flat-bottom tissue culture plate. 50µL purified mAb at 20µg/mL was added to the first column and serial dilutions were performed. This was followed by 50µL/well of recombinant influenza virus titrated pre-hand for plateau infection (1-4 HAU/50µL). Control wells contained influenza virus-only in VGM (no antibody), MDCK-SIAT-1 cells in VGM (no virus and no antibody), and positive control wells with mAb P6 and mAb 040. Plates were incubated at 37°C for 2 hours.

MDCK-SIAT-1 cells were washed in PBS, trypsinised (5mL trypsin at 37°C for 15 minutes) and resuspended at $3x10^5$ cells/mL in VGM. 100µL MDCK-SIAT-1 cells were added to each well and plates were incubated at 37°C for 24 hours.

The following day, supernatant was discarded and cells were fixed with 100µL 10% formaldehyde. Plates were incubated at 4˚C for 30 minutes and washed with 200µL PBS. Fluorescent intensity was read on a CLARIOstar plate reader with the following settings: 4mm detection diameter, 50 flashes per second, excitation 483, emission 515, with both bandwidth of 8nm, dichroic filter 499, focal length 5-6 and gain 3000. Wells were also viewed under the Axiovert S100 inverted fluorescent microscope and images were captured with the Axiovision digital microphotography software.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software and Graphpad QuickCalc online calculators. Statistical significance was considered at $P \leq 0.05$.

3. Results

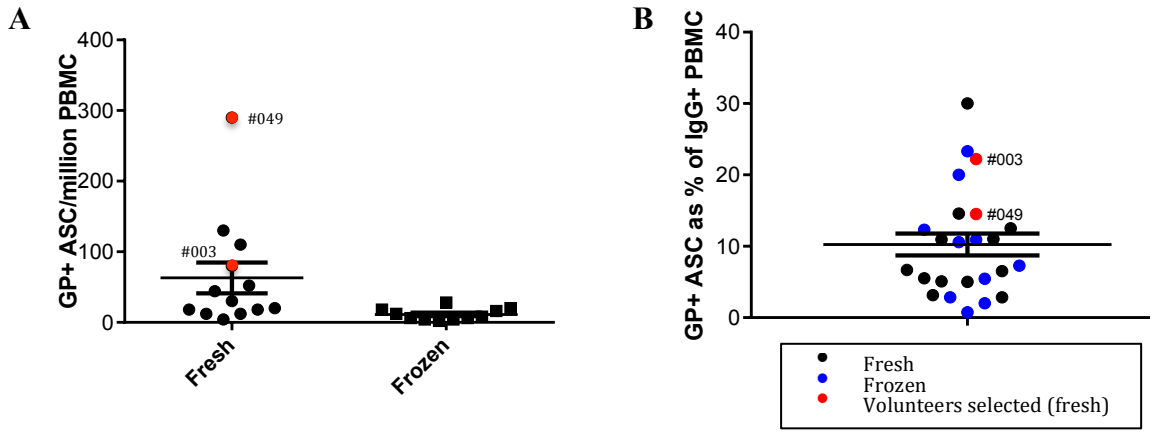
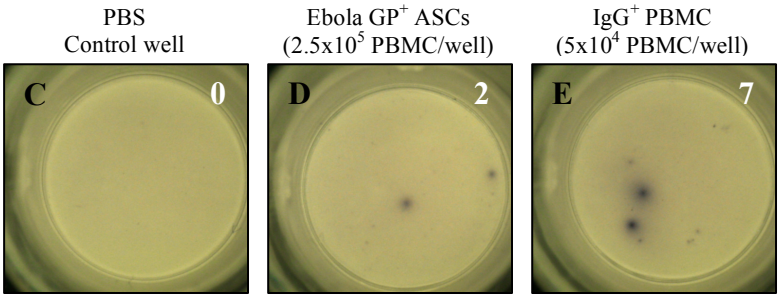


Figure 10. ASC ELISPOTs. Comparison of ASC ELISPOTs performed on fresh and frozen samples. (A) Ebola GP-specific ASCs per million PBMCs and, (B) as a percentage of the IgG$^+$ PBMC population. Wells containing (C) PBS control, (D) Ebola GP, and (E) goat anti-human IgG are shown.



3.1. ASC ELISPOT

Figure 10A shows a comparison between fresh and frozen *ex-vivo* ASC ELISPOT. Frozen PBMC had significantly lower numbers of Ebola GP-specific ASC/million PBMC than the fresh samples (mean difference=51.73; *z=2.64,* p=0.008[*]). However, the proportion of Ebola GP-specific ASCs in the IgG$^+$ PBMC population was comparable (mean difference=2.06%; z=0.96, p=0.337[*]) in both fresh and frozen samples (Figure 10B).

Volunteers 003 and 049 respectively had 22.22% and 14.50% Ebola GP-specific ASCs in their IgG$^+$ PBMC population at day seven post MVA boost. Volunteer 049 had a significantly higher count of Ebola GP-specific ASCs than volunteer 003 (290 vs. 80 per million PBMCs). On the basis of these ASC data, these two volunteers were selected for RT-PCR and isolation of mAbs.

[*] Mann-Whitney *u* Test

3.2. Generation of human mAbs

One plate containing 48 wells of single-sorted ASCs was processed from each volunteer: plate 109 from volunteer 003, and plate 136 from volunteer 049. The initial RT-PCR, amplification PCR and nested PCR steps yielded 91 matching pairs (from 96 wells) of Ig heavy and light chain cDNA (Table 11). After gel extraction and purification, the cDNA was digested and ligated onto matching Ig chain backbone. The plasmids were directly used to transform *E. coli*. Cloning had a 96% success rate, with 87 of the 91 matching pairs screening positive for both heavy and light chains. The positive clones were purified and used to co-transfect HEK293A cells. The transfection supernatant was screened by ELISA. 79 of the 87 transfections (91%) successfully produced IgG, and 15% (n=12) of these were Zaire Ebola GP-specific (Figure 11). The antigen-specific mAbs were then bulked up by doing a large-scale transfection of HEK293E cells in suspension and purifying the product. The purified antigen-specific mAbs were then characterised by indirect ELISA, IFA, *in vitro* influenza MN assay and sequencing of the plasmids.

Table 11. Success rates of PCR and transfection from each volunteer

Plate (volunteer):	#109 (003)	#136 (049)	Total
Wells PCR screen positive	46	45	91
Transformation colony screen positive, matching pairs (% of positive PCR wells)	42/46 (91%)	45/45 (100%)	87/91 (96%)
IgG^+ mAbs by ELISA (% of positive transformations)	40/42 (95%)	39/45 (87%)	79/87 (91%)
EBL GP^+ mAbs by ELISA (% of IgG^+ mAbs)	5/40 (13%)	7/39 (18%)	12/79 (15%)

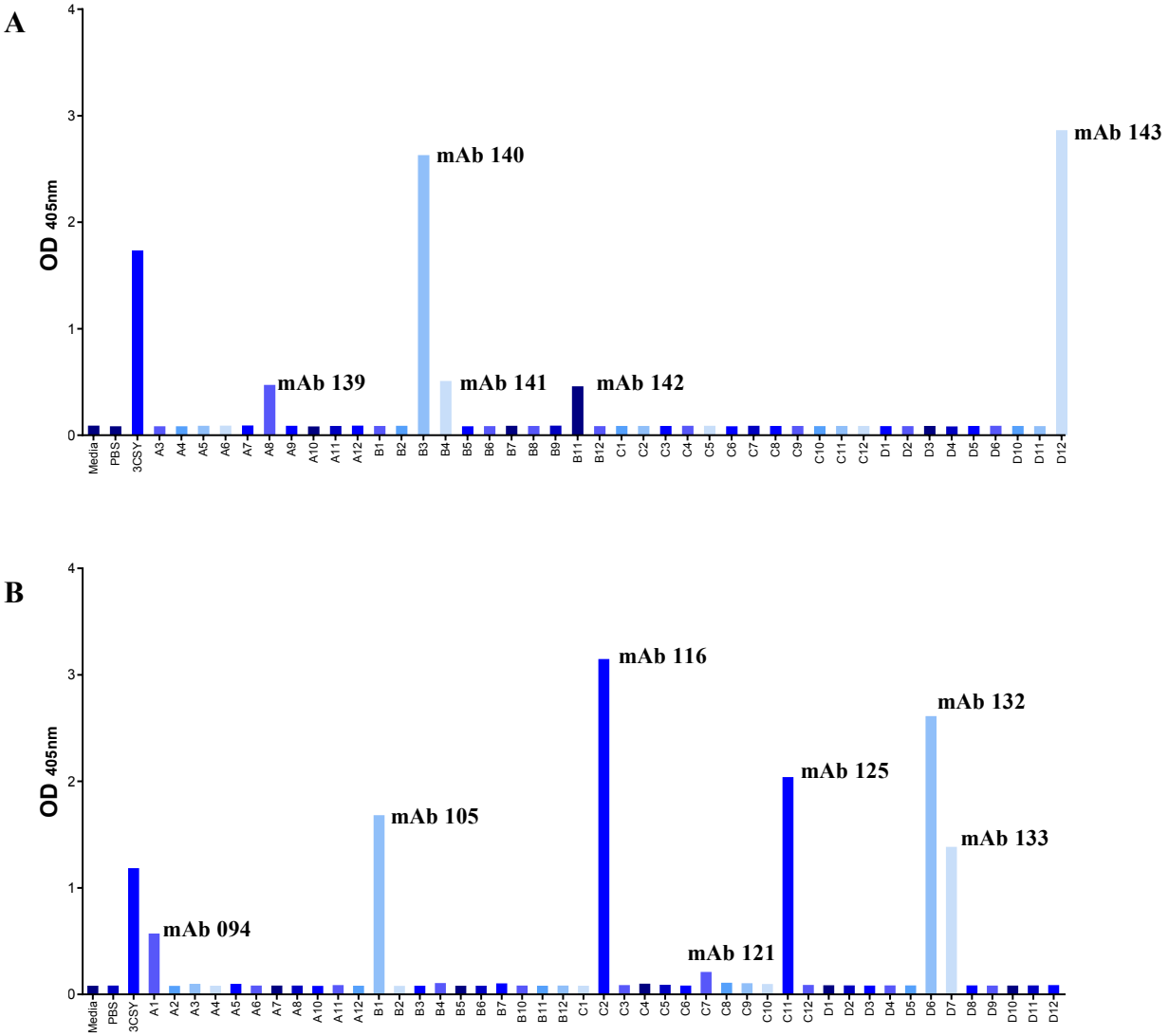


Figure 11. Indirect ELISA with Zaire Ebola GP trimer screening supernatant from (A) plate 109 (volunteer 003), and (B) plate 136 (volunteer 049).

3.3. Antibody binding affinity

Figure 12 demonstrates the relative affinity by indirect ELISA of the Ebola GP-specific mAbs at varying concentrations for the Zaire Ebola GP trimer. Relative binding strength was calculated using the half maximal effective concentrations (EC50s). Only three of the mAbs (mAbs 116, 140 and the control, mAb 040) exhibit strong binding (defined as an EC50 between 0.01-0.1µg/mL). Seven mAbs show medium binding (EC50 between 0.1-1µg/mL), and three mAbs bind weakly (EC50 between 1-10µg/mL). mAbs116 and 143 have the highest affinity at concentrations of 1-10µg/mL, but mAb 040 has the highest affinity at lower concentrations.

Antibody affinity for Zaire Ebola GP trimer was also assessed by fluorescence intensity readings of indirect immunofluorescence of MDCK-SIAT-1 cells expressing the Zaire Ebola GP (Figure 13). With this assay, mAbs 116, 125, 140 and 141 exhibit the strongest binding at a concentration of 10µg/mL. It is worth noting that fluorescence intensity readings may be influenced by the confluency of MDCK-SIAT cells in each well. Though care was taken to ensure consistency, this variable should be considered when interpreting these results.

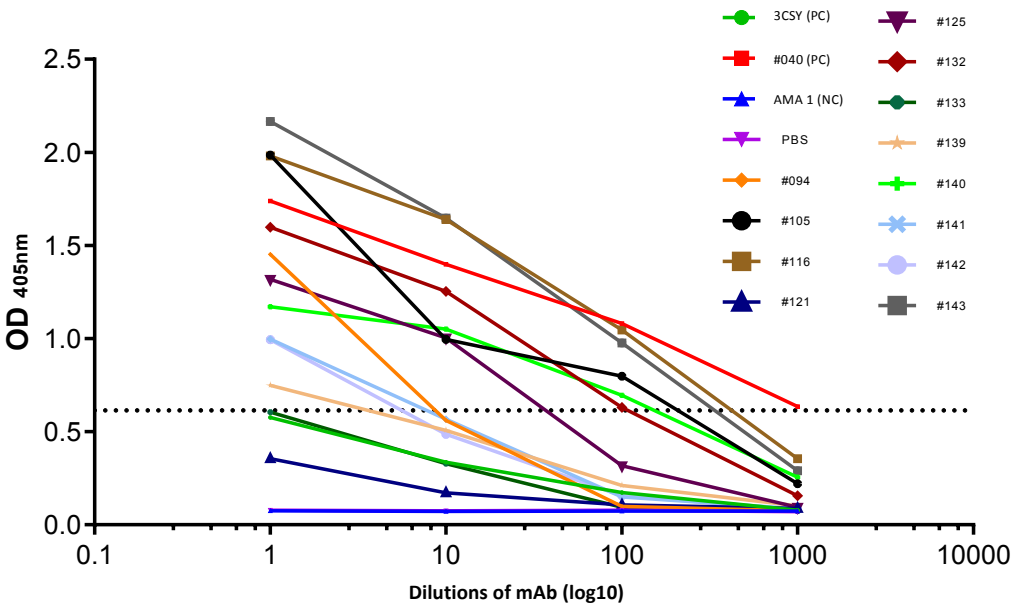


Figure 12. Binding curves determined by indirect ELISA with serial mAb dilutions (starting at 10µg/mL) against the Zaire Ebola GP trimer. Control wells contained, 3CSY (positive control), mAb 040 (positive control), and AMA 1 (negative control).

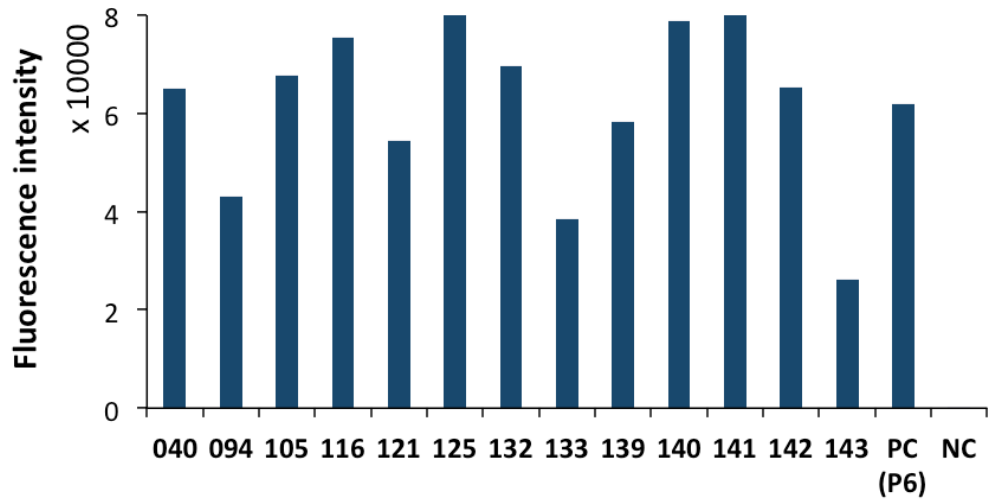


Figure 13. Indirect immunofluorescence. Fluorescence intensity readings of Ebola GP-specific mAbs bound to MDCK-SIAT-1 cells stably expressing Zaire Ebola GP Control wells contained P6 (positive control), a non-specific influenza antibody (negative control), and mAb 040 (positive control).

3.4. Cross-reactivity with Ebola GPs

All of the antibodies bind with higher affinity by ELISA to the trimeric forms of the Zaire and Sudan Ebola GPs (Figure 14), with the exception of mAb 141, which has a higher affinity for the monomeric form of the Sudan GP. Four of the antibodies (mAbs 040, 094, 133 and 141) exhibit cross-reactivity with the Sudan Ebola GP trimer and monomer. Antibody 141 has the highest affinity to Sudan GP. However, the affinity of these antibodies for the Sudan GPs is weak in comparison to affinity to the Zaire Ebola GPs. The ELISA also demonstrates a weaker affinity of all the mAbs for the Townsend Ebola GP, which is a crude unfractionated protein (containing both Zaire Ebola GP monomer and trimer).

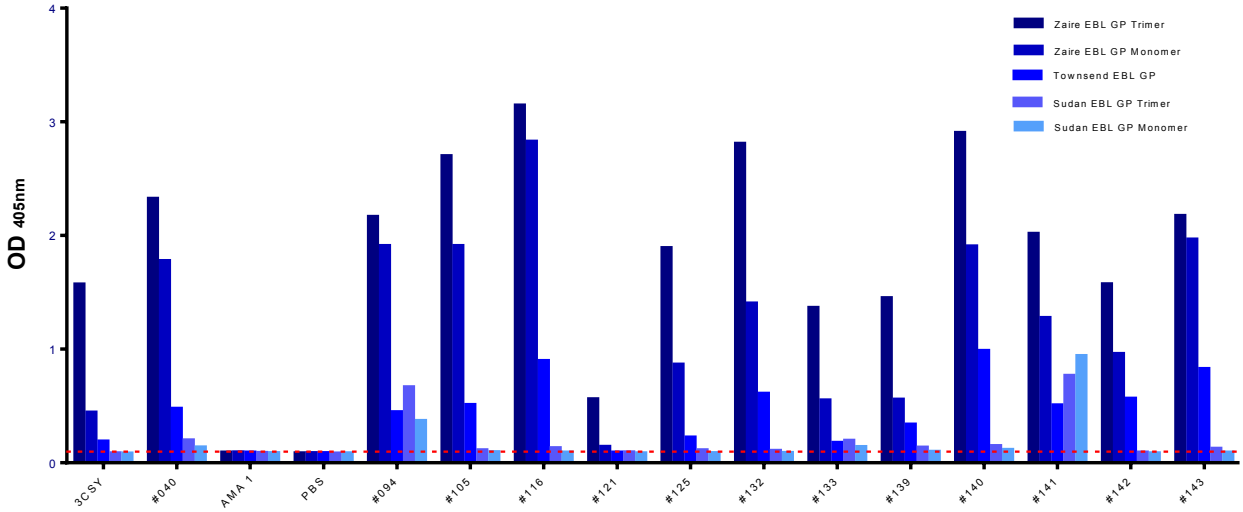
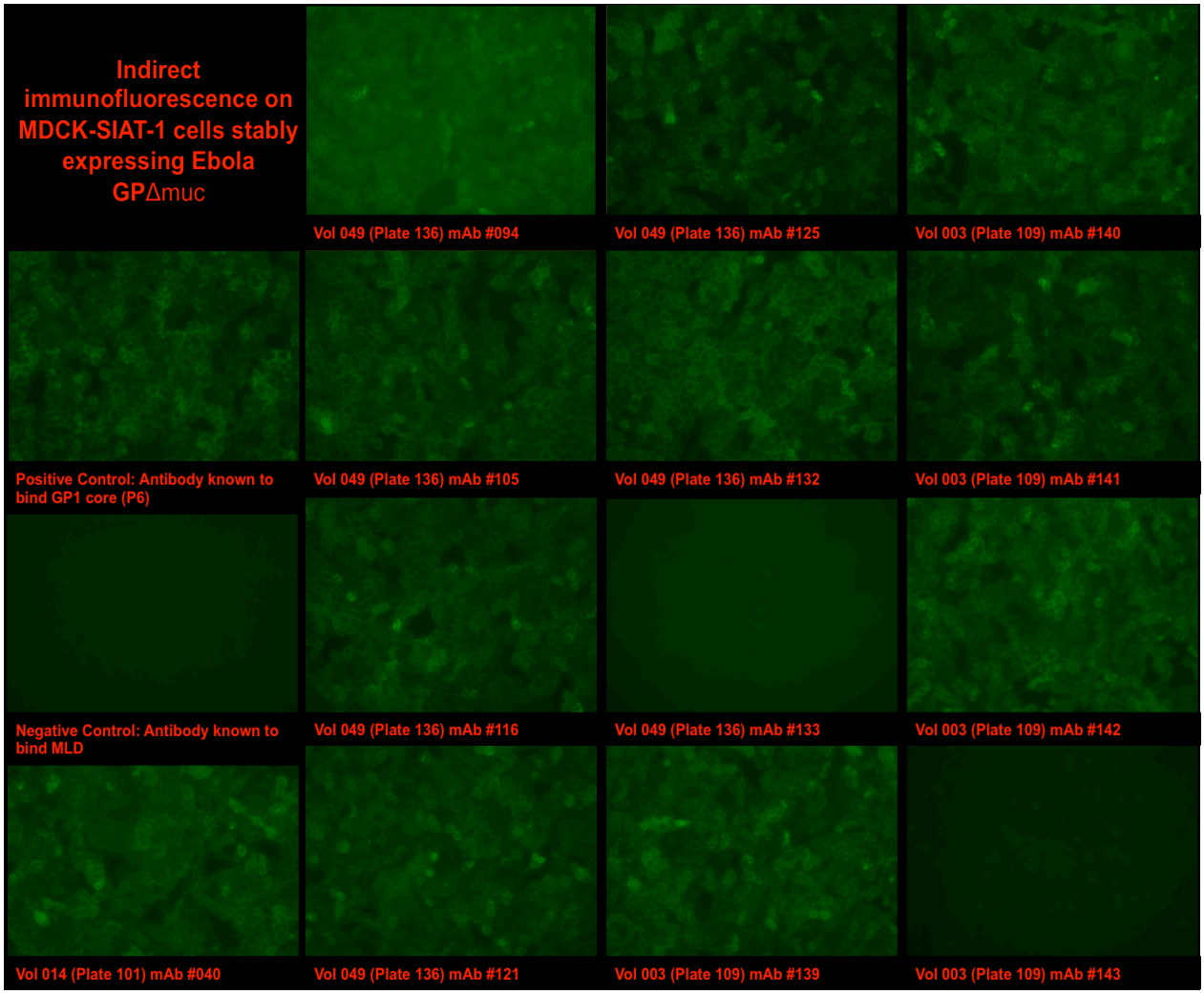


Figure 14. Indirect ELISA. Purified Ebola GP-specific mAbs against the Zaire and Sudan Ebola GP trimer and monomer, and a Zaire Ebola GP produced by the Townsend group at the WIMM.

3.5. Antibody binding site

Indirect immunofluorescence with MDCK-SIAT-1 cells expressing Zaire Ebola GPΔmuc revealed two antibodies (mAbs 133 and 143) that bind exclusively to the MLD of the GP1 protein (Figure 15). These two antibodies also display the weakest affinity on the IFA with the Zaire Ebola GP (Figure 13). Most of the other mAbs bound with similar affinity (less than 1.5 fold difference) to both the Ebola GP and GPΔmuc. We can infer that the other Ebola-specific mAbs bind to sites on the GP1 core or GP2 proteins. The exceptions were mAbs 132 and 094, which respectively showed a 1.8 and 2.4 fold decrease in affinity to GPΔmuc. This may indicate that part of their epitopes lie in the MLD. Another antibody of interest is mAb 133 as it is cross-reactive with the Ebola Sudan strain, indicating that its MLD epitope may be shared across strains.

A



B

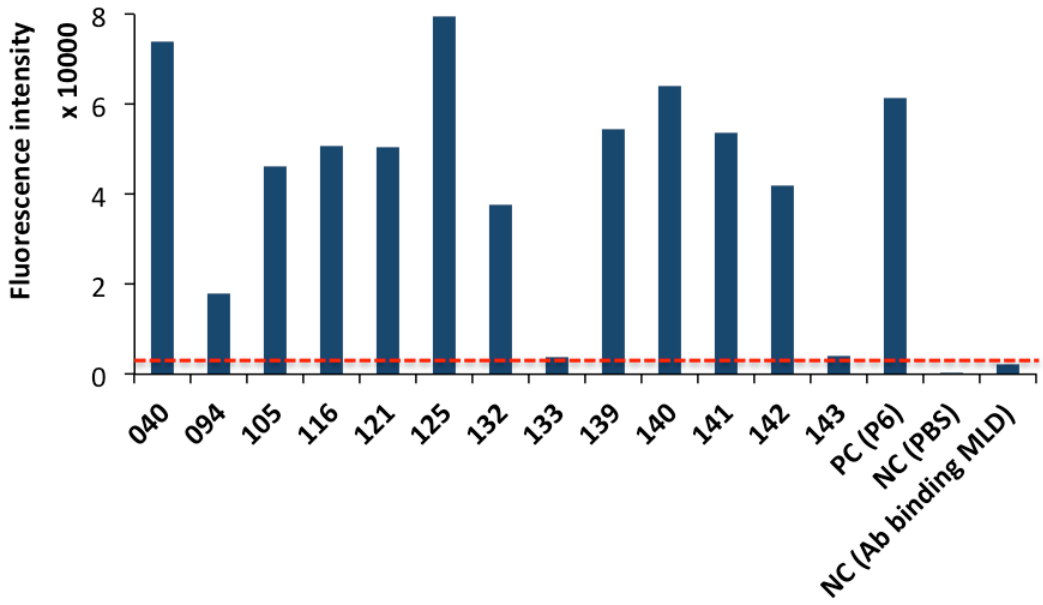


Figure 15. Indirect immunofluorescence demonstrating binding of Ebola GP-specific mAbs to MDCK-SIAT-1 cells stably expressing Zaire Ebola GPΔmuc. (A) Antibody that binds the surface antigen can be detected with a FITC-conjugated secondary antibody. This results in a characteristic (green) bright-ring pattern of staining around the MDCK-SIAT cell. Images captured at x200 magnification. (B) Fluorescence intensity readings.

3.6. *In vitro* neutralisation

The mAbs were screened for *in vitro* neutralisation with a recombinant influenza virus expressing Zaire Ebola GP on its surface with a cytoplasmic hemagglutinin domain (S-Flu-EH) and containing GFP RNA. Fluorescence indicates non-neutralisation of the influenza virus by the Zaire Ebola GP-specific mAbs and expression of GFP by the influenza infected SIAT cells (Figure 17). Conversely, neutralised influenza virus does not infect the SIAT cells and GFP is not expressed resulting in absent or limited fluorescence. Entry efficiency at a mAb concentration of 10µg/mL was calculated as a percentage of influenza viral entry (GFP fluorescence) in the wells containing mAbs over the virus-only control wells (mean). Five of the twelve antibodies (094, 116, 133, 139 and 143) are non-neutralising according to fluorescence intensity readings (Figure 16D) and fluorescent microscopy imaging (Figure 17). In-fact, at least two of these non-neutralising mAbs (116, 139) appear to enhance pseudotyped viral entry into SIAT cells (Figure 16A), with progressively lower fluorescence intensity readings with serial dilutions. This is in contrast to neutralising mAbs, which demonstrate progressively increasing fluorescence intensity readings with serial dilutions, which indicates decreased neutralisation at lower concentrations. Three of the mAbs exhibit partial neutralisation (mAbs 121, 132 and 140), reducing viral entry and expression of GFP by 30-60% (Figure 16A,C) at the highest concentration (10µg/mL). Four of the mAbs are neutralising (mAbs 105, 125, 141, 142), reducing viral entry by >70% at the highest concentration tested (Figure 16A,B). The positive control mAb 040 (a human Ebola-specific mAb produced by collaborators in the same group prior to this project) exhibited the highest *in vitro* neutralisation reducing viral entry by 95%. Neither of the antibodies that bind the MLD are neutralising, however one antibody that is cross-reactive with Sudan GP (mAb 141) neutralises the Zaire pseudotyped virus. Whether it would neutralise an equivalent virus expressing Sudan GP is yet to be established.

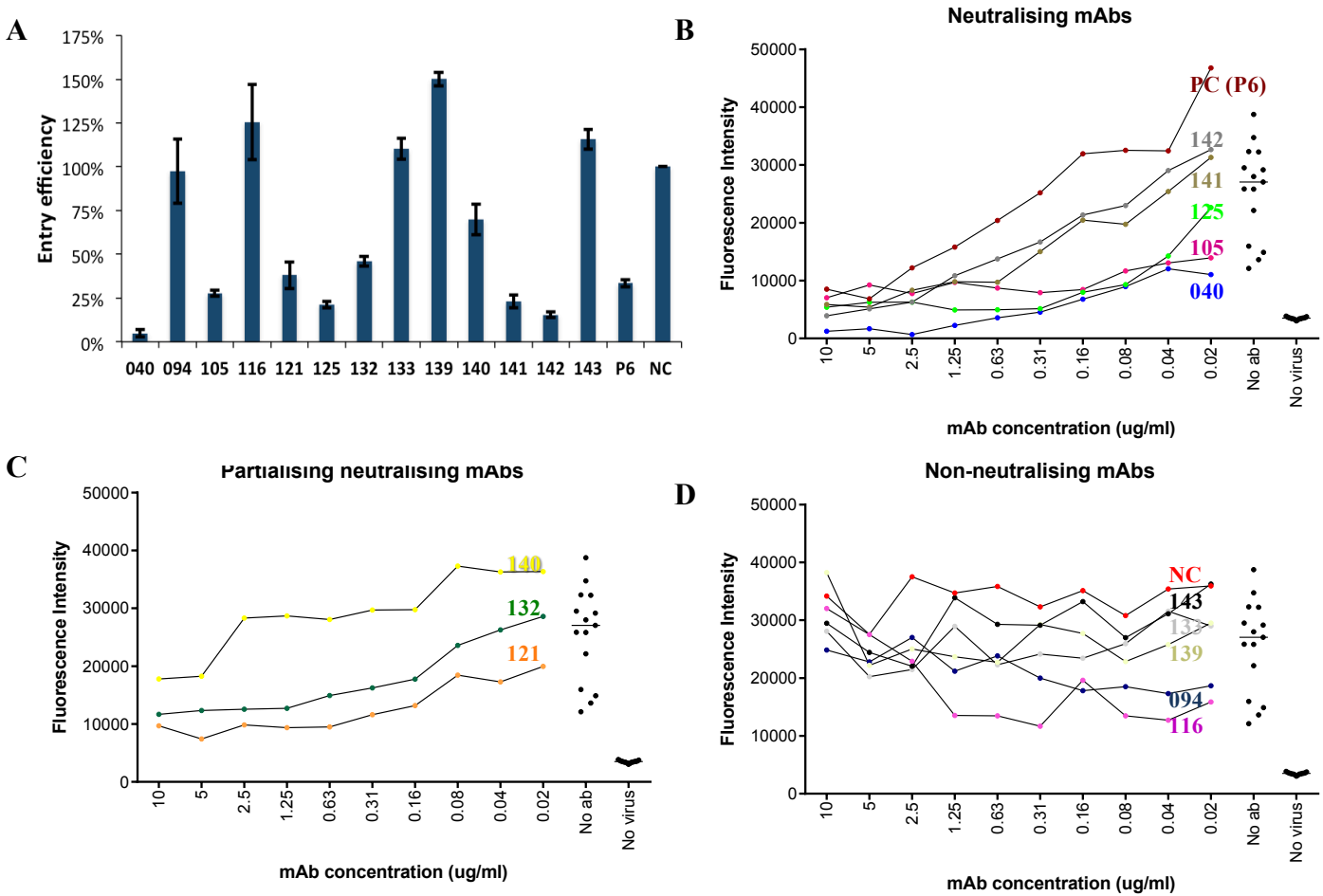


Figure 16. Influenza MN assay with Ebola GP-specific mAbs. (A) Entry efficiency at mAb concentration 10µg/mL was calculated as a percentage of influenza viral entry (GFP fluorescence) in the wells containing mAbs over the virus-only control wells (mean value). Fluorescence intensity readings of (B) neutralising, (C) partially neutralising, and (D) non-neutralising Zaire Ebola GP-specific mAbs. Control wells contained SIAT cells with no antibody (virus only) or SIAT cells with no virus (blank). Known neutralising mAbs from vaccinees, mAbs 040 and P6, were used also used as positive controls, the negative control well (NC) contained a non-specific (influenza) antibody.

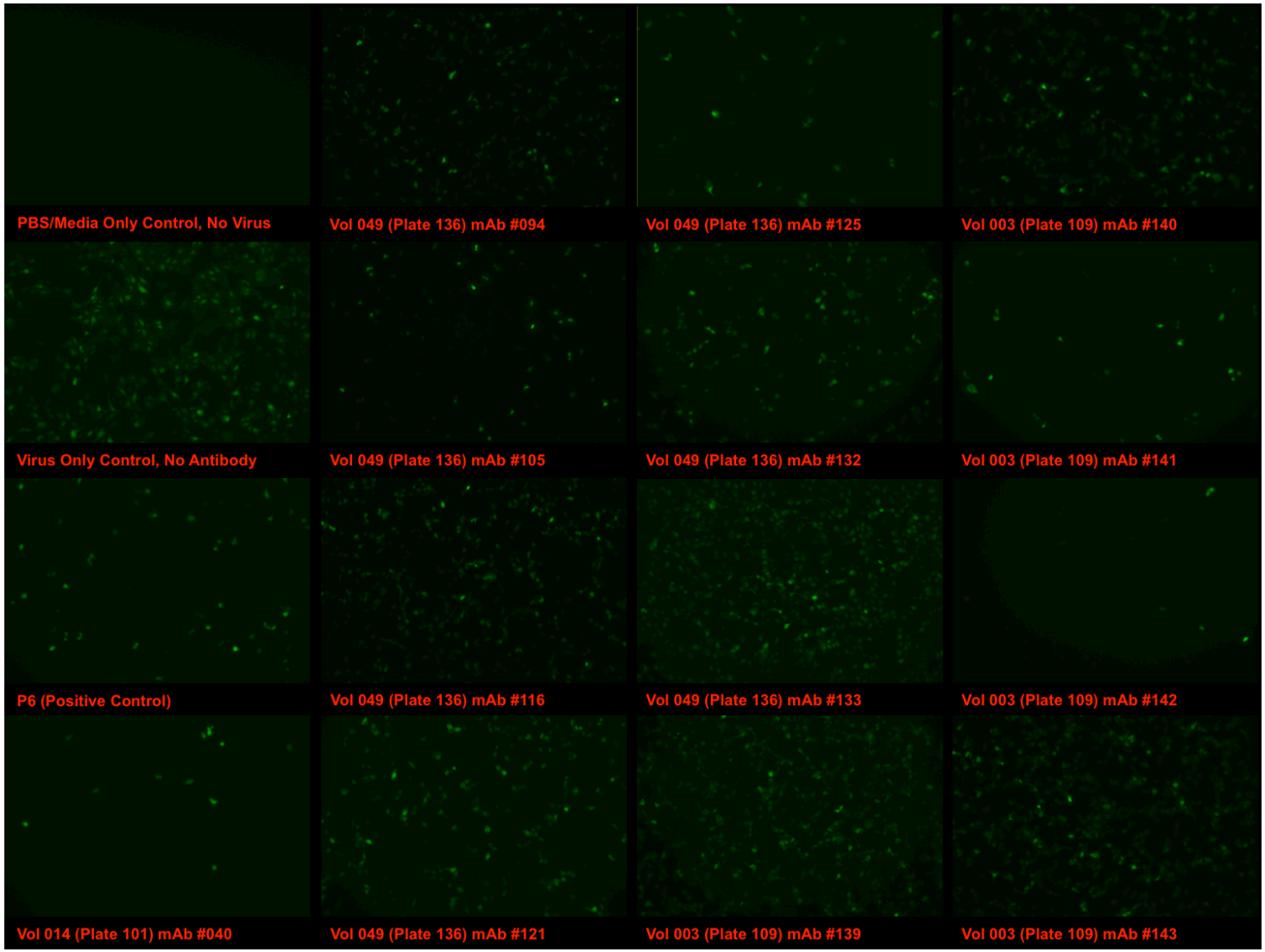


Figure 17. Influenza MN assay with Ebola GP-specific mAbs at 10µg/mL. The fluorescent green (GFP expression) reflects influenza viral entry. Images captured at x100 magnification.

3.7. Sequence analysis

Fourteen Ebola GP-specific mAbs (including two additional mAbs produced by other investigators in the same lab group) were sequenced by GATC-Biotech (Figure 18 and Table 12). Although the sample size is not large enough to draw any significant correlations, several observations were made. Overall, only a small number of somatic mutations/mismatches are observed in both mAb heavy and light chains. All of the Ebola-specific mAb heavy chains are ≥95.5% germline configuration (≤13 somatic mutations). The light chain sequences of the eight neutralising and partially neutralising mAbs have four or fewer somatic mutations (≥98.6% germline), as opposed to non-neutralising antibodies that have six or more mutations (≤98.3% germline).

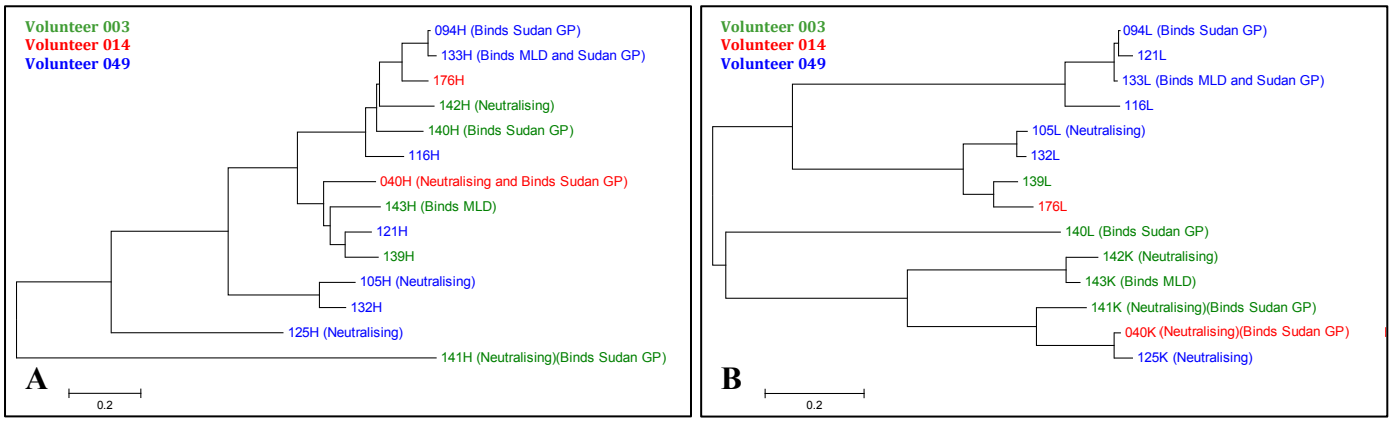


Figure 18. Immunoglobulin (A) heavy and (B) light chain phylogenetic trees for the whole Ig coding region including framework regions.

Heavy chain sequences:

mAb	Heavy V	Heavy D	Heavy J	Somatic mutations	% Germline	CDR1 Length	CDR2 Length	CDR3 Length
#040	3-23*04	3-22*01	6*02	13	95.5	24	24	5
#094	1-3*01	6-6*01	6*03	2	99.3	24	24	7
#105	3-15*07	3-3*02/01	6*04	9	97	24	30	5
#116	1-2*04	6-19*01	4*02	6	97.9	24	24	3
#121	3-33*01	2-15*01	4*02	3	99	24	24	8
#125	4-30-4*01	2-15*01	2*01	3	99	30	21	7
#132	3-15*07	3-10*01/02	4*02	4	98.7	24	30	6
#133	1-3*01	6-6*01	6*03	10	96.6	24	24	7
#139	3-33*01	2-2*01/03	6*02	3	99	24	22	7
#140	1-18*01	3-22*01	4*02	2	99.3	24	24	6
#141	3-21*01	3-3*02/01	6*03	12	95.9	24	24	8
#142	1-69*01	6-13*01	6*03	4	98.6	24	24	3
#143	3-74*01	2-8*01/02	6*03	5	98.3	24	24	7
#176	1-3*01	3-10*01	6*02	7	97.6	24	24	7

Light chain sequences:

Light Chain	Light V	Light J	Somatic mutations	% Germline	CDR1 Length	CDR2 Length	CDR3 Length
κ	1-17*01	1*01	2	99.3	18	9	20
λ	3-1*01	2*01/3*01	6	97.9	18	9	21
λ	1-40*01	3*01	3	99	27	9	25
λ	3-25*02	2*01/3*01	6	98.3	19	9	29
λ	3-1*01	2*01/3*01	3	98.9	18	9	22
κ	1-9*01	4*01	4	98.6	18	9	20
λ	1-40*01	1*01	4	98.6	27	9	23
λ	3-1*02	2*01/3*01	7	97.5	18	9	21
λ	2-23*01/03	1*01	6	98	27	9	26
λ	3-25*02	2*01/3*01	4	98.6	18	9	27
κ	3-15*01	1*01	1	99.6	18	9	19
κ	3-20*01	1*01	2	99.3	21	9	18
κ	3-20*01	4*01	7	97.6	21	9	22
λ	2-8*01	1*01	0	100	27	9	26

Table 12. Heavy and light chain sequences. IgG heavy and light chain sequences were analysed with the online analysis programme IGBLAST. Neutralising and partially neutralising antibodies are highlighted in yellow. mAb 176 (grey) had not been screened by IFA or MN assay.

4. Discussion

4.1. Isolation and characterisation of mAbs

In this dissertation I have cloned and characterised the first fully human Ebola-specific mAbs derived from vaccinated individuals. Previously described Ebola GP-specific mAbs have been isolated from human survivors of Ebola and Marburg viruses (Flyak et al., 2015; Hashiguchi et al., 2015; Maruyama et al., 1999) or are murine derived (Marceau et al., 2014; Olinger et al., 2012; Qiu et al., 2012). Both mice and human study participants were exposed to the same vaccine antigen (Ebola virus GP), although different vaccine constructs were used. It is therefore possible that the mAbs described here could bind to sites similar to the murine-derived chimeric mAb's binding sites.

Human mAbs were generated from single sorted ASCs at day seven post boost by RT-PCR and expression vector cloning using restriction enzymes. This process had a high efficiency with a yield of 91% IgG^+ mAbs out of all transfection supernatants screened, of which 15% were Ebola GP^+. This is in line with the literature, which reports an 85% yield of IgG^+ mAbs (Tiller et al., 2008). However, this mAb isolation process is expensive and laborious. One limiting factor is that we are not able to use antigen baiting of the ASCs at day seven post boost to increase the yield of Ebola-specific mAbs. Typically biotinylated antigen is used which binds B cells via the BCR and then can be detected using a streptavin-conjugated fluorophore during cell sorting. This method has been used successfully for HIV-1 antigens (Scheid et al., 2009b; Wilson and Andrews, 2012) but has proved more difficult for antigens from other pathogens. It should be noted that non site-specific biotinylation may obscure BCR binding sites on the Ebola GP. As we have not resolved all the antibody binding sites on the Ebola GP, we may fail to isolate crucial Ebola-specific mAbs binding new epitopes. In addition, plasmablasts have decreased expression of the BCR on their surface, which would make their detection difficult. To overcome such limitations more advanced techniques will be investigated in future, including antigen tetramers and targeted biotinylation.

Natural variations in the immune response of vaccinees to Ebola GP results in a variable yield of antigen-specific antibodies. It has been demonstrated that the ASC ELISPOT can be used to predict the yield of Ebola-specific antibodies from vaccinees, as has been suggested by Smith et al. (2009). The mean ASC ELISPOT result of the two plates from which mAbs were isolated was 18.36% Ebola GP-specific ASCs in the IgG^+ PBMC population, which is similar to the 15.19% of Ebola GP-specific mAbs isolated*. These results support the theory that there is an increase in antigen-specific plasmablasts in the blood around day seven post boost, when they transit between the secondary lymphoid organs and bone marrow or spleen (Bernasconi et al., 2002; Wrammert et al., 2008). This assay is also a valuable tool early for selecting priority candidates for mAb isolation, at least in terms of yield per plate of single sorted cells.

Characterisation using ELISA, IFA, and MN assays yielded a panel of twelve mAbs with a range of binding characteristics and neutralising abilities. I demonstrate that two of the non-neutralising antibodies bind the MLD, whilst the remainder of the mAbs bind the GP core. The lack of neutralisation by the MLD-binding mAbs is consistent with the idea that the "glycan shield" protects the receptor-binding site (hidden beneath the glycan cap) from antibody binding (Lee et al., 2008). I show that four of the antibodies are neutralising and another three are partially neutralising with the *in vitro* influenza MN assay (in the absence of complement). The neutralising activity of these mAbs confirms that antibodies elicited in a vaccine response are able to neutralise the Ebola virus. These findings would support the theory that the humoral immune response has a functional role in protection against Ebola virus disease. However, *in vitro* neutralisation does not necessarily predict improved *in vivo* survival of NHPs infected with lethal doses of Ebola virus (Oswald et al., 2007).

As demonstrated by the *in vitro* MN assay, some mAbs may actually increase viral entry—a mechanism known as antibody-dependent enhancement of viral infection (Takada and Kawaoka, 2003). One theory posits that complement protein C1q mediates the enhancement by forming a complex with the Fc portion of the antibodies bound to

* Sample size (n=2) is too small to perform the Spearman Rank Correlation test.

specific Ebola virus GP epitopes (Takada et al., 2003). This complex attaches to cellular C1q receptors resulting in enhanced binding of the Ebola virus to virus-specific receptors. However, this theory does not explain the increased viral entry of certain mAbs with the *in vitro* MN assay (without the addition of complement). Two samples of convalescent serum from Ebola patients during the 1995 Kikwit outbreak was shown to actually increase Ebola virus infectivity (Takada et al., 2003). Treatment of Ebola virus disease with serum transfusions should be performed with caution as they may enhance viral entry and worsen Ebola virus disease survival. Using well-characterised mAbs may reduce the risks associated with polyclonal serum. The ability of the Ebola virus to induce an antibody response that actually enhances infectivity may help to explain its pathogenicity (Takada et al., 2007).

The ELISA results also demonstrate that four of the mAbs are cross-reactive with the Sudan Ebola GP. It is possible that the epitopes for the four cross-reactive antibodies are conserved across the Zaire and Sudan Ebola viruses. They may also be conserved on the Tai Forest and Bundibugyo strains, though cross reactivity to these strains has not yet been shown. The cross-reactive efficacy of ZMapp with other Ebola virus strains is unknown. The cross-reactive antibodies may be valuable in a mAb cocktail because the conserved epitope may be less prone to antigenic escape. Neutralising mAb 141 has the highest cross-reactivity with the Sudan Ebola GP, raising the possibility that it shares a unique epitope with both strains. The other antibody of particular interest is mAb 040, which has the highest affinity binding at low concentrations, is the best *in vitro* neutraliser of influenza virus expressing Zaire Ebola GP and is weakly cross-reactive with the Sudan Ebola GP. Cocktails containing non-neutralising mAbs may still provide *in vivo* protection by improving presentation of the constant regions of neutralising mAbs, improving immune effector function (Murin et al., 2014). Certain mAbs also function by fixing complement for Ebola invasion-inhibitory activity *in vivo* (Wilson et al., 2000).

Gene sequencing of the mAbs allows me to make several observations. All of the Ebola-specific antibodies were close to germline configuration, particularly the light chain sequences. The three antibodies with the most somatic mutations (≥10) on their heavy chains (mAbs 040, 133, and 141) are also cross-reactive with the Sudan strain. This suggests that the MVA boost (which includes Sudan Ebola GP) results in affinity

maturation of the antibody repertoire. Interestingly, the other antibody that is strongly cross-reactive with the Sudan strain (mAb 094), has the fewest somatic mutations in its heavy chain (n=2), and is the closest to germline configuration (99.3%). Together with two mAbs that bind the MLD (mAbs 133 and 143), this mAb also exhibits the weakest affinity binding on IFA to Zaire E-SIAT cells. Sequence alignment of heavy chains (Figure 18A) reveal that mAbs 094 and 133 (also both cross-reactive with the Sudan GP) are clonally related. Sequence alignment of light chains (Figure 18B) broadly groups the antibodies into kappa and lambda chains. The majority of lambda chain Ebola GP-specific mAbs are from volunteer 049 and conversely most kappa chains belong to volunteer 003.

Antigen-specific B lymphocytes in the germinal centres of lymph nodes undergo clonal expansion and BCR diversification by somatic hypermutation in response to the priming antigen (McHeyzer-Williams and McHeyzer-Williams, 2005). Through positive selection, the germinal centre B cells with higher affinity BCRs avoid apoptosis by antigen and T follicular helper cell co-stimulation, and differentiate into long-lived memory B cells (Tarlinton and Good-Jacobson, 2013). When challenged with a secondary booster antigen (such as the Ebola MVA quadrivalent boost), primed memory B cells outside the germinal centre can rapidly differentiate into plasmablasts, capable of secreting large volumes of antibody, with further somatic hypermutation. This may result in the production of antibodies that recognise the new boosted antigen while retaining high affinity for the original priming antigen (Sasaki et al., 2011). Theoretically, Ebola-specific mAbs isolated from ASCs on day seven post quadrivalent boost should have more somatic hypermutations than mAbs derived from pre-boost memory B cells, recognise other Ebola strains, and yet retain a high affinity for the Zaire Ebola GP. Collaborators are currently investigating the difference in somatic hypermutation between memory B cell and ASC derived mAbs.

4.2. Future direction and conclusion

Ebola-specific mAbs that bind the membrane-bound GP but not the sGP "decoy" isoform could potentially result in improved *in vivo* neutralisation and survival. As such, the affinity of the mAbs to recombinant sGP should be measured. The sGP may compete for mAb binding with the membrane-bound GP, since they share structural proteins. To further ascertain which of the mAbs will be clinically useful, we will

need to map their epitopes with X-ray crystallography, competition assays using mAbs with defined epitopes, target-antigen mutagenesis, and escape mutant-analysis. An *in vitro* neutralisation assay with the addition of complement may demonstrate that some non-neutralising mAbs neutralise in the presence of complement, while others may enhance infectivity of the Ebola virus. It would also be prudent to assess the cross-reactivity of the mAbs with other Ebola virus strains, as the GP is relatively conserved across the *Filoviridae* family (Flyak et al., 2015; Fusco et al., 2015; Hashiguchi et al., 2015). Both the IFA and *in vitro* MN assay can be adapted to different Ebola virus strains.

It has long been understood that combinations of two or more mAbs can act additively or synergistically (Elliott et al., 1978; Ziegler-Heitbrock et al., 1986). Increasing antibody density on the viral surface by occupying several epitopes may amplify the antibodies neutralising ability (Marasco and Sui, 2007). Certain mAbs that bind the Ebola MLD and glycan may also potentially improve the binding of neutralising mAbs and enhance immune effector function (Murin et al., 2014). In addition, treatment with single neutralising mAbs can result in antigenic escape in infections with highly variant RNA viruses (Goswami et al., 2013). This problem may be avoided by using a cocktail of mAbs that binds several epitopes or a conserved region.

Once fully characterised, these antibodies may potentially be used as part of a mAb cocktail for post exposure prophylaxis and treatment of Ebola virus disease. Ideally we would want to compare the efficacy and safety of this fully human mAb cocktail head-to-head with the murine-derived ZMapp. This would allow us to conclude whether fully human Ebola-specific mAbs are better than mAbs originating from mice. The neutralising efficiency of the mAbs, either individually or in combinations, will ultimately need to be determined by *in vivo* animal models using lethal challenges with Ebola virus.

There is critical need for an effective treatment to halt the spread of the current Ebola epidemic and to prevent future outbreaks of such magnitude of any Ebola virus species. The isolation of Ebola-specific mAbs from vaccinees may bring us closer to that goal.

5. Authorship statement

The work presented in this dissertation is part of a collaborative effort to generate human mAbs at the Jenner Institute and Weatherall Institute of Molecular Medicine (WIMM), University of Oxford. The ELISPOTs and cell sorting of ASCs from fresh volunteer samples were performed during the phase 1 clinical trials of the Oxford EBL01 vaccine trial in November to December 2014 and not by the author of this dissertation. Only assays from frozen samples were performed by the author.

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